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Validation of CZECANCA (CZEch CAncer paNel for Clinical Application) for targeted NGS-based analysis of hereditary cancer syndromes

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Abstract

Background

Carriers of mutations in hereditary cancer predisposition genes represent a small but clinically important subgroup of oncology patients. The identification of causal germline mutations determines follow-up management, treatment options and genetic counselling in patients' families. Targeted next-generation sequencing-based analyses using cancer-specific panels in high-risk individuals have been rapidly adopted by diagnostic laboratories. While the use of diagnosis-specific panels is straightforward in typical cases, individuals with unusual phenotypes from families with overlapping criteria require multiple panel testing. Moreover, narrow gene panels are limited by our currently incomplete knowledge about possible genetic dispositions.

Methods

We have designed a multi-gene panel called CZECANCA (CZEch CAncer paNel for Clinical Application) for a sequencing analysis of 219 cancer-susceptibility and candidate predisposition genes associated with frequent hereditary cancers.

Results

The bioanalytical and bioinformatics pipeline was validated on a set of internal and commercially available DNA controls showing high coverage uniformity, sensitivity, specificity and

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accuracy. The panel demonstrates a reliable detection of both single nucleotide and copy number variants. Inter-laboratory, intra- and inter-run replicates confirmed the robustness of our approach.

Conclusion

The objective of CZECANCA is a nationwide consolidation of cancer-predisposition genetic testing across various clinical indications with savings in costs, human labor and turnaround time. Moreover, the unified diagnostics will enable the integration and analysis of genotypes with associated phenotypes in a national database improving the clinical interpretation of variants.

Introduction

Hereditary cancer syndromes are heterogeneous diseases characterized by the development of various cancer types in carriers of rare germline mutations in cancer susceptibility genes. These genes dominantly code for tumor suppressor proteins negatively regulating mitotic signals and cell cycle progression, activating apoptotic pathways, or executing DNA repair processes [1].

In general, it is considered that around 5% of all cancer diagnoses arise in hereditary cancer form. However, the percentage of hereditary cancers varies by cancer type, ranging from less than 3% in lung cancer to over 30% in pheochromocytoma [2, 3]. Important features distinguishing hereditary and sporadic cancers include an increased lifetime cancer risk with early disease onset, an increased risk of cancer multiplicity, the accumulation of cancer diagnoses in affected families, and a 50% risk of disease trait transmission to the offspring [1]. Considering these attributes and their consequences in terms of decreased life expectancy, decreased quality of life and increased medical expenses, patients carrying mutations in cancer susceptibility genes and their relatives represent a medically important subgroup with specific needs for increased cancer surveillance, a tailored follow-up and therapy, and rational prevention. However, the primary need is an unequivocal identification of the causative germline variant.

Although cancer inheritance has been suggested for over 150 years, the first gene conferring an increased cancer risk (*Rb*) was discovered only 30 years ago [4]. Hundreds of predisposing or candidate genes have been characterized since then, including the clinically most important "major" cancer susceptibility genes with high penetrance representing a subset of genes whose germline variants confer a high cancer risk (with relative risk (RR) > 5.0) in a substantial proportion of hereditary cancer patients. Pathogenic germline variants in "major" genes occur most commonly in patients with breast, ovarian, and colorectal cancers with variable proportions across populations worldwide. The group of cancer susceptibility genes with moderate penetrance is more extensive and growing steadily [5]. However, the clinical utility for many moderate penetrance genes is currently limited by the insufficient evidence about the degree of cancer risks associated with their germline variants.

The rapid improvement and availability of next-generation sequencing (NGS) technologies enable efficient simultaneous analyses of many cancer susceptibility genes in oncology patients or asymptomatic individuals at risk in routine diagnostics. NGS offers multiple approaches for the investigation of cancer predisposition, including the sequencing of whole genomes, exomes or transcriptomes. At present, however, the most widely used method of detecting clinically informative genetic alterations in the clinical setting is targeted panel NGS, analyzing selected

subsets of genes of interest [6]. Nevertheless, the numbers of genes included in panels differ substantially among laboratories and depend on healthcare systems. While some cancer-specific or multi-cancer panels include only the "major" predisposition genes for which substantial literature exists with regard to their diagnostic relevance, others include larger gene sets consisting of all clinically relevant genes and additional genes for which the evidence of cancer predisposition is still unclear.

NGS-based cancer testing has been rapidly adopted by routine clinical laboratories [7]. Their primary choice resides in the decision whether to use a commercially available NGS panel, or to design custom-made systems. The decision is influenced by clinical demand determining the set of targeted genes, by the spectrum of cancer diagnoses that will be analyzed, by the expected number of analyzed samples, and by costs of the analyses.

Our aim was to develop a universal diagnostic approach suitable for contributing genetic laboratories and allowing sample batching across multiple cancer indications. We focused on i) designing a custom-made multi-cancer panel with the desired sequencing quality and uniformity permitting a reliable variant identification, ii) the development of a robust analytical procedure limiting inter-run and inter-laboratory differences, and iii) the optimization of the bioinformatics pipeline enabling unified variant calling and annotation. The data collected from analyses of high-risk individuals performed in contributing laboratories will be used to create a nationwide genotype–phenotype database improving clinical variant interpretation in high-risk individuals.

Methods

Validation samples

Patient DNA samples. Validation of CZECANCA pipeline included analyses of 389 samples previously tested for the presence of germline variants available from DNA repository of the Institute of Biochemistry and Experimental Oncology. First Faculty of Medicine, Charles University. Of these, 137 samples carried pathogenic SNVs or short indels (in *BRCA1/2*, *PALB2*, *CHEK2*, *ATM*, *NBN*, *DPYD*, *PPM1D*, *RAD51C*, *RAD51D*, or *TP53*), 217 had been tested negatively using previous gene-by-gene analyses based on Sanger sequencing or a protein truncation test (PTT) [8–16], and 35 samples carried intragenic rearrangements in *BRCA1*, *CHEK2*, *PALB2*, or *TP53*, identified by the MLPA (multiplex ligation-dependent probe amplification) analysis [10, 17, 18]. All blood-isolated DNA samples were obtained from individuals that gave their written informed consent with mutation analyses of cancer susceptibility genes and who agreed to use their genetic material for research purposes. The study was approved by Ethics Committee of the First Medical Faculty, Charles University and General University Hospital in Prague. All used samples were anonymized prior analysis.

Human genome reference standards. Five commercially available DNA reference standards (NA12878, NA24149, NA24385, NA24631 and NA24143) were obtained from Coriell Institute for Medical Research. Well described genotypes, including high confident calls for variant and wild-type alleles, is the major advantage of these reference standards. The genotypes and variants in reference samples identified by CZECANCA analysis and obtained from reference variant-call format (VCF) files (available from the Genome in a Bottle (GIAB) website; http://jimb.stanford.edu/giab/), respectively, were compared to compute CZECANCA sensitivity, specificity, and accuracy, as described by Hardwick et al. [19].

Panel design

The multi-cancer panel CZECANCA was designed using the online NimbleDesign software utility (NimbleGen, Roche; http://sequencing.roche.com/products/software/nimbledesign-

software.html). For enrichment, we selected genes with a known predisposition for hereditary breast, ovarian, colorectal, pancreatic, gastric, endometrial, kidney, prostate and skin cancers, together with known DNA repair genes associated (or potentially associated) with cancer susceptibility (a list of 219 selected genes is provided in S1 Table), considering the results of our previous NGS analysis with a broad panel of 581 genes [20]. The primary gene target for probe coverage was represented by all exons (in case of known cancer susceptibility genes) or all coding exons (in other genes), including 10 bases from adjacent intronic regions. The design considered all transcription variants of selected genes available at UCSC website (https://genome.ucsc.edu/; accessed 2015-05-21). The promoter regions of the *BRCA1* and *BRCA2* genes were included into the primary target. The probes were designed using *continuous design* under strict conditions–minimal and maximal *close matches* (number of times in which a probe sequence matches the genome with either $\leq$ 5 insertions or deletions, or gap of $\leq$ 5 bp) were one and three, respectively, allowing us to hybridize the probes up to three targets across the genome. Because of the strict design conditions, some clinically relevant regions were left untargeted for technical reasons such as repeats and homologous regions (see S1 Table). The final panel target size reached 628,069 bases.

Library preparation

Five hundred ng of genomic DNA isolated from peripheral blood and dissolved in TE buffer was used for preferred ultrasound shearing using Covaris E220 (Covaris Inc). As an alternative DNA fragmentation method, we tested enzymatic digestion using Fragmentase (KAPA Biosystems, Roche) with incubation for 25 min at 37˚C according to the manufacturer's instruction. The mean average size of DNA fragments targeted 200 bp. Sizing and quality was controlled using the Agilent High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer System (Agilent).

Libraries were prepared using the KAPA HTP Library Preparation kit (for ultrasound-sheared DNA samples) or KAPA HyperPlus Kit (for Fragmentase-digested DNA samples) according to the manufacturer's instructions (KAPA Biosystems, Roche) with minor modifications including the use of universal in-house prepared adapters, double-indexing primers for ligation-mediated polymerase chain reaction (LM-PCR), and primers for post-capture PCR, as described further. The adapters [Adapter#1: `5′-ACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3′` ("*" denotes for phosphothiolate bond) and Adapter#2: `5′-pGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3′` ("p" denotes for 5' phosphate)] were hybridized in Tris:NaCl buffer mix (50 mM Tris:HCl pH 7.5; 50 mM NaCl) in 97˚C for 2 min, followed by 72 cycles involving incubation at 97˚C for 1 min (-1˚C per cycle) and 25˚C for 5min. The barcoding of size-selected DNA fragments enabling subsequent sample pooling was performed during LM-PCR with indexing primers [Primer#1: `5′-AATGATACGGCGACCACCGAGATCTACACxxxxxxxxACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3′` and Primer#2: `5′-CAAGCAGAAGACGGCATACGAGATxxxxxxxxGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C-3′` ("*" denotes for phosphothiolate bond; "xxxxxxxx" denotes for a sequence of particular indices same as the Illumina Truseq HT index i7 and i5)]. The number of LM-PCR cycles was reduced to six to limit the presence of PCR duplicates. Sizing and quality after the double-sided size selection and LM-PCR were controlled using the Agilent High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer System.

To reach the targeted mean coverage (100X), 30 individual barcoded samples (33 ng each) were pooled for the enrichment (usually two overnight hybridizations; tested for 16–72 hours without a significant effect on enrichment efficacy) using the CZECANCA (NimbleGen SeqCap EZ Choice, Roche) to create a sequencing library. After the enrichment, the library was amplified using Primer 1: `5′-AATGATACGGCGACCACCGAGATCTACAC-3′` and Primer 2:

5′ -CAAGCAGAAGACGGCATACGAGAT-3′ . The number of post-capture PCR cycles was reduced to 11 to reach the optimal library concentration (2 ng/μl) and to minimalize the number of PCR duplicates.

After the enrichment control using qPCR (NimbleGen SeqCap EZ Library SR User's Guide), the final 18 pM libraries were sequenced on the MiSeq system using MiSeq Reagent Kit v3, 150 cycles (Illumina).

Bioinformatics

Single nucleotide variants (SNVs). The NGS data obtained from sequencing with the CZECANCA were processed using an analysis pipeline based on standard tools. FASTQ files were generated by MiSeq. The quality of raw data was controlled using FastQC v0.11.2 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). FASTQ files were subsequently mapped using Novoalign v2.08.03 to hg19 (http://www.novocraft.com/products/novoalign/) to generate sequence alignment map (SAM) files. SAM files were transformed to binary form (BAM files) using Picard tools v1.129 (https://broadinstitute.github.io/picard/). Raw BAM files were further processed to eliminate PCR duplicates of mapped reads. The quality of mapped bases was checked and recalibrated according to default settings using Genome Analysis Toolkit (GATK) v3.3 (https://software.broadinstitute.org/gatk/). The finalized BAM file was converted using a GATK pipeline to a variant-call format (VCF) containing alternative variants only. ANNOVAR was used to annotate VCF files generated using GATK [21, 22] and to check the presence of each variant in external databases (ExAC, 1000Genome or ClinVar) [23–25]. Predictive values from selected prediction algorithms (for example SIFT [26], Mutation Analyzer [27], MutationTaster [28], LRT [29], PolyPhen-2 [30], phyloP [31], GERP [32], CADD [33] or spidex (https://www.deepgenomics.com/spidex) were added to the annotated alternative variants.

For a comparison with CZECANCA sequencing, the data from routine analyses using the TruSight cancer panel (Illumina), performed in a laboratory of the Masaryk Memorial Cancer Institute in Brno were analyzed by an identical bioinformatics pipeline [34].

The Integrative Genomics Viewer (IGV) was used for visualization and manual inspection of individual BAM files [35].

Medium-size indels. The detection and exact sequence determination of medium-size insertions and tandem duplications (involving approximately half of the sequence reads, depending on the sequencing chemistry used) is very challenging. The identification of these alterations was based on the method of soft-clipped bases using Pindel (http://gmt.genome.wustl.edu/packages/pindel/) [36]. The finalized BAM files served as an input for the analysis. In our case (with mean read size of 75 bp; MiSeq Reagent Kit v3, 150 cycles chemistry) insertion or duplication exceeding 35 bp was considered as a medium-size indel.

Copy number variations (CNVs). An analysis CNVs was performed using the CNVkit (https://pypi.python.org/pypi/CNVkit). The CNVs analysis is coverage-based and therefore required good coverage uniformity. Raw BAM files served as the input for this analysis.

Coverage visualization. The visualization of sequence coverage of the individual samples, enabling a fast visual inspection of coverage limit >20X (for a reliable identification of heterozygotes) across the analyzed genes, was performed by an in-house "Boudalyzer" script written in R language. The coverage is visualized from the finalized BAM files. This tool was used for the generation of manuscript figures showing coverages of the analyzed genes.

Variant interpretation. We used the scoring scheme outlined in ENIGMA guidelines (https://enigmaconsortium.org/) for variant interpretation to classify SNVs and indels as benign (Class 1), likely benign (Class 2), variant of unknown significance (Class 3), likely pathogenic (Class 4) and pathogenic (Class 5) [37]. Identified variants of unknown significance

(VUS) were further prioritized if their minor allele frequency was lower than 1% in ExAC, 1000Genome databases, or in a two sets of population-matched controls containing anonymized genomic data from 530 non-cancer controls analyzed by CZECANCA NGS and from 780 unselected Czech individuals analyzed by an exome sequencing (provided by the National Center for Medical Genomics; http://ncmg.cz). Potentially deleterious VUSes were selected based on concordant results obtained from above-mentioned *in silico* prediction algorithms. These priorized VUS variants were enrolled into the list of variants for subsequent segregation analyses or functional *in vitro* testing performed in selected genes.

The CZECANCA contains 22 genes that are listed in the ACMG recommendation (S1 Table) for the reporting of secondary findings [38].

Results

Target gene coverage

The NGS analysis with CZECANCA targeting the coding sequences of 219 genes (S1 Table) displayed high coverage uniformity. Under standard conditions for routine analyses, we targeted sequencing coverage 100X. In these settings, more than 85% of the targeted regions were covered 100X, 98% of the targeted regions were covered at least 50X and less than 0.2% of targeted regions had coverage below 20X (Fig 1A). The entire coding sequence was fully covered at least 100X in 144/219 targeted genes (65.8%), at least 50X in 190/219 genes (86.8%), and at least 20X in 207/219 targeted genes (94.5%; Fig 2). Coverage did not exceed 300X in any of the captured targets.

Coverage was uniform among samples independently analyzed in the participating laboratories using the described protocol (Fig 3), and also among samples sequenced using separately-synthesized CZECANCA lots (data not shown). The equal coverage uniformity was independent of coverage depth (Fig 1B). The coverage uniformity was partially influenced by the DNA fragmentation approach with better results obtained by ultrasound fragmentation in comparison with enzymatic DNA cleavage. The improved results (more random DNA shearing) obtained with the ultrasound fragmentation protocol were indicated by an analysis of terminal (di)nucleotides in reads from samples prepared by both DNA fragmentation methods, regardless of the laboratory site (Figs 1C and 3). The CZECANCA coverage uniformity substantially surpassed that of the Illumina TruSight Cancer Panel (Fig 3F).

Low-covered regions (uncovered or with coverage $\leq$20X) were constantly observed in 12/219 genes (5.5%; Fig 2, S1 Table). In nine genes, the low–covered regions were mostly limited to a single exon (typically the first exon) representing usually a small fraction of the coding sequence. In three incompletely covered genes (*CHEK2*, *MDC1*, *NF1*), single or several exons were omitted from the CZECANCA design (see Panel design in Methods). The remaining low-covered regions were GC-rich regions with mean GC content of 76.88% (S2 Table) while the average GC content of the CZECANCA targets is 47%.

Sequencing quality was partially influenced by the particular MiSeq sequencer. In standard runs, more than 99% of bases reached a Phred score >20 (i.e. 99% accuracy) and approximately 97% of bases overcame a Phred score of 30 (i.e. 99.9% accuracy). A decrease in PCRs cycles during library preparation reduced the number of PCR duplicates, which finally represented 7–9% of reads. The mean off-target (reads mapped to distance exceeding 250 bp from the nearest bait) across the performed runs was constantly less than 12% of reads.

Reproducibility, specificity and sensitivity analysis

The reproducibility of variant calls was tested using intra-, inter-run, and inter-laboratory replicates. During the sequencing of intra-run replicates, we also evaluated the impact of coverage depth on coverage uniformity and reproducibility.

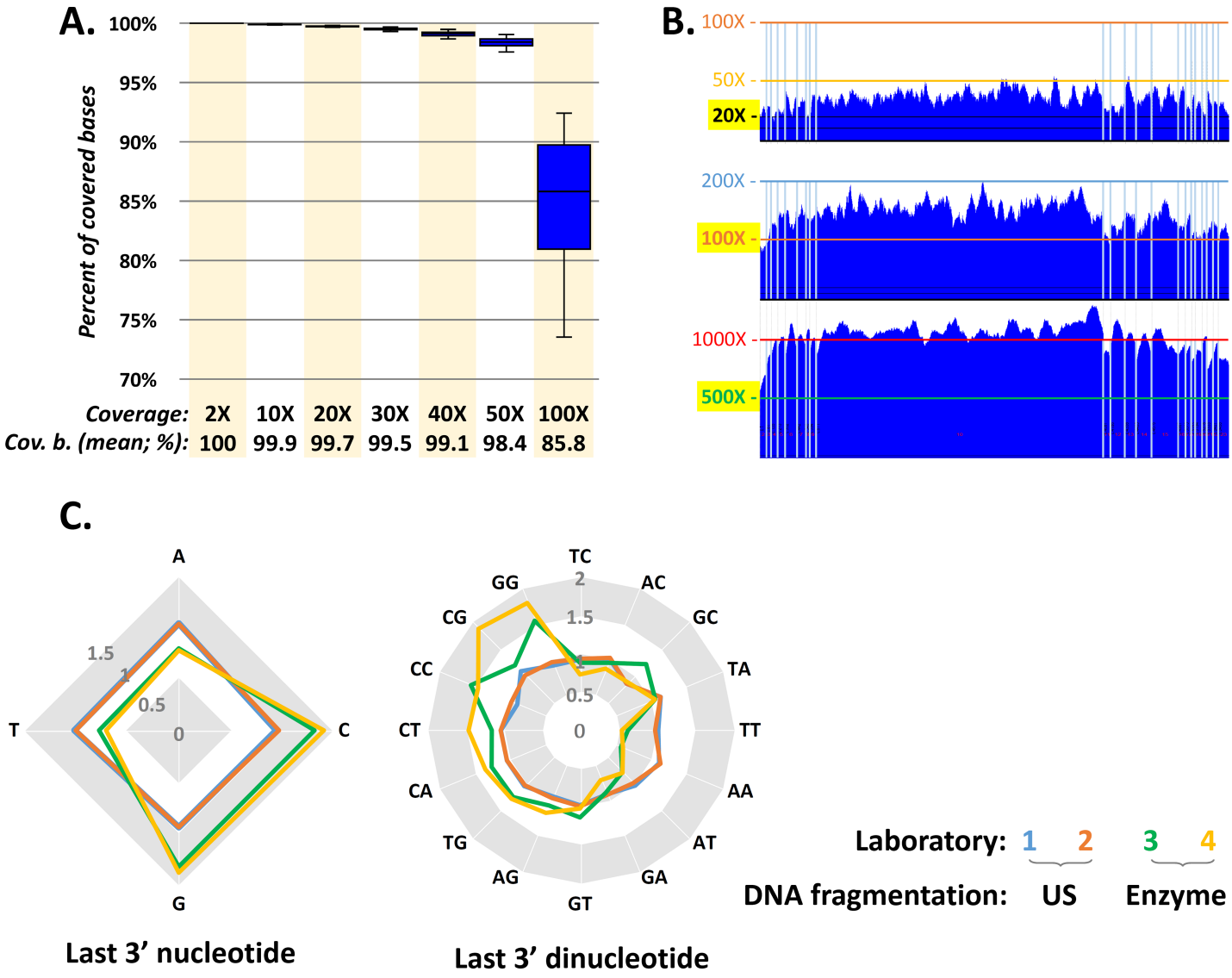

Fig 1. Coverage parameters from CZECANCA sequencing. (A) The chart expresses the percentages of covered target bases (cov. b.) obtained from 25 analyzed samples from a standard run targeting sequencing coverage 100X. (B) The coverage (at y-axis) of *BRCA1* coding sequence (NM_007294; x-axis; vertical lines represent exon boundaries) in three independent runs targeting sequencing coverages 20X, 100X, or 500X demonstrates coverage uniformity, not influenced by coverage depth. (C) The "randomness" of the DNA shearing approach using ultrasound (US) and enzymatic cleavage was compared by an analysis of the distribution of ending nucleotides and dinucleotides in reads completely mapped to the large exon 11 (chr17:41243452–41246877; 3426bp) in the *BRCA1* gene, representing one of the largest continuous genomic fragments targeted by CZECANCA probes. The chart displays the relativized distribution of terminal nucleotides and dinucleotides in the analyzed region from 12 samples from each laboratory normalized to the average nucleotide and dinucleotide content of the analyzed region. The distribution of last nucleotides and dinucleotides in fragments from samples processed by US oscillate closer to a normalized value (1) than in fragments of samples prepared by the enzymatic cleavage.

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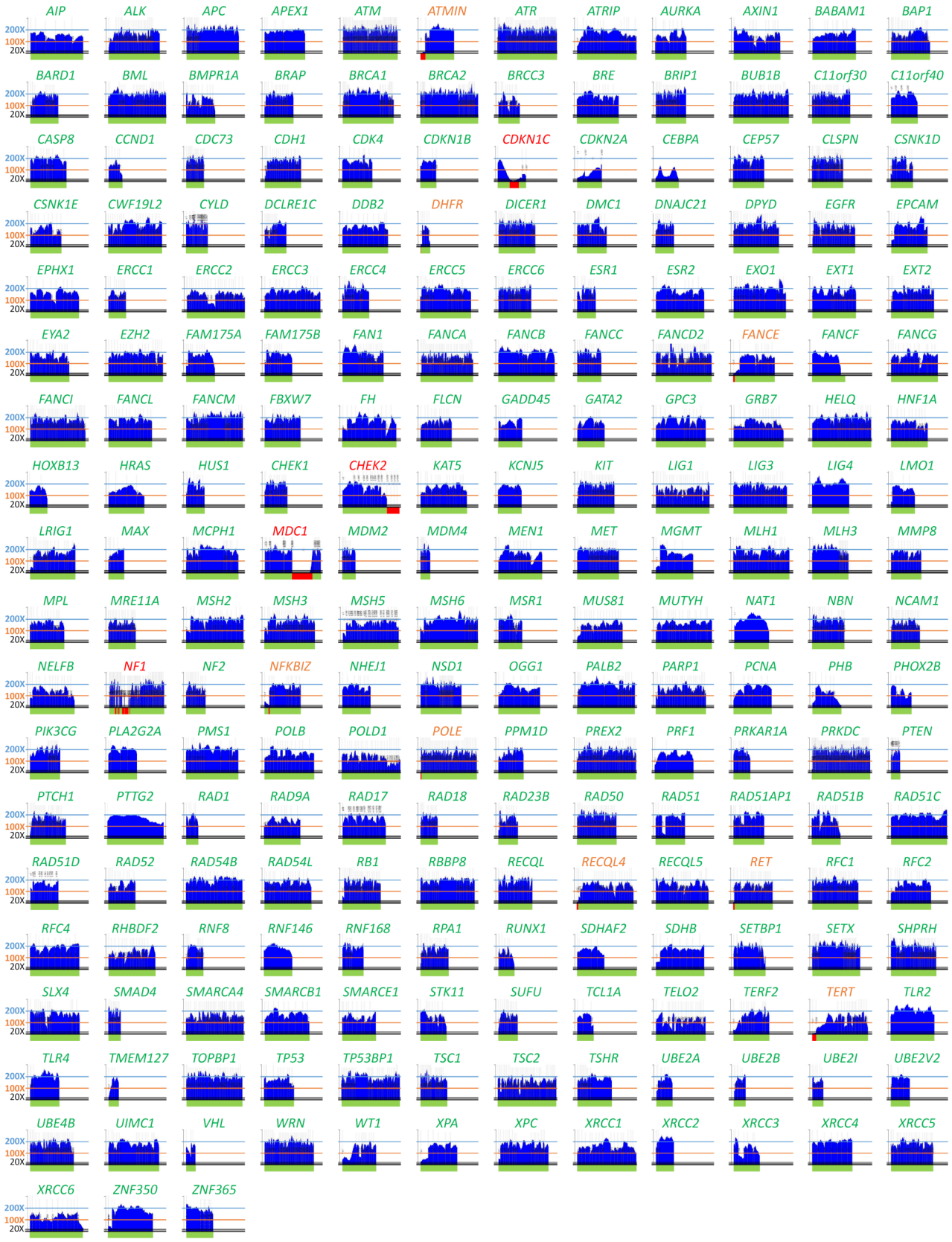
Three individually bar-coded replicates were pooled for enrichment in amounts corresponding to 33 ng (considered as 100%), 24.75 ng (75%), and 16.5 ng (50%), respectively. The subsequent bioinformatics of these samples, considering variants with GATK quality >100 in the targeted regions (exon sequences with 12 bp from adjacent introns), revealed 293 (100%), 292 (99.7%) and 290 (99.0%) variants, respectively (S3 Table). Altogether, 289/293 (98.6%) variants were identified in all replicates, while four variants not detected in DNA-reduced samples were variant homozygotes located in low-covered regions or had GATK quality <100. The


AIP
ALK
APC
APEX1
ATM
ATMIN
ATR
ATRIP
AURKA
AXIN1
BABAM1
BAP1
BARD1
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BRAP
BRCA1
BRCA2
BRCC3
BRE
BRIP1
BUB1B
C11orf30
C11orf40
CASP8
CCND1
CDC73
CDH1
CDK4
CDKN1B
CDKN1C
CDKN2A
CEBPA
CEP57
CLSPN
CSNK1D
CSNK1E
CWF19L2
CYLD
DCLRE1C
DDB2
DHFR
DICER1
DMC1
DNAJC21
DPYD
EGFR
EPCAM
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ERCC1
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EXO1
EXT1
EXT2
EYA2
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FAM175A
FAM175B
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FANCB
FANCC
FANCD2
FANCE
FANCF
FANCG
FANCI
FANCL
FANCM
FBXW7
FH
FLCN
GADD45
GATA2
GPC3
GRB7
HELQ
HNF1A
HOXB13
HRAS
HUS1
CHEK1
CHEK2
KAT5
KCNJ5
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LRIG1
MAX
MCPH1
MDC1
MDM2
MDM4
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MMP8
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MSH5
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NSD1
OGG1
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PHB
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POLB
POLD1
POLE
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PREX2
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PRKAR1A
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RAD18
RAD23B
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RAD51B
RAD51C
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RAD54B
RAD54L
RB1
RBBP8
RECQL
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RFC2
RFC4
RHBDF2
RNF8
RNF146
RNF168
RPA1
RUNX1
SDHAF2
SDHB
SETBP1
SETX
SHPRH
SLX4
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SMARCA4
SMARCB1
SMARCE1
STK11
SUFU
TCL1A
TELO2
TERF2
TERT
TLR2
TLR4
TMEM127
TOPBP1
TP53
TP53BP1
TSC1
TSC2
TSHR
UBE2A
UBE2B
UBE2I
UBE2V2
UBE4B
UIMC1
VHL
WRN
WT1
XPA
XPC
XRCC1
XRCC2
XRCC3
XRCC4
XRCC5
XRCC6
ZNF350
ZNF365
200X
100X
20X

Fig 2. Coverage (y-axis) of coding sequences (x-axis) of 219 CZECANCA target genes from a routine, randomly selected run targeting 100X coverage. Note: Fully covered genes are depicted in green letters, genes with coverage <20X in a single exon are in orange letters, and genes with uncovered regions exceeding single exon or >10% of coding sequence are in red letters. Green horizontal bars (below individual graphs constructed using "Boudalyzer" script) indicate coverage ≥ 20X; red horizontal bars indicate regions covered <20X and uncovered regions.

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analysis demonstrated that alternative nucleotides could still be reliably detected in samples with reduced overall coverage, showing the robustness of the analysis in samples with unequal DNA input (Fig 4A).

A subsequent analysis of inter-run replicates (performed with another DNA sample analyzed in two independent runs) revealed 356 unique variants with GATK quality >100 in at least one replicate (S4 Table). Overall, 354 (99.4%) variants were identified in both inter-run replicates with a strong coverage correlation (Fig 4B).

In addition, the inter-laboratory performance was tested by an NGS analysis of an identical DNA control sample in four laboratories participating in the panel validation (Fig 4C), which revealed 332 unique variants with GATK quality >100 in at least one laboratory, from which we identified 331 (99.7%), 327 (98.5%), 329 (99.1%), and 329 (99.1%) variants in the particular laboratory, respectively. The discordant findings were caused by variants in low-covered regions, with low base Phred quality, or GATK quality <100 (S5 Table).

Sensitivity and specificity were assessed in 354 samples previously tested for the presence of germline variants. All 137 previously identified pathogenic germline mutations in *BRCA1/2* and other susceptibility genes were detected by CZECANCA (S6 Table). Moreover, an analysis

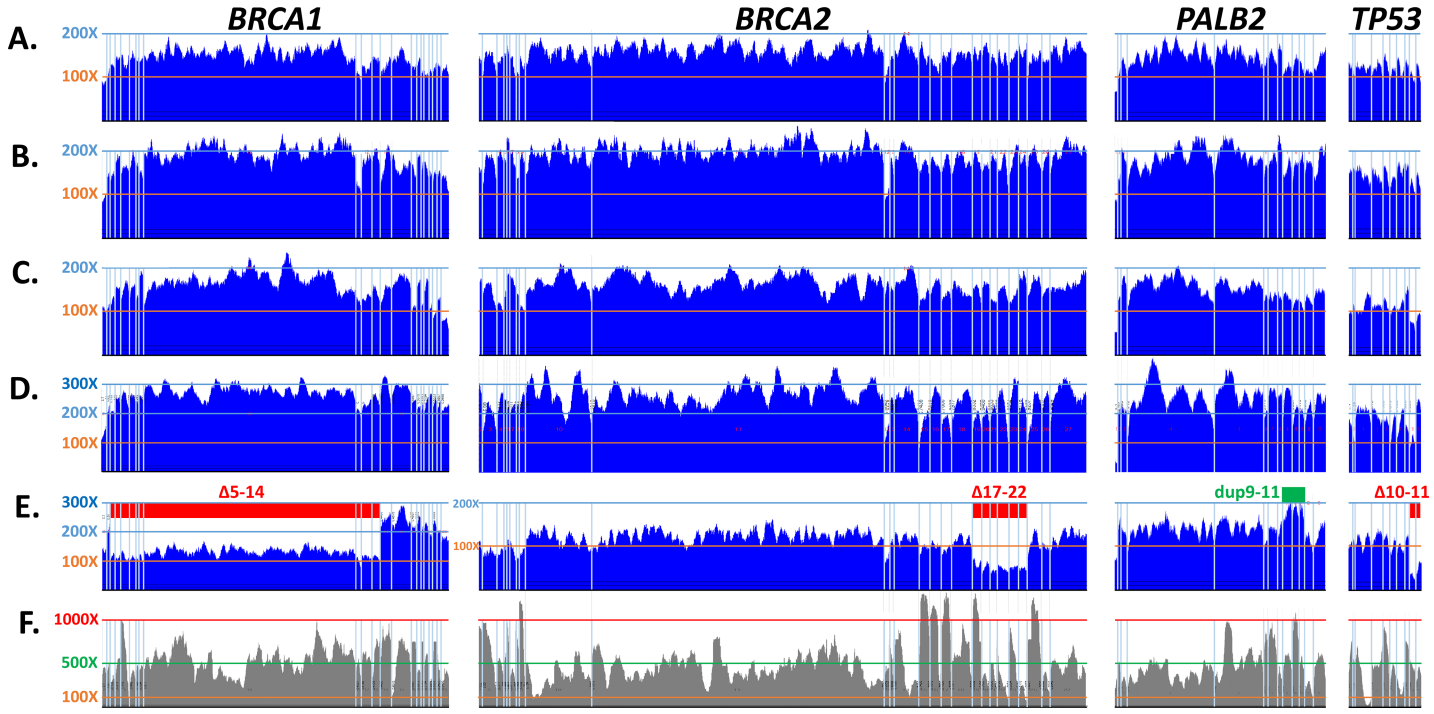

Fig 3. Coverage of selected genes from the CZECANCA (A-E) and TruSight Cancer sequencing (F) panels. The pictures show coverage (at y-axis) alongside the coding sequences of *BRCA1* (NM_007294), *BRCA2* (NM_000059), *PALB2* (NM_024675), and *TP53* (NM_000546), the vertical lines represent exon boundaries. Panels A–D show results obtained from a CZECANCA NGS analysis of various samples performed in four participating laboratories using the ultrasound (A, B) or enzymatic (C, D) DNA fragmentation protocol. Examples of the identified CNV aberrations in the depicted genes (deletions in *BRCA1*, *BRCA2* and *TP53* and duplication in *PALB2*) are shown in panel E. For comparison, panel F demonstrates the uneven coverage of the depicted genes by sequencing using the TruSight Cancer panel (Illumina).

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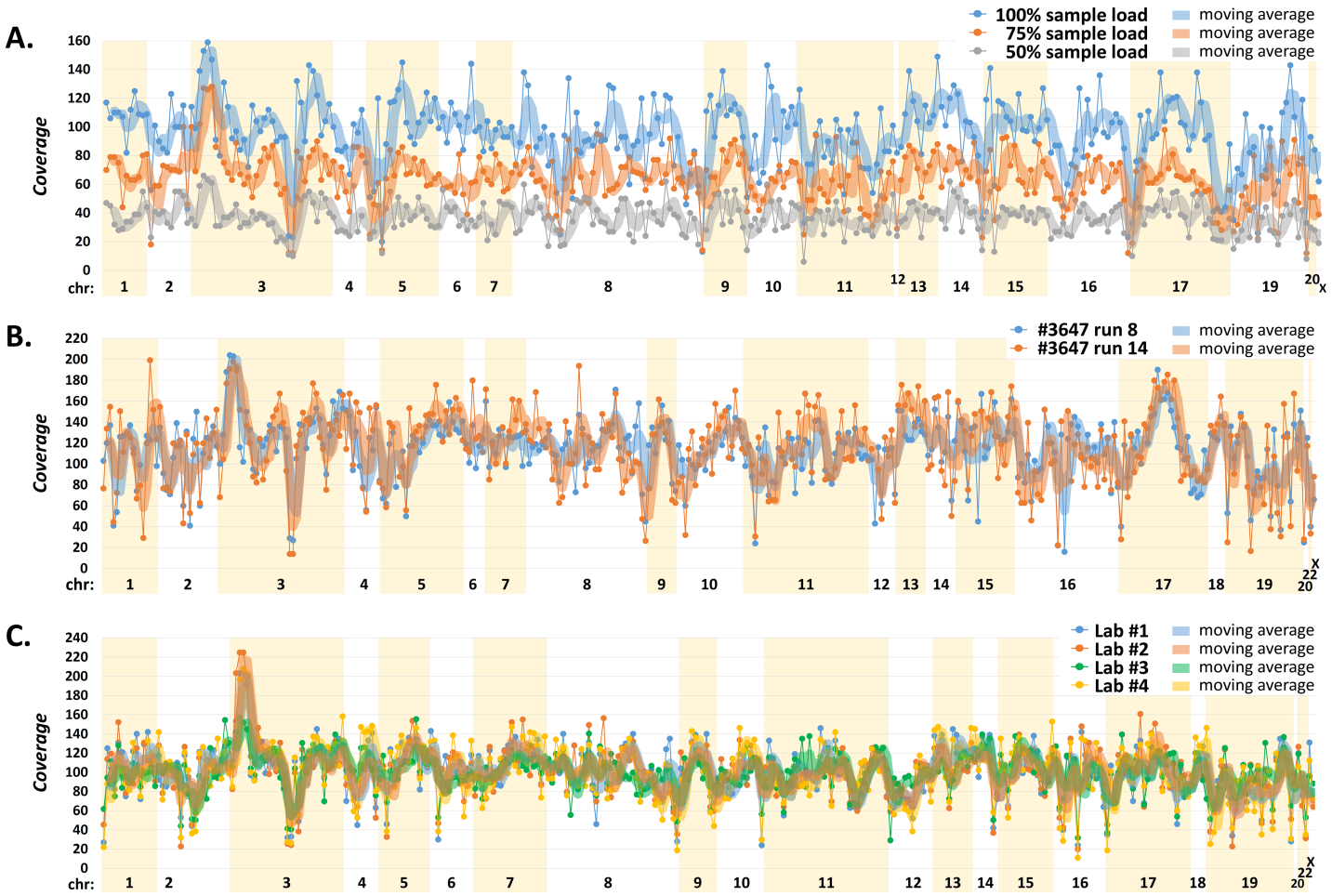

Fig 4. Analysis of intra-run (A), inter-run (B), and inter-laboratory (C) replicates. The panels show sequencing coverages (y-axis) of the identified variants arranged according to chromosomal localizations (x-axis). We used moving average curves (average of 3 values) to compare trends in coverages. Panel (A) describes the results of an analysis of three independently processed intra-run replicates from an identical DNA sample pooled in 33 ng (considered as 100%), 24.75 ng (75%), and 16.5 ng (50%), respectively. Panel (B) demonstrates variant coverages identified in two independent inter-run (run 8 and 14) replicates. All coverage values of sample #3647 in run 14 were corrected by a factor of 1.3880 to normalize coverages between samples (see S4 Table). Panel (C) shows coverages of variants identified in an inter-laboratory control sequenced in four laboratories (Lab) participating in panel validation (see S5 Table). The coverages of variants identified in Lab 2, 3, and 4 were normalized to the average coverage of Lab 1 for better comparisons of coverages.

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revealed nine additional *BRCA1* or *BRCA2* mutations. Of these, seven mutations were identified in samples previously tested by cDNA sequencing (they had not been detected previously, probably because of nonsense-mediated decay). The pathogenic missense mutation c.3G>A in *BRCA2* was found in a sample negatively analyzed using PTT and the pathogenic *BRCA2* mutation c.5645C>A was found in the carrier of c.5266dupC in *BRCA1* in whom the identification of a pathogenic *BRCA1* variant discontinued subsequent *BRCA2* testing.

Further, we validated the sensitivity of CNVs detection on 35 samples tested positively using the MLPA analysis (S7 Table). All CNVs including 18 samples with large *BRCA1* deletions or duplications, 12 CNVs in *CHEK2*, four in *PALB2* and one in *TP53* were detected using CNVkit software in routine settings targeting 100X coverage (Fig 5A; S8 Table). This analysis also enabled to setup CNVkit thresholds indicating the presence of a deletion or a duplication. To estimate the number of false positive and true positive CNV calls obtained from CNVkit, we further analyzed aggregated results from four consecutive runs performed in two

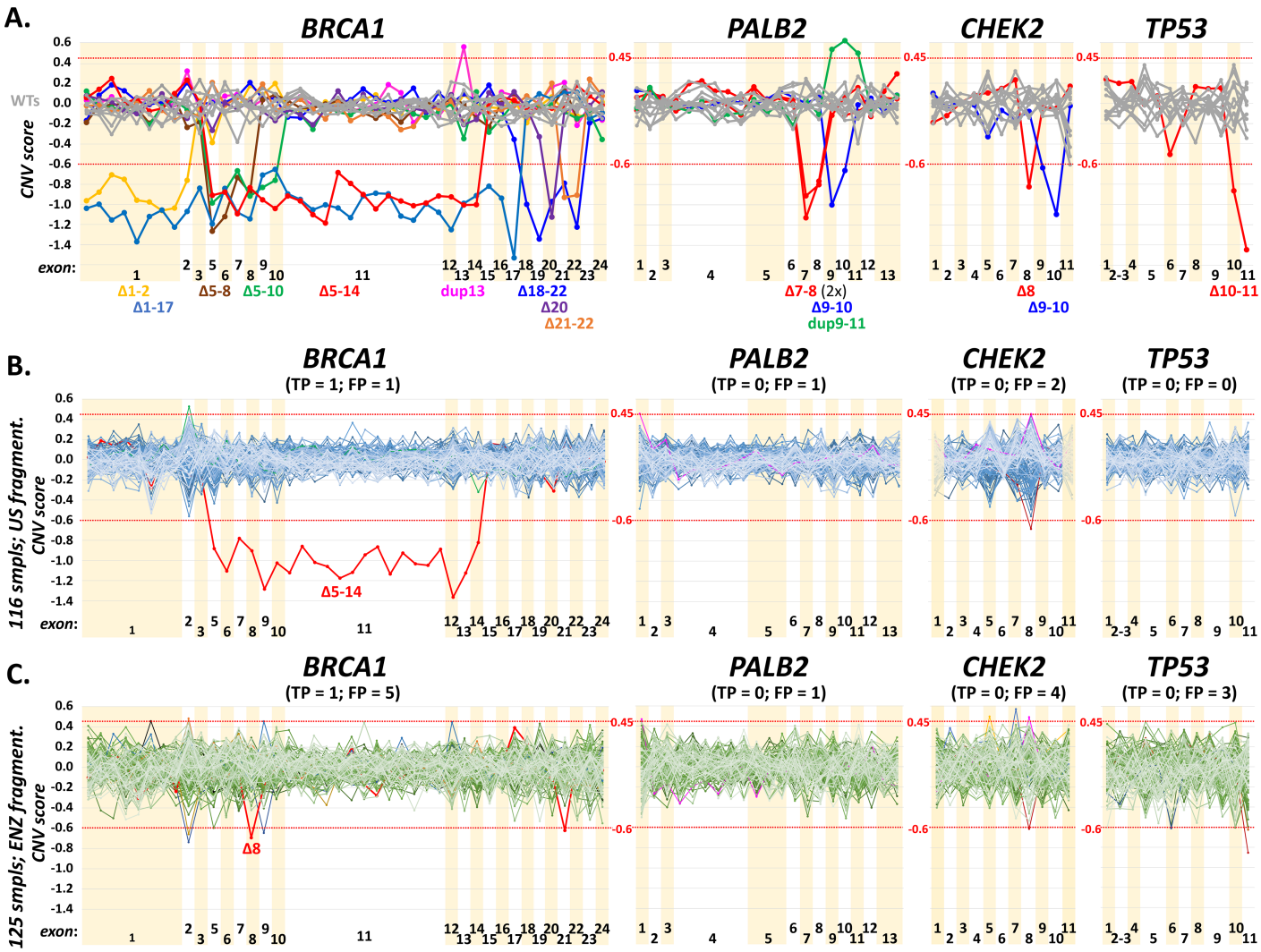

Fig 5. The panel A show results of CNV analysis revealing large deletions or duplications in four genes in a testing set of 35 samples with previously identified CNVs. The charts show median-normalized values of CNV scores for particular gene bins (default settings in CNVkit software; S8 Table). Values <-0.6 and >0.45 (red dotted lines) were assumed as thresholds indicating a deletion or a duplication, respectively. All shown CNVs were confirmed by MLPA previously (S7 Table). The panels B and C demonstrate frequency of true positive (TP) and false positive (FP) CNV signals from analyses performed in two participating laboratories (laboratory 1 in B and laboratory 3 in C). While 116 samples analyzed in four consecutive runs in B were prepared using the ultrasound (US) fragmentation, 125 other samples in four consecutive runs in C were prepared using the enzymatic (ENZ) fragmentation method. Samples in vivid colors highlight suspected samples that were further analyzed by MLPA analysis and samples in *BRCA1* Δ5–14 (B) and Δ8 (C) denote for true positives. The presence of putative CNVs in *PALB2*, *CHEK2*, and *TP53* were excluded by analysis that revealed heterozygotes in regions with suspected deletions or by an MLPA analysis.

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participating laboratories preparing sequencing libraries by ultrasound shearing and enzymatic digestion, respectively (Fig 5B and 5C). The CNV analysis in *BRCA1* gene revealed that two out of 116 (1.7%) ultrasound-sheared samples (from laboratory 1) and five out of other 125 (4%) enzymatically-digested samples (from laboratory 3) were scored as the samples with suspected deletion or duplication. The *BRCA1* MLPA analysis performed in all samples revealed that one suspected sample from each laboratory was true positive (exon 5–14 del in laboratory 1 and exon 8 del in laboratory 3), remaining suspected samples (one from laboratory 1 and four from laboratory 3) were false positive, and 114/116 in laboratory 1 and 120/125 in laboratory 3 were true negative *BRCA1* samples.

While the minimum coverage for a reliable detection of SNVs was estimated at 20X, the minimum coverage required for a reliable detection of CNVs is higher [39]. However, we have noticed that coverage uniformity is at least of the same importance. While the type of the DNA fragmentation protocol (ultrasound vs. enzymatic digestion) did not influence the sensitivity of SNVs detection (Fig 4C), enzymatic digestion caused difficulties in reliable CNVs detection (with an increased number of CNVkit false positives) when comparing samples with the same coverage. We suppose that the main problem of a CNVs coverage-based analysis of enzymatically fragmented samples is worse coverage uniformity caused by non-random DNA cleavage, as discussed above (Fig 1C). To evaluate the sensitivity of CNVs detection in other targeted genes and to better address the influence of DNA fragmentation protocol on the CNV analysis, we compared results of CNVkit analysis in remaining 20 ACMG genes (except *BRCA1* and *TP53* discussed above) covered by CZECANCA target (Fig 6).

The analysis revealed relative low rate of suspected CNVs (0–4 and 0–23 carriers per gene in samples prepared by ultrasound DNA fragmentation and enzymatic DNA digestion, respectively) and demonstrated that preparation of sequencing libraries using ultrasound digestion substantially decreased the need for subsequent MLPA analyses. With the exception of *BRCA2* in which MLPA analysis was performed in all suspected samples, application of MLPA analysis in remaining genes were directed by the phenotype characteristics of analyzed probands. The only CNV identified in remaining ACMG genes was exon 17 deletion in the tuberin (*TSC2*) gene in a patient with typical skin affections. The CNV analysis of the entire set of CZECANCA target genes is provided in S11 Table. The data indicate that deviations of median-normalized CNVkit values in a run of consecutive bin sets could indicate highly probable presence of a large intragenic deletion or duplication (S1 Fig). The extreme case of such situation provides the analysis of genes localized on X chromosome in male and female probands (S2 Fig) that also demonstrates the dynamic range of analysis in detection of real deletion.

For the detection of medium-size insertions and tandem duplications, we added the Pindel tool to the bioinformatics pipeline in order to identify the 64 bp tandem duplication in *BRCA1* (c.5468-11_5520dup64; NM_007294; Chr17: 41197765–41197830 on Assembly GRCh37) not detected by GATK. The sensitivity of a Pindel analysis was recently confirmed by another GATK-omitted variant, the 38 bp duplication in *CHEK2* (c.845_846+36dup38; NM_007194; Chr22: 29105958–29105995 on Assembly GRCh37), confirmed by Sanger sequencing.

Five DNA reference standards (NA12878, NA24149, NA24385, NA24631 and NA24143) with well-described genotypes were analyzed by CZECANCA pipeline to benchmark the overall workflow performance [19]. Comparison between genotypes identified in CZECANCA analysis and available as reference VCFs showed a high concordance in identification of homozygotes and heterozygotes and also high sensitivity, specificity and accuracy of CZECANCA NGS analysis (Fig 7; S9 Table). Totally, 1,722 true positive variants (332–355 per sample), 252 false positive variants (42–57 per sample), and 13 false negative variants (0–5 per sample) were scored in all analyzed DNA reference standards considering 628,069 bases of CZECANCA target region. All were localized in 84 short genomic regions that comprised in majority homopolymeric or repetitive non-coding sequences creating recurrent sequencing errors in currently used sequencing platforms, as indicated by 7/13 not identified (false negative) variants flanking to position of false positive variants. The subsequent manual IGV inspection revealed that the remaining six false negative variants (all indels) were present with allelic fraction below 15% (filtered out through the bioinformatics pipeline).

Finally, an external quality assessment of CZECANCA was performed using the pilot NGS germline mutations scheme provided by the European Molecular Genetics Quality Network (EMQN; www.emqn.org). This external quality assessment showed a 100% sensitivity of variant detection (S10 Table).

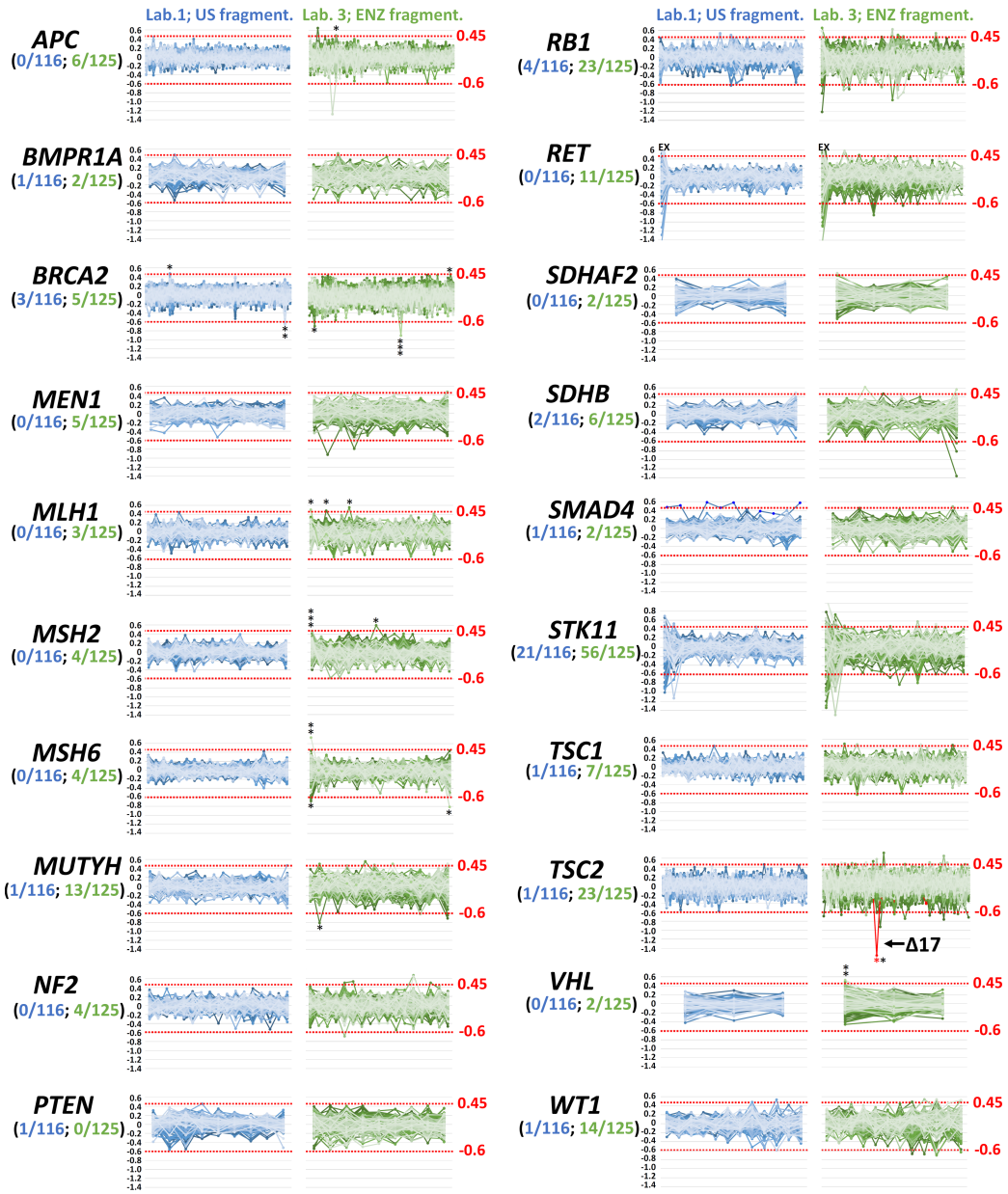

Fig 6. CNV detection is influenced by a DNA preparation method. Panels show analyses of remaining ACMG genes (not shown in Fig 5B and 5C) from four runs performed in laboratory 1 (116 DNA samples fragmented by ultrasound) and laboratory 3 (125 DNA samples fragmented enzymatically). The numbers in parentheses express number of samples with possible CNVs from all analyzed samples in contributing laboratories. *indicate samples analyzed by MLPA negatively (FP–black) or positively (TP–red). Bin set covering exon 1 in *RET* was excluded from the analysis due to the large coverage variability.

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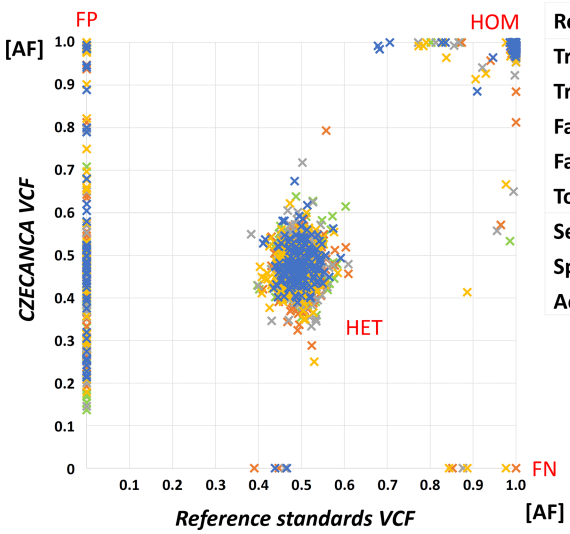
Discussion

Multi-gene panel NGS has changed the genetic landscape for hereditary cancer syndromes. At present, clinical testing prioritizes the use of smaller cancer-specific panels, usually up to 30 cancer susceptibility genes. A large number of panels is available particularly for breast/ovarian and colorectal cancers, which represent frequent diagnoses with a high contribution of genetic components influencing the disease onset, progression and treatment outcomes [40]. Analyses



Reference standard no.	NA12878	NA24143	NA24149	NA24385	NA24631
True positive (TP)	355	341	332	351	348
True negative (TN)	627,672	627,674	627,678	627,658	627,671
False positive (FP)	42	49	56	57	48
False negative (FN)	0	5	3	3	2
Total	628,069	628,069	628,069	628,069	628,069
Sensitivity [TP/(TP+FN)]	100.000%	98.555%	99.104%	99.153%	99.429%
Specificity [TN/(TN+FP)]	99.993%	99.992%	99.991%	99.991%	99.992%
Accuracy [(TP+TN)/Total]	99.993%	99.991%	99.991%	99.990%	99.992%

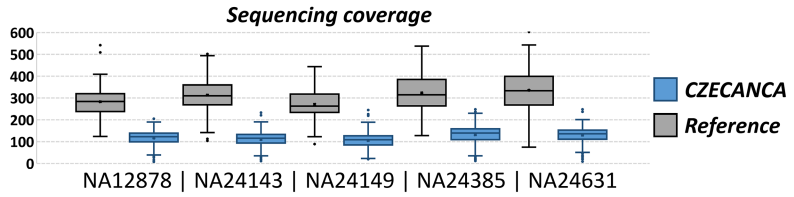


Fig 7. Comparison of variant detection (shown as values of variant allelic fraction; AF) in DNA reference standards (NA12878, NA24149, NA24385, NA24631 and NA24143) obtained from CZECANCA analysis (x-axis) and AF from VCF files for these standards downloaded from http://jimb.stanford.edu/giab/ (y-axis). The graph shows all variants with GATK quality >100 reached in CZECANCA analysis (including FP variants) and undetected (FN) variants. Heterozygote variants clustered in the center, while homozygote variants in right upper corner. Variant distribution was partially influenced by the differences in mean sequencing coverage targeting 100X and 300X in CZECANCA and DNA reference standards VCFs, respectively. The number of TP, TN, FP, FN, and total number of variant (= CZECANCA target) was used to calculate of sensitivity, specificity, and accuracy of CZECANCA analysis.

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based on smaller panels mainly simplify the clinical interpretation of the identified genotypes with a reduction of incidental findings. While their use is beneficial in clearly indicated patients with typical phenotype characteristics for a given cancer syndrome, the selection of a proper cancer-specific gene panel is not trivial in individuals with less characteristic features (e.g. patients from multi-cancer families). Moreover, our current knowledge of many cancer syndromes is based on the analyses of mostly prototypical cases, the testing criteria are changing dynamically, and the list of cancer predisposition genes with clinical utility is far less complete. Recently, Pearlman et al. analyzed 450 early-onset colorectal cancer patients and showed that a third (24/72) of mutation-positive patients did not meet the established genetic testing criteria for the gene(s) in which they had a mutation [41]. An analysis of mismatch repair (MMR) genes (traditionally linked to hereditary non-polyposis colorectal cancer) in a set of 34,981 cancer patients in a study by Espenschied et al. revealed that out of 528 patients with MMR mutations, 63 (11.9%) had breast cancer only and thus *MSH6* and *PMS2* mutation carriers may manifest with a hereditary breast and ovarian cancer phenotype [42]. In an analysis of *BRCA1* and *BRCA2* in 1,371 unselected breast cancer cohorts, Grindedal et al. showed that common guidelines identified only 45–90% of mutation carriers [43]. The ultimate solution to identify cancer risks would be an analysis of the whole exome (or even better genome) in all cancer patients; however, the implementation of such a strategy is not realistic at present [44]. We suppose that the use of larger multi-cancer panels (containing hundreds of genes) for an analysis of genetic risk in cancer patients is beneficial for several reasons. i) Such an analysis reveals a complex variation landscape of target genes in different cancers [7]. ii) It reveals carriers of concurrent pathogenic mutations and iii) it enables the testing of affected individuals from multi-cancer families with reasonable costs and turnaround time. Finally, iv) combining all genes of interest in a single panel simplifies and unifies laboratory procedures in a single workflow even if testing for different syndromes.

We have developed the custom-designed CZECANCA multi-cancer panel targeting the coding sequence of 219 cancer susceptibility or candidate genes, enabling the identification of a genetic predisposition in the most frequent hereditary cancer syndromes. Besides the established cancer susceptibility genes, we have decided to include also a subset of genes with low, clinically still unconfirmed utility, although their variants cannot be reported until their clinical evidence is known. These genes code for known interactors of established cancer susceptibility gene products, whose mutations may result in a similar phenotypic outcome. However, we suppose that knowledge obtained through the association of the identified genotypes with the phenotypic characteristics of the analyzed patients may substantially accelerate the process of clinical utility evaluation. Moreover, a subsidiary genetic report could be easily generated from the stored data in case of the approval of new cancer susceptibility genes included in CZECANCA. From the technical point of view, a larger genomic target has a favorable impact on panel complexity, improving its coverage uniformity [45].

The validation of the CZECANCA analytic workflow together with the bioinformatics pipeline is necessary for its implementation into routine diagnostics [46]. The presented analytical workflow was optimized for sequencing using MiSeq Illumina, representing the most frequently used NGS platform currently available in diagnostic laboratories. Genetic testing using gene panels is a cost-effective strategy [47]. The material costs for library preparation and sequencing (chemicals, kits, and disposables) using CZECANCA do not exceed €150 per patient in the standard settings (targeting sequencing coverage 100X). The CZECANCA workflow was intended mainly for medium throughput laboratories. As a universal panel, CZECANCA significantly reduces the turnaround time. The sequencing data for 30 analyzed DNA samples in one sequencing MiSeq run might be available in four days (three days for DNA fragmentation and library preparation, depending on hybridization time, and one day for MiSeq sequencing). We are aware that the low-covered or uncovered regions (affecting 12/219 CZECANCA-targeted genes) may require additional effort and time, when requested for genetic assessment.

The validation showed CZECANCA's high sensitivity, specificity, analytical robustness, and accuracy. We have demonstrated that SNVs and small/medium-size indels could be detected with high confidence. Moreover, we have shown that the uniform coverage (targeting to mean 100X coverage) of a target sequence enabled a robust identification of CNVs without the need of routine MLPA, serving as the method for independent CNVs confirmation or exclusion of false positivities. However, despite that the number of false positive calls was low and we detect no false negative sample in ACMG genes, we are aware that with caution needs to be interpreted positive CNV calls in genes for which MLPA assay (or other method) are not routinely available for confirmatory purposes. When required, presence of false positive signals can be reduced by the use of ultrasound fragmentation providing unbiased DNA shearing over enzymatic lysis and/or increased sequencing coverage.

Another advantage of NGS (over Sanger sequencing) is its ability to identify *cis* or *trans* positions of compound, closely localized heterozygous SNVs. For example, the position of double substitution in the *PALB2* gene creating a stop codon (c.661_662delinsTA; p.Val221*; NM_024675), which required further analyses (e.g. PTT) before the NGS era [10], can be identified directly from sequencing reads (Fig 8). The identification of additional pathogenic mutations during the validation procedure in negatively pre-tested samples indicated that a re-analysis is warranted for at least high-risk patients negatively tested by historical analyses based on indirect prescreening methods (e.g. PTT) or cDNA sequencing [48].

CZECANCA (CZEch CAncer paNel for Clinical Application) is intended to unify cancer predisposition testing in the Czech Republic, helping diagnostics laboratories transform the gene-by-gene strategy to NGS, even if is not a population-specific panel *per se*. NGS-based

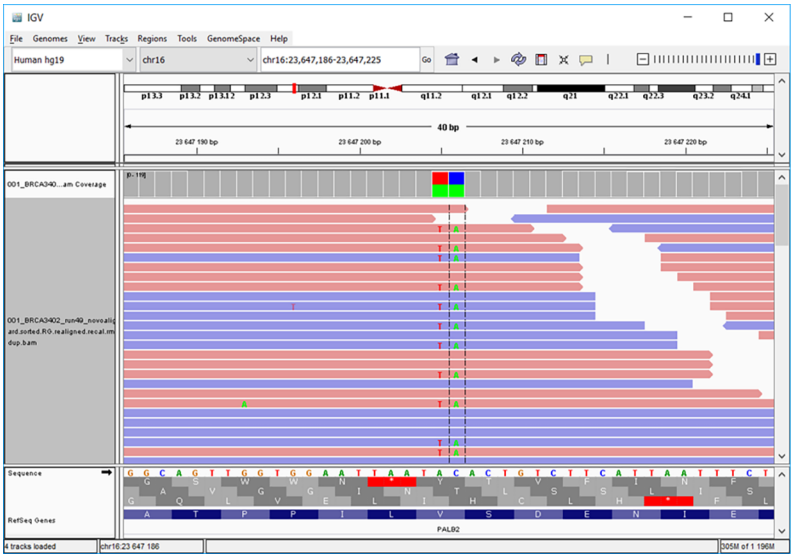

Fig 8. Identification of c.661_662delinsTA double substitution (p.Val221*) in *PALB2* (NM_024675). The BAM file displayed in IGV shows the *cis*-position of both substitutions in approximately 50% of forward (pink bars) and reverse (blue bars) reads, respectively.

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technologies bring new challenges including technological aspects, bioinformatics processing, the management of large datasets, and clinical interpretation of results [46]. The use of a uniform analytical and bioinformatics approach improves the identification of technical and platform-specific sequencing errors, as we demonstrated in inter-run and intra-run comparisons. Moreover, validation of the panel using reference standard DNA samples with known genotypes enabled identification of genomic loci (dominantly homopolymeric regions) providing these recurrent sequencing errors, which could be subsequently easily eliminated by bioinformatics. The use of CZECANCA will help generate a global view of constitutional variants from the perspective of known cancer predisposition and candidate genes in the population. Simultaneously with the sequencing of cancer patients, we aim to sequence non-cancer controls in order to identify and establish the frequency of population-specific neutral variants. The introduction of patients' and control genotypes with associated phenotypes into a nationwide database currently being created will simplify the interpretation of variants, which remains the main challenge at present. In general, NGS-based analyses result in an increased number of incidental findings or variants of unknown significance. The patient must be informed about this possibility before the testing and must have the opt in / opt out possibility clearly formulated in the informed consent. Consensus on what incidental information should be disclosed has yet to be reached. Currently, there is general agreement on reporting mutations in known high-penetrant genes in patients with a typical personal and family cancer history [38]. However, there is no agreement on pathogenic mutations in genes with lower penetrance or on mutations related to autosomal-recessive syndromes. These questions are currently being tackled in cooperating centers on a rather individual basis, depending on the formulation of the informed consents obtained, and on the clinical experience of the indicating geneticists [49].

In conclusion, CZECANCA allows comprehensive testing for a majority of frequent hereditary cancer syndromes while mitigating potential difficulties of incidental findings in non-cancer genes as seen in exome or genome sequencing. The reliability of the procedure enables an unbiased identification of variants present in patients, which together with a correct interpretation of variants is key for the effective management of hereditary cancer patients and their relatives.

Supporting information

S1 Table. List of 219 CZECANCA targeted genes with basic characteristics of their protein products. The primary gene target for the probe coverage was represented by coding sequences (cds) representing all exons (in case of known cancer susceptibility genes) or all coding exons (in other genes), including 10 bases from adjacent intronic regions. The promoter regions of the *BRCA1* and *BRCA2* genes were included into the primary target. Because of the strict design conditions, some clinically important regions were left untargeted (highlighted) for technical reasons such as repeats and homologous regions. (The characteristics of protein products were obtained from string.embl.de and/or genecards.org).
(XLSX)

S2 Table. Regions of interest with low coverage ≤20X. The average coverage is the mean from 10 randomly selected samples.
(XLSX)

S3 Table. Comparison of identified variants in the targeted exonic regions and 12 bp from adjacent introns with GATK quality >100 in three intra-run replicates of sample #2268. The DNA sample pooled for the enrichment in amounts corresponding to 33 ng (e.g. 1/30; considered as 100%), 75% and 50% of this amount, respectively. (Cov = coverage; Q = quality; discordant variants are highlighted).
(XLSX)

S4 Table. Comparison of identified variants in the targeted exonic regions and 12 bp from adjacent introns with GATK quality >100 in two independent run replicates of sample #3647. All values of coverages (Cov) of sample #3647 in run 14 were corrected by a factor of 1.3880 to normalize coverages between samples for presentation in Fig 4B. (Q = quality; discordant variants are highlighted).
(XLSX)

S5 Table. Comparison of identified variants in the targeted exonic regions and 12 bp from adjacent introns with GATK quality >100 in sample #3582 analyzed independently in four participating laboratories(Lab). All values of coverages (Cov) in Lab2, Lab3, and Lab4 were corrected to the coverage of Lab1 by a factor shown in line 336 to normalize coverages between samples for Fig 4C. (discordant variants are highlighted).
(XLSX)

S6 Table. List of variants used for the validation of SNVs detection.
(XLSX)

S7 Table. List of CNVs used for the validation of a large genomic rearrangements analysis.
(XLSX)

S8 Table. CNV scores (from CNVkit software) of bins in *BRCA1*, *PALB2*, *CHEK2*, and *TP53*. The numbers of samples with previously characterized CNVs are highlighted in red. The table show raw values obtained from CNVkit as well as median-normalized values. The normalized values >0.5 (highlighted in green) were indicative for the presence of a duplication, while values <-0.6 (highlighted in yellow) were indicative for a deletion. Data from this table were used for creation of Fig 5.
(XLSX)

S9 Table. Variants identified in five Coriell Institute reference samples sequenced using CZECANCA pipeline and their comparison with VCF files obtained from GIAB website.

The considered targeted region encompasses 628,069 bases of CZECANCA target region. False negative variants are highlighted.
(XLSX)

S10 Table. Variant consensus analysis report from EMQN (NGS pilot 2016) for CZECANCA sequencing of a reference sample.
(XLSX)

S11 Table. Results of CNV analysis performed in two validation sets consisting of four runs from Laboratory 1 (116 samples prepared using the ultrasound DNA fragmentation on Covaris) and four runs from Laboratory 3 (125 other samples prepared using the enzymatic DNA cleavage by Fragmentase). To estimate number of false positive (FP) and false negative (FN) samples, data for CNV analysis of Coriell Institute reference samples (Coriell; 10 samples analyzed in Laboratory 1 and prepared using the ultrasound DNA fragmentation on Covaris) were added. The values in cells represent differences of CNV scores for a given cell (i.e. sample in the coordinate) from the median value of signals from particular sample group (i.e. Coriell—columns Q-Z, Laboratory 1—columns AB-EM, Laboratory 3—columns EO-JI) in a given CNVkit_bin_set_coordinate (column A). Values in cells showing individual analyzed samples from particular sample group exceeding the given CNVkit threshold value for deletion (<-0,6) and duplication (>0,45) are highlighted as red and green cells, respectively. The columns C-O provide several aggregated metrics, that include number of individual samples in which deletion (columns G-I), duplication (J-L), or deletion+duplication (M-O) was found in a given coordinate in particular sample group. Columns C-E enable identification of non-informative bin sets with suspected false positive (FP) signals (indicated by the value = 1) that include regions on X chromosome called in male samples as deletions (highlighted in blue in column B), regions with insufficient coverage or containing pseudogenes (highlighted in orange and yellow, respectively; in column B), or bin sets containing the improbable number of deletions+duplications exceeding the 4% of analyzed samples in a particular sample group.
(XLSX)

S1 Fig. Run of consecutive bin set coordinates with values indicating a deletion (< -0.6; red) or a duplication (> 0.45; green) increases the probability of a real rearrangement. The *BRCA1* and *BRIP1* deletions were confirmed by MLPA analyses, which are currently no available for confirmation of secondary findings in *MSR1* or *ZNF350*. (The graphs expressed normalized CNVkit values shown in S11 Table).
(TIF)

S2 Fig. CNV analysis of genes *BRCC3*, *FANCB*, *GPC3*, and *UBE2A* localized on X chromosome enabled to demonstrate differences in normalized CNVkit values in samples carrying a real 'deletion' in samples prepared by ultrasound DNA fragmentation or enzymatic DNA lysis. The XX and X indicates areas of samples obtained from female and male probands, respectively. (The graphs expressed normalized CNVkit values shown in S11 Table). Upper panel shows normalized CNVkit values in 116 samples analyzed in four runs in laboratory 1. Lower panel shows normalized CNVkit values in 125 other samples analyzed in four runs in laboratory 3.
(TIF)

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RESEARCH ARTICLE

WILEY HGVS HUMAN GENOME VARIATION SOCIETY

BRCA1 and *BRCA2* 5′ noncoding region variants identified in breast cancer patients alter promoter activity and protein binding

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Abstract

The widespread use of next generation sequencing for clinical testing is detecting an escalating number of variants in noncoding regions of the genome. The clinical significance of the majority of these variants is currently unknown, which presents a significant clinical challenge. We have screened over 6,000 early-onset and/or familial breast cancer (BC) cases collected by the ENIGMA consortium for sequence variants in the 5′ noncoding regions of BC susceptibility genes *BRCA1* and *BRCA2*, and identified 141 rare variants with global minor allele frequency < 0.01, 76 of which have not been reported previously. Bioinformatic analysis identified a set of 21 variants most likely to impact transcriptional regulation, and luciferase reporter assays detected altered promoter activity for four of these variants. Electrophoretic mobility shift assays demonstrated that three of these altered the binding of proteins to the respective *BRCA1* or *BRCA2* promoter regions, including NFYA binding to *BRCA1*:c.-287C>T and PAX5 binding to *BRCA2*:c.-296C>T. Clinical classification of variants affecting promoter activity, using existing prediction models, found no evidence to suggest that these variants confer a high risk of disease. Further studies are required to determine if such variation may be associated with a moderate or low risk of BC.

KEYWORDS
breast cancer, BRCA1, BRCA2, promoter, transcription, variants of unknown clinical significance (VUS)

1 | INTRODUCTION

Genetic susceptibility to breast cancer (BC) is complex. Multiple germline variants have been identified over the past 25 years that are broadly categorized as high, moderate, and low risk. High-risk variants are generally rare, have a major deleterious effect on gene function, are sufficient to confer a high risk of disease, and are highly penetrant within a family. Nonsense, splicing, large deletions, and some missense changes in *BRCA1* and *BRCA2* fall into this category (reviewed in Walsh et al., 2006). There is also evidence that some alleles confer a moderate risk of cancer. These can include hypomorphic variants in known "high-risk" cancer syndrome genes (Shimelis et al., 2017; Spurdle et al., 2012), or clear loss-of-function alleles in other genes such as *CHEK2*, *PALB2*, and *ATM* (Couch et al., 2017). Low-risk variants, largely identified by genome-wide association studies, are usually common and cause subtle functional effects, such as small but significant changes in gene expression due to altered activity of proximal and distal regulatory elements (reviewed in Bogdanova, Helbig, & Dork, 2013; Ghoussaini, Pharoah, & Easton, 2013; Skol, Sasaki, & Onel, 2016). Evidence suggests that combinations of low, moderate, and high-risk variants could confer a clinically significant risk of disease (Ding et al., 2012; Kuchenbaecker et al., 2017; Sawyer et al., 2012). Identification and evaluation of all such variants is therefore crucial for accurately predicting BC risk.

Use of next generation sequence analysis for germline clinical testing of cancer cases is identifying an increasing number of variants in noncoding regions of cancer susceptibility genes, including promoters, untranslated regions (UTRs), and introns. There are currently no firm recommendations for assessing the relevance of noncoding region variants to clinical testing of Mendelian disease genes, and so the vast majority of such variants are deemed of uncertain clinical significance. This adds to the clinical challenge presented by variants of uncertain significance, namely that they complicate test reporting and genetic counseling, limit patient eligibility for intensive surveillance and gene-targeted therapies, and prevent gene testing and guided management of relatives (reviewed in Amendola et al., 2015; Eccles et al., 2013; Plon et al., 2011). It is therefore essential that the functional and clinical significance of variants mapping to noncoding regions of the genome is determined.

Gene expression is controlled at many levels with key regulatory elements being housed in noncoding regions of the genome, such as gene promoters, introns, long-range elements, and 5′ and 3′ UTRs. The *BRCA1* gene is regulated at the transcriptional and posttranscriptional levels, with functional proximal and distal regulatory elements being described in the promoter, introns, and UTRs, by us and others (Brewster et al., 2012; Brown et al., 2002; Santana dos Santos et al., 2017; Saunus et al., 2008; Tan-Wong, French, Proudfoot, & Brown, 2008; Wardrop, Brown, & kConFab, 2005; Wiedemeyer, Beach, & Karlan, 2014). Although less studied, the *BRCA2* promoter has also been mapped and characterized (reviewed in Wiedemeyer et al., 2014).

Common and rare variations in regulatory elements upstream of genes have been shown to alter gene expression and be associated with disease risk (reviewed in Betts, French, Brown, & Edwards, 2013; Diederichs et al., 2016; Millot et al., 2012). We and others have described germline cancer-associated variants in the regulatory regions, including large deletions in the *BRCA1* promoter (Brown et al., 2002), and single nucleotide variants in the promoter and/or

5′ UTR of *BRCA1* and *BRCA2* (Evans et al., 2018; Santana dos Santos et al., 2017), *MLH1* promoter (Hitchins et al., 2011), *POLG* promoter (Popanda et al., 2013), *PTEN* promoter (Heikkinen et al., 2011), *TERT* promoter (Horn et al., 2013), *KLHDC7A* and *PIDD1* promoters (Michailidou et al., 2017), *BRCA1* 3′ UTR (Brewster et al., 2012), and BC-associated Single Nucleotide Polymorphisms (SNPs) in long-range enhancers of *CCND1* (French et al., 2013).

Cancer risk-associated variants within regulatory regions are anticipated to mediate an effect on trans-acting regulatory factors (e.g., transcription factors [TFs] and miRNAs), by disrupting binding of regulatory factors and interactions between regulatory elements, such as promoter–enhancer interactions. For example, a variant in a *Cyclin D1* transcriptional enhancer has been associated with altered binding of the ELK4 TF (French et al., 2013) and a variant within the *BRCA1* 3′UTR has been shown to introduce a functional mir-103 binding site (Brewster et al., 2012). In addition, a dominantly inherited 5′ UTR *BRCA1* variant was recently shown to be associated with *BRCA1* promoter hypermethylation, which is known to impact TF binding, and associated allelic loss of *BRCA1* expression in two families affected by breast and ovarian cancers (Evans et al., 2018).

In this paper, we describe 141 germline variants in the *BRCA1* and *BRCA2* promoter, identified by members of the ENIGMA consortium in early onset or familial BC patients with no known pathogenic variants in the coding region of these genes. Using a combination of bioinformatic and experimental analyses, we have prioritized and analyzed a subset of variants that are most likely to affect the regulation of *BRCA1* and *BRCA2* and thus have the most potential to contribute to BC risk. TF binding site affinity changes resulting from these variants were subsequently analyzed by information theory (IT)-based analyses. In parallel, we have assessed if these variants exhibited the features expected for a high-risk pathogenic *BRCA1* or *BRCA2* variant, on the basis of available clinical and population data.

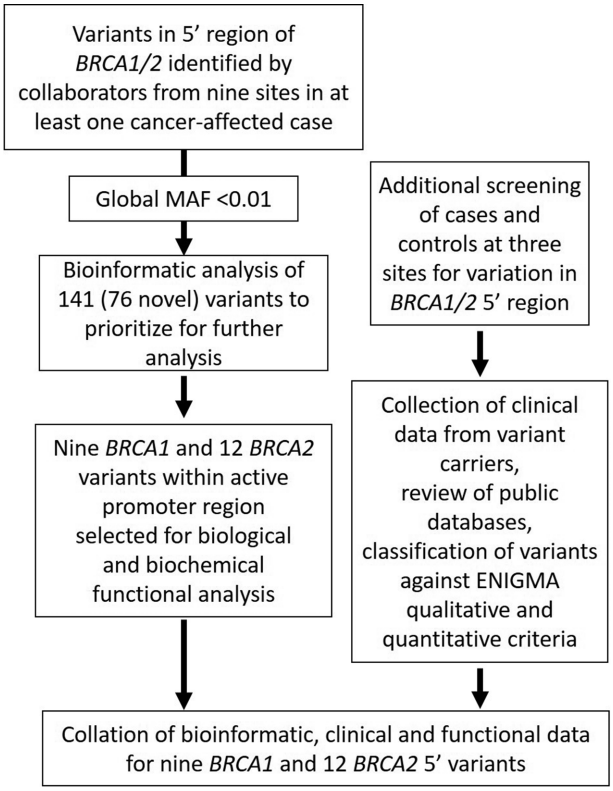

FIGURE 1 Overview of study design. Outline of the workflow of variant collection, prioritization and analysis

2 | MATERIALS AND METHODS

2.1 | Study design

An overview of the study design is shown in Figure 1. Collection of variants at all sites enabled an initial catalogue of variants from which variants were prioritized for functional analysis. Additional screening was carried out at three sites, Maastricht (M), Santiago (S), and Prague (Pr), that included additional patients (M, S, and Pr) and controls (Pr) that expanded the list of variants (Pr), the number of patients (M, S, and Pr), and included control subjects (Pr).

2.2 | Clinical and control samples

Clinical and genetic data were collected and analyzed in accordance with local human ethics guidelines of the institutions contributing to this study. All participating individuals provided informed consent for their data to be used for research purposes. An overview of the samples analyzed is shown in Table 1. Clinical samples were collected from nine European sites and were originally selected for *BRCA1* and *BRCA2* testing using ascertainment criteria that included family history and young age of BC diagnosis. Female patients who did not carry a pathogenic variant in *BRCA1* or *BRCA2* coding regions or splice junctions were selected for testing of variation in the *BRCA1* and *BRCA2* 5′ regions. The controls were as follows: 661 healthy female individuals recruited through the Immunohematology and Transfusion Medicine Service of INT and Associazione Volontari Italiani Sangue (AVIS) of Milan; 312 healthy females above 60 years of age and with no malignancy in the first filial generation recruited through First Faculty of Medicine, Charles University in Prague (Lhota et al., 2016; Soukupova, Zemankova, Kleiblova, Janatova, & Kleibl, 2016); and 130 healthy females without cancer diagnosis recruited in Santiago de Compostela.

2.3 | Identification of variants

Regions containing the *BRCA1* and *BRCA2* promoter and 5′ UTR were sequenced using a range of standard DNA sequencing technologies, and bioinformatic filtering pipelines. Variants mapping to the 2,400 bp region (hg19; chr17:41,278,514 – 41,276,114) of *BRCA1* and the 2,000 bp region (hg19; chr13: 32,888,597-32,890,597) of *BRCA2* were considered for further analysis. The identified variants in *BRCA1* and *BRCA2* 5′ noncoding regions are numbered whereby the first translated nucleotide of the translation initiation codon is +1 (https://varnomen.hgvs.org/) using the Mutalyzer website (https://mutalyzer.nl/). *BRCA1* is described using NC_000017.10 (hg19 genomic sequence) and NM_007294.3 (transcript). *BRCA2* is described using NC_000013.10 (hg19 genomic sequence) and NM_000059.3 (transcript).

TABLE 1 Samples used in this study

Location	Institution	Samples	Gene region
Paris	Institut Curie, Saint Cloud	686 cases	*BRCA1* 5′region, *BRCA2* 5′region
Milan	IFOM, Fondazione Instituto FIRC di Oncologia Molecolare	772 cases 661 controls	*BRCA1* 5′region
Pisa	Department of Translational Research and New Technologies in Medicine, University of Pisa	80 cases	*BRCA1* 5′region, *BRCA2* 5′region
Santiago de Compostela	Fundación Pública Galega de Medicina Xenómica-SERGAS, Grupo de Medicina Xenómica-USC, CIBERER, IDIS	270 cases 130 controls	*BRCA1* 5′region, *BRCA2* 5′region
Copenhagen	Center for Genomic Medicine	1157 cases	*BRCA1* 5′region, *BRCA2* 5′region
Ghent	Center for Medical Genetics, Ghent University Hospital	357 cases	*BRCA1* 5′region, *BRCA2* 5′region
Barcelona	Vall d'Hebron Institute of Oncology	192 cases	*BRCA1* 5′region, *BRCA2* 5′region
Prague	CZECANCA – CZEch CAncer panel for Clinical Aplication, Institute of Biochemistry and Experimental Oncology	2961 cases 312 controls	*BRCA1* 5′region, *BRCA2* 5′region
Maastricht	Department of Clinical Genetics, Maastricht University Medical Centre	900 cases	*BRCA2* 5′region

2.4 | Bioinformatic analysis of variants

As an initial screen, each variant submitted for study was assessed for population frequency using intersection of the variants with dbSNP (version 138 or 150, as the study progressed) within the UCSC Genome browser and Variant Effect Predictor at ENSEMBL (https://www.ensembl.org/info/docs/tools/vep/index.html). Variants with a global minor allele frequency (MAF) of < 0.01 were included in subsequent bioinformatic analyses. Further details of bioinformatics analyses to map active regulatory elements and prioritize variants for functional assays are contained in Supporting Information Methods. Variants were considered to be high priority for experimental analysis if they contained all of the following features: (1) resided in DNaseI or formaldehyde-assisted isolation of regulatory elements (FAIRE) peaks, (2) coincided with high scores for DNaseI (Base Overlap Signal > 40) or FAIRE (Base Overlap Signal > 10) in a breast cell line, (3) resided in a region of breast cell specific TF binding, (4) overlapped with a TF consensus motif, and (5) were within an evolutionarily conserved element with a high Phastcons score (>0.75). Medium priority variants lacked one or two of these features, whereas low priority variants had only one or none of these features.

2.4.1 | *In silico* TF binding analysis

All rare variants were analyzed *in silico* using an IT-based method (Caminsky et al., 2016; Mucaki et al., 2016) and a modified version of the Shannon pipeline utilizing TF information models built from ENCODE ChIP-seq datasets (Lu, Mucaki, & Rogan, 2017) to assess potential effects of variants on TF binding. Details of analyses are contained in Supporting Information Methods.

2.5 | Experimental analysis of variants

2.5.1 | Promoter reporter assays

The 499 bp *BRCA1* (chr17:41,277,787-41,277,289) and 750 bp *BRCA2* (chr13:32,889,230-32,889,979) promoter regions were cloned into pCR-Blunt vector (Thermo Fisher, Waltham, MA). Site-directed mutagenesis was used to introduce variants using the primers listed in Supporting Information Table S1. Plasmids were purified using the QIAprep miniprep kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Plasmid preparations were validated using restriction digest and DNA sequencing and inserts were shuttled into pGL3-Basic luciferase reporter vector (Promega, Madison, WI). All plasmids for transfection were analyzed for DNA conformation on a 1% w/v agarose gel and only plasmids possessing a supercoiled conformation were used for transfections. Transfection details are described in Supporting Information Methods.

The luciferase-based reporter assay was performed as described previously (Brewster et al., 2012). Positive controls were B1-Ets, *BRCA1*:c.-330_-329delinsTT, that decreases *BRCA1* promoter activity in MCF7 cells (Atlas, Stramwasser, Whiskin, & Mueller, 2000) and B2-Ets (E2Fmut1: *BRCA2*:c.-282_-281delinsAA), that has been shown to decrease *BRCA2* promoter activity in MCF7 cells (Davis, Miron, Andersen, Iglehart, & Marks, 1999). Statistical analyses were performed in GraphPad Prism using one-way analysis of variance followed by Tukey's post hoc test and values $P < 0.05$ were deemed statistically significant.

2.5.2 | Electrophoretic mobility shift assays

Nuclear proteins were extracted as described in Supporting Information Methods and electrophoretic mobility shift assays (EMSAs) were carried out using a Pierce LightShift Chemiluminescent EMSA Kit (Thermo Fisher, Waltham, MA) with modifications described in Supporting Information Methods. For competition and supershift studies, nuclear extracts were initially incubated with unlabeled double-stranded (ds) competitor probes or antibodies in binding buffer before addition of the biotinylated probe and incubation at room temperature. Positive controls for *BRCA1* and *BRCA2* DNA binding were sequences surrounding the B1-Ets and B2-Ets mutations described above.

2.6 | Qualitative and quantitative classification of variants

Variants were classified according to the ENIGMA classification criteria for variation in *BRCA1* and *BRCA2* (https://enigmaconsortium.org/) to determine whether any of the prioritized variants were associated with a high risk of disease. See Supporting Information Methods for further details.

3 | RESULTS

3.1 | Identification and prioritization of sequence variants in *BRCA1* and *BRCA2* 5′ noncoding regions

The 5′ noncoding regions of *BRCA1* and *BRCA2* in early onset or familial BC patients with no known *BRCA1* or *BRCA2* germline pathogenic variant were sequenced at nine different sites as part of an approved ENIGMA (https://enigmaconsortium.org/) project. For the *BRCA1* 5′ region, 6,475 patients were sequenced at eight different sites along with 1,103 controls. For the *BRCA2* 5′ region, 6,603 patients were sequenced at eight different sites as well as 442 controls.

After excluding variants with global MAF > 0.01 at time of variant identification, a total of 141 unique single nucleotide variants and short insertions/deletions were identified, 81 in *BRCA1* and 60 in *BRCA2* (Supporting Information Tables S2 and S3). Theses variants have been submitted to the LOVD databases, www.lovd.nl/BRCA1 and www.lovd.nl/BRCA2. To evaluate the potential of these rare variants to impact gene regulation, we initially undertook a comprehensive bioinformatic analysis. Promoter regions of *BRCA1* and *BRCA2* were defined by bioinformatic predictors including chromatin marks (Figure 2). These regions show the characteristic histone H3 epigenetic marks, including H3K4me3, H3K27ac, and H3K9ac, as well as occupancy by multiple TFs. Of the variants identified in cases only, 22 *BRCA1* and 23 *BRCA2* variants resided within the minimal promoter regions.

To predict the potential impact of variants on promoter activity, we prioritized variants using breast cell specific data for chromatin accessibility and TF occupancy along with evolutionary conservation. Due to the limited breast cell specific TF ChIP-seq data, we also included ENCODE TF ChIP-seq and TF consensus motif data from all cell lines. A total of nine *BRCA1* and 12 *BRCA2* variants were selected for further functional analysis (Figure 2; Tables 2 and 3).

3.2 | *BRCA1* and *BRCA2* promoter activity is altered by 5′ noncoding sequence variants

To examine the potential effect of the 21 prioritized *BRCA1* and *BRCA2* 5′ noncoding variants on regulatory activity, promoter activity was measured using luciferase assays in MCF7 and MDA-MB-468 BC cell lines. Two of the nine prioritized *BRCA1* variants decreased *BRCA1* promoter activity relative to the wild-type (WT) construct (Figure 3a and 3b). *BRCA1*:c.-315del significantly decreased the *BRCA1* promoter luciferase activity in both cell lines, whereas *BRCA1*:c.-192C decreased luciferase activity in the MCF7 cell line. Furthermore, one variant, *BRCA1*:c.-287T, displayed increased activity relative to the WT construct in the MCF7 cell line. For *BRCA2*, one of the 12 variants, *BRCA2*:c.-296T, decreased *BRCA2* promoter activity relative to the WT construct in the MCF7 cell line (Figure 3c and 3d).

3.3 | *In silico* analyses of *BRCA1* and *BRCA2* 5′ variants predict alterations in TF binding

BRCA1 and *BRCA2* promoters are regulated by a complex array of DNA-binding proteins and transcriptional coactivators and corepressors (reviewed in McCoy, Mueller, & Roskelley, 2003; Mueller & Roskelley, 2003; Wiedemeyer et al., 2014). In silico analysis was carried out to examine whether the *BRCA1* and *BRCA2* promoter variants shown to alter luciferase activity (see above) are likely to affect binding of trans-acting protein factors in breast cells.

Interrogation of ENCODE ChIP-seq datasets derived from breast cell lines show that, although the number of datasets is limited, TFs bind to regions encompassing the prioritized variants (Figure 2 and Supporting Information Figure S1). ENCODE ChIP-seq data from other cell lines indicate that some variants are located within consensus motifs for specific TFs associated with these regions (Tables 2 and 3; Supporting Information Figure S1). *BRCA1*:c.-287C>T overlaps with the consensus binding motif for CCAAT Box binding factors and *BRCA2*:c.-296C>T is located within the consensus motif for PAX5.

IT analysis of the prioritized variants showed that the binding strengths of several TFs are predicted to be altered by the *BRCA1* and *BRCA2* variants (Table 4 and Supporting Information Table S4). All of the variants that altered promoter activity were predicted to have consequences on TF binding. *BRCA1*:c.-287C>T and *BRCA2*:c.-296C>T are predicted to disrupt binding of CCAAT Box binding factors and PAX5, respectively. *BRCA1*:c.-315del is predicted to disrupt the binding of TCF7L2 but creates a POU2F2 (also known as Oct-2) binding site. *BRCA1*:c.-192T>C is predicted to strengthen a RFX5 site and creates an ETS1 site.

3.4 | 5′ variants in *BRCA1* and *BRCA2* alter protein–DNA interactions in EMSA analyses

To examine potential alterations in the binding of nuclear proteins from breast cells by the *BRCA1* and *BRCA2* promoter variants that altered luciferase activity, we carried out EMSA analysis. For *BRCA1*, two of three analyzed variants, c.-315del and c.-287C>T, displayed allele-specific protein binding (Figure 4). For probes containing the region surrounding the *BRCA1*:c.-315del variant, changing the WT sequence to the variant sequence resulted in the enhanced binding of a slower migrating band (Figure 4a and 4b). For probes containing the region surrounding the *BRCA1*:c.-287C>T variant, introduction of the variant sequence resulted in almost complete loss of protein binding to the probe (Figure 4a).

To determine if the DNA-protein interactions were specific, competition experiments were performed. In the case of *BRCA1*:c.-315del, all bands were competed by both the WT and the variant containing probes in two cell lines (Figure 5a and 5b). For *BRCA1*:c.-287C>T, only the WT probe was able to compete for binding (Figure 5c). The nonspecific probe from an unrelated region of the *BRCA1* promoter

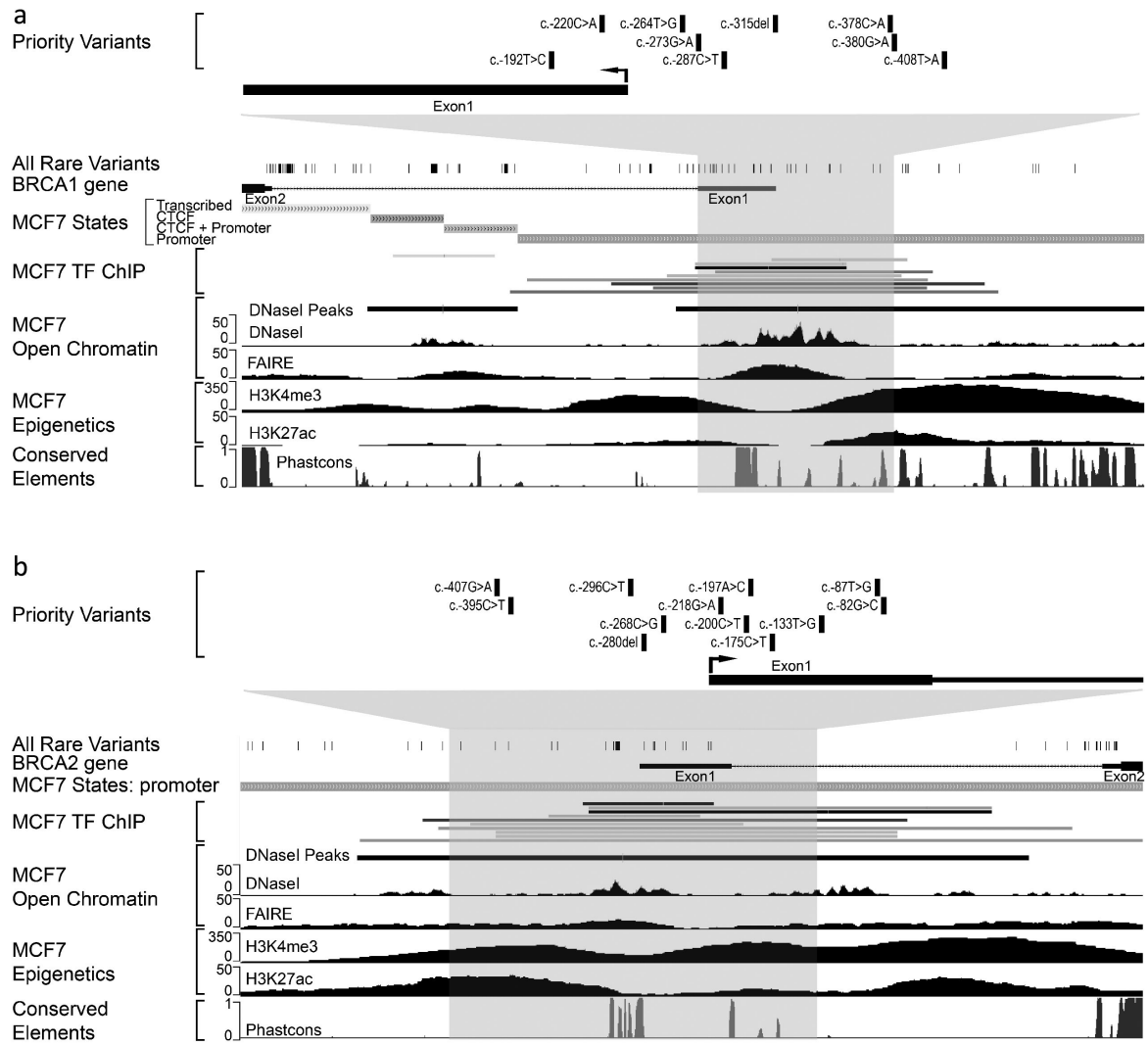

FIGURE 2 Variants identified in the 5′ regions of *BRCA1* and *BRCA2* map to predicted regulatory elements. Snapshots of the UCSC genome browser showing regions of *BRCA1* (a) and *BRCA2* (b) analyzed by targeted sequencing with available ENCODE regulatory marks derived from MCF7 cells. Chromatin segregation states from regulatory region annotation are shown (MCF7 states). The *BRCA1* and *BRCA2* genomic regions used for functional analyses are highlighted in grey. Prioritized variants within these regions are indicated

TABLE 2 *BRCA1* prioritized variants

Gene	hg19 position (chr17)	Variant name[a]	rsID	Global MAF in dbSNP	TF motif (ENCODE)[b]	Bioinformatic priority
BRCA1	g.41277676A>T	c.-408T>A	Novel		CEBPB	High/medium
BRCA1	g.41277648C>T	c.-380G>A	Novel		RXRA	High/medium
BRCA1	g.41277646G>T	c.-378C>A	rs186775935	0.00040	RXRA	High/medium
BRCA1	g.41277583del	c.-315del	rs901029407	0.00003	ATF1,2,3, CREB1[c]	Medium
BRCA1	g.41277555G>A	c.-287C>T	Novel		NFYA, NFYB	High/medium
BRCA1	g.41277541C>T	c.-273G>A	rs112960339	0.00499		Medium
BRCA1	g.41277532A>C	c.-264T>G	rs904148166	0.00003		Medium
BRCA1	g.41277488G>T	c.-220C>A	Novel			Medium
BRCA1	g.41277460A>G	c.-192T>C	rs113323025	0.00519		Medium

TF, transcription factors.
[a]Based on NM_007294.3.
[b]Overlap with TF motif in ENCODE TF-ChIP datasets from all cells.
[c]Variant overlaps this motif, but the deletion does not alter the motif sequence.

TABLE 3 *BRCA2* prioritized variants

Gene	hg19 Position (Chr13)	Variant name[a]	rsID	Global MAF in dbSNP	TF motif (ENCODE)[b]	Bioinformatic priority
BRCA2	g.32889437G>A	c.-407G>A	rs36221751	0.0018		Medium
BRCA2	g.32889449C>T	c.-395C>T	Novel			Medium
BRCA2	g.32889548C>T	c.-296C>T	rs563971900	0.0004	PAX5	High/medium
BRCA2	g.32889564delG	c.-280del	Novel		ELF1, GABPA, ELK1,4	High
BRCA2	g.32889576C>G	c.-268C>G	Novel			High/medium
BRCA2	g.32889626G>A	c.-218G>A	Novel			Medium
BRCA2	g.32889644C>T	c.-200C>T	Novel		MAZ	Medium
BRCA2	g.32889647A>C	c.-197A>C	rs370721506	NA	MAZ	Medium
BRCA2	g.32889669C>T	c.-175C>T	rs55880202	0.0058		Medium
BRCA2	g.32889711T>G	c.-133T>G	Novel			Medium
BRCA2	g.32889757T>G	c.-87T>G	Novel			Medium/low
BRCA2	g.32889762G>C	c.-82G>C	Novel			Medium/low

NA, no data available, TF, transcription factors.
[a]Based on NM_000059.3.
[b]Overlap with TF motif in ENCODE TF-ChIP datasets from all cells.

did not compete any bands showing that the bands seen in the EMSA were specific.

Analysis of the regions of the *BRCA2* promoter using EMSA revealed that region containing the *BRCA2*:c.-296C>T variant bound nuclear proteins from MCF7 nuclear extracts and that this interaction was dramatically reduced by introduction of the variant sequence (Figure 6a). Competition experiments showed that these interactions were specific and not competed by a nonspecific probe from an unrelated region of the *BRCA1* promoter (Figure 6a).

To determine the effect of these variants on the binding of specific TFs, competition and supershift analyses were performed. *BRCA1*:c.-287C>T overlaps with the consensus binding motif for CCAAT Box binding factors, NFYA and NFYB (Table 2 and Supporting Information Figure S1a), and IT analysis predicts that the variant disrupts binding of these TFs (Table 4). Consistent with these predictions, supershift experiments show that *BRCA1*:c.-287C>T disrupts binding of NFYA to this region (Figure. 5d). In addition, we analyzed *BRCA2*:c.-296C>T, which maps within the consensus binding motif for PAX5 (Table 2 and Supporting Information Figure S1b), and is predicted by IT analysis to disrupt binding of PAX5 (Table 4), by cross-competition experiments using known PAX5 binding sites from *hCD19* (Kozmik, Wang, Dorfler, Adams, & Busslinger, 1992) and *hDAO* (Tran et al., 2015) genes. These experiments show that known PAX5 binding sites compete efficiently for binding of nuclear proteins to the *BRCA2* promoter region, indicating that PAX5 binding is reduced as a consequence of the nucleotide sequence change (Figure. 6b). In contrast, supershift experiments for POU2F2 (Oct-2) showed no evidence for *BRCA1*:c.-315del causing a change in binding of POU2F2 in the cell line used (data not shown).

3.5 | Clinical classification of *BRCA1* and *BRCA2* 5′ noncoding sequence variants

Variants were classified according to the ENIGMA guidelines, which are calibrated for classification of variants as high risk, using available population frequency and/or clinical data (Supporting Information Tables S5 and S6). In this context, the term pathogenicity refers to a variant that confers a high risk of disease. Importantly, these classification guidelines do not identify those variants that confer a moderate or low risk of disease.

Of those variants identified in cases only, 26/70 (37%) of BRCA1 variants had been reported in dbSNP at study initiation (maximum global frequency = 0.006; Supporting Information Table S2), and 22/54 (41%) of *BRCA2* variants observed in cases only were identified in dbSNP (maximum global frequency = 0.006; Supporting Information Table S3). Review of variant frequency in public reference groups identified 21 variants that were classifiable, as Not Pathogenic, based on frequency in control groups (Supporting Information Table S5): six *BRCA1* and five *BRCA2* variants were observed at >1% frequency in population subgroups (stand-alone evidence against pathogenicity, when detected in a nonfounder outbred population group); six *BRCA1* and four *BRCA2* variants occurred at frequency 0.001–0.01 (range 0.0014–0.0076) in at least five individuals in the reference set, which combined with a low assumed prior is considered sufficient as evidence against pathogenicity (Supporting Information Table S5). Frequency data from controls screened for this study also supported the frequency-based classifications for eight of these 21 variants (Supporting Information Table S5).

Segregation analysis for seven informative families aided classification for six variants, whereas histopathology likelihood ratios (LRs) derived for 24 tumors altered classification for 10 variants (Supporting Information Table S6). Combining findings from qualitative and quantitative methods, most variants (113/141; 80%) remained Class 3 Uncertain, largely due to a lack of data.

A total of 27/141 (19%) variants were classified as Not Pathogenic or Likely Not Pathogenic. Of the 21 variants prioritized for functional analysis, eight variants (38%) were classified as Not Pathogenic or Likely Not Pathogenic based on frequency information and/or multifactorial analysis (Table 5), including two variants (*BRCA1*:c.-192T>C

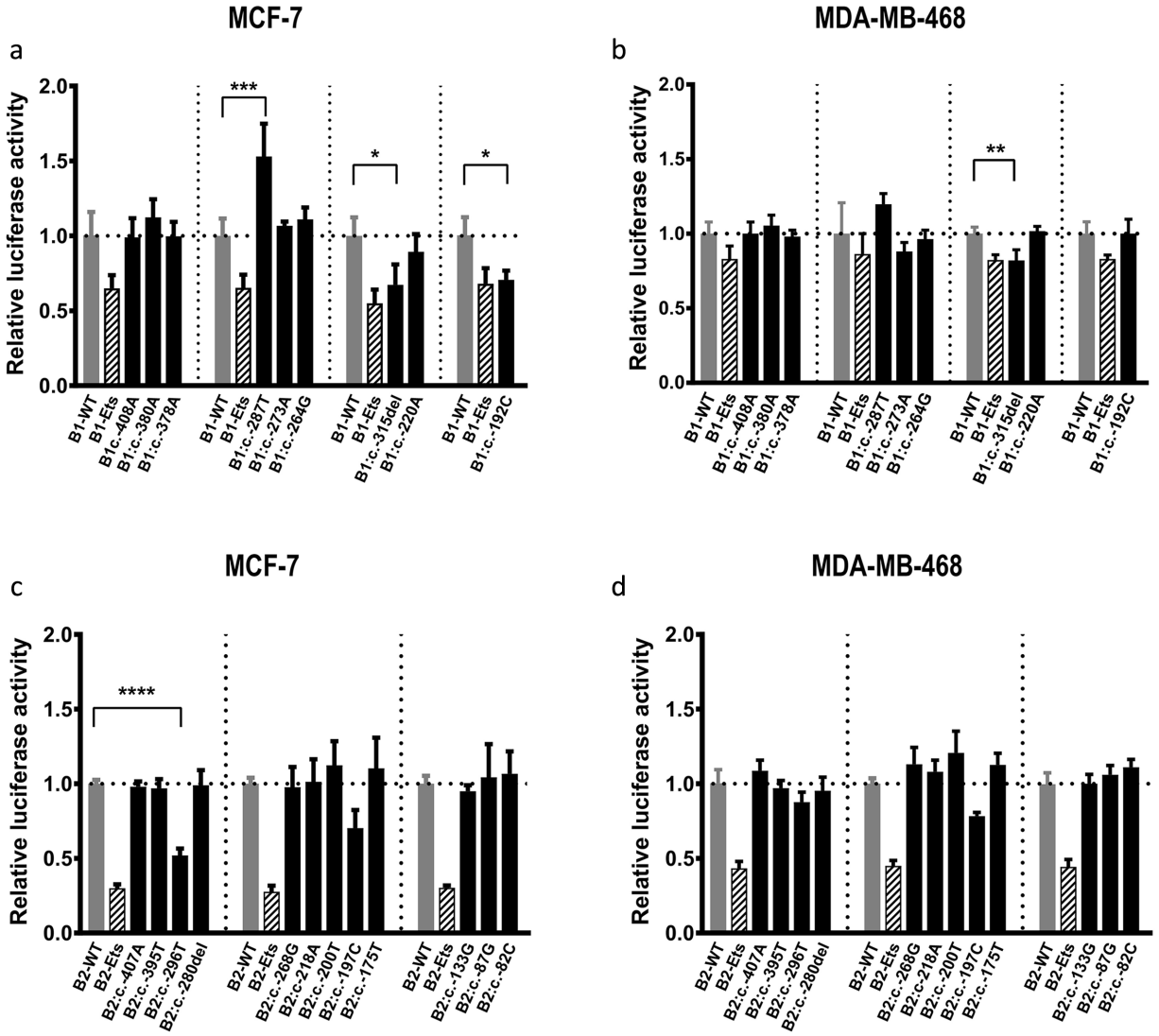

FIGURE 3 Variants mapping to the 5′ regions of *BRCA1* and *BRCA2* alter promoter activity in MCF7 and MDA-MB-468 breast cancer cells. MCF7 (a and c) and MDA-MB-468 cells (b and d) were transfected with pGL3 vectors where luciferase expression is controlled by a portion of the *BRCA1* (B1) (a and b) or *BRCA2* (B2) (c and d) promoter. Cells were transfected with plasmids containing the wild-type (WT) promoter sequence (grey bars), positive control (B1-Ets or B2-Ets; striped bars) or the indicated variants (black bars). Luciferase expression was normalized to a cotransfected pRL-TK plasmid. Data represent the average of three independent biological replicates ± standard deviation (*SD*). The horizontal dotted line represents WT promoter activity set at 1.0-fold. The vertical dotted lines demarcate individual experiments that include WT, positive control, and variant containing plasmids. (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$)

and *BRCA2*:c.-296 C > T) that were shown to decrease promoter activity and in the case of *BRCA2*:c.-296 C>T also resulted in perturbed TF binding. Taken together this analysis indicates that none of the variants shown to affect function in this study are associated with a high risk of disease. This analysis is silent, however, on whether these variants may confer a moderate or low risk of disease.

4 | DISCUSSION

Next generation sequencing and gene panel testing enable rapid analysis of gene regions that have previously not been included in standard screening procedures, including promoters, UTRs, introns, and extragenic regions. It is hypothesized that variants in these regions have potential to modulate gene expression (Stranger et al., 2005; Stranger et al., 2007) and impact on relative disease risk, possibly in collaboration with multiple other low-, moderate-, and high-risk variants (Manolio et al., 2009). This extends and validates our previous study (Santana dos Santos et al., 2017) by using a larger number patients analyzed over nine geographical locations, identifying additional BC-associated variants, and showing that a subset of these variants modulate binding of specific TFs. Further, we have compared results from our bioinformatics and functional analysis to variant classifications based on ENIGMA *BRCA1/2* guidelines for high-risk variation in these genes.

Through targeted sequencing of over 6,000 early onset/familial BC patients, we identified 141 single nucleotide variants and small indels mapping to the 5′ noncoding regions of BRCA1 and BRCA2. Of these,

TABLE 4 Information theory analysis of prioritized *BRCA1/2* variants

Variant name	TF motif (ENCODE)	Consequences
BRCA1:c.-408T>A	CEBPB	CEBPB site weakened (did not meet stringent filtering thresholds)
BRCA1:c.-380G>A	RXRA	Weak RXRA and IRF3 sites weakened, HNF4G site weakened.
BRCA1:c.-378C>A	RXRA	RXR unchanged, HSF1 site lost and GR site created
BRCA1:c.-315del	ATF1,2,3, CREB1[a]	TCF7L2 site lost and POU2F2 created
BRCA1:c.-287C>T	NFYA, NFYB	NFYA and NFYB sites lost, weak PBX3 site created
BRCA1:c.-273G>A		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA1:c.-264T>G		BHLHE32 and MYC sites created.
BRCA1:c.-220C>A		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA1:c.-192T>C		ETS1 site created, weak RFX5 site strengthened.
BRCA2:c.-407G>A		Weak MEF2A site strengthened, GATA2 site lost.
BRCA2:c.-395C>T		TEAD4 site lost.
BRCA2:c.-296C>T	PAX5	PAX5 site weakened.
BRCA2:c.-280del	ELF1, GABPA, ELK1,4	GABPA site unchanged, MXI1 andTCF3 sites lost.
BRCA2:c.-268C>G		Altered TF strength did not meet filtering thresholds[b]
BRCA2:c.-218G>A		Altered TF strength did not meet filtering thresholds[b]
BRCA2:c.-200C>T	MAZ[c]	KLF1 site abolished.
BRCA2:c.-197A>C	MAZ[c]	SP4 weakened, GR site weakened, TCF3 site created
BRCA2:c.-175C>T		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA2:c.-133T>G		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA2:c.-87T>G		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA2:c.-82G>C		Altered TF strength did not fulfill stringent filtering thresholds[b]

[a]Variant overlaps this motif, but the deletion does not alter the motif sequence.
[b]Change in information did not fulfill stringent filtering criteria, where [A] site $R_i < R_{sequence} - 1$ standard deviation of TF model, or [B] where $\Delta R_i < 4$ bits.
[c]No MAZ binding model available.

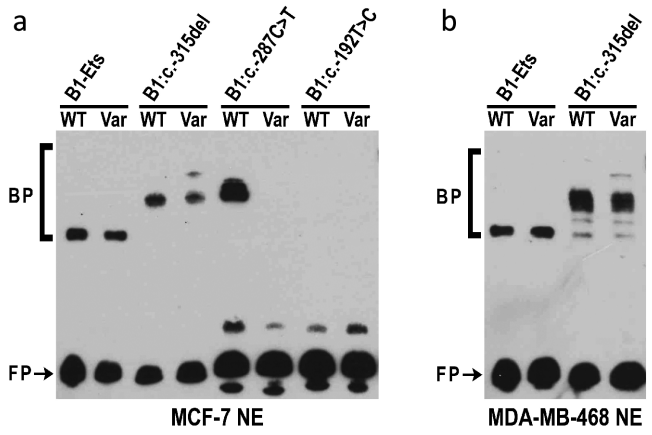

FIGURE 4 Variants in the 5′ regions of *BRCA1* alter DNA:protein complex formation. Electrophoretic mobility shift assay (EMSA) reactions were performed with 3′ biotinylated double-stranded DNA probes from the *BRCA1* 5′ region and nuclear extracts (NE) from (a) MCF7 or (b) MDA-MB-468 cells. DNA probes contained either wild-type (WT) or variant (Var) sequences. Free unbound probe (FP) and probe bound by nuclear proteins (BP) are indicated

four (*BRCA1*:c.-315del, *BRCA1*:c.-287C>T, *BRCA1*:c.-192T>C, and *BRCA2*:c.-296C>T) caused a significant change in promoter activity. The observed alterations in *BRCA1* and *BRCA2* promoter activity are of a similar magnitude to that seen with other germline variants associated with BC risk (Michailidou et al., 2017), including a variant in the *TERT* promoter, which creates a new binding site for Ets factors and results in a 1.2–1.5-fold increase in luciferase activity in a promoter reporter assay (Horn et al., 2013), and variants in the promoters of *KLHDC7A* and *PIDD1* (Michailidou et al., 2017). Although this supports the hypothesis that moderate change in promoter activity can be associated with disease risk, further work is needed to confirm this.

One of the four variants significantly altered luciferase activity in both tested cell lines, whereas the remaining three variants only affected luciferase activity in MCF7 cells. This may reflect the differential availability of crucial TFs in MDA-MB-468 cells (Kao et al., 2009) and highlights the importance of undertaking that assays for functional activity of variants in more than one cell line. Three variants, *BRCA1*:c.-380G>A, *BRCA2*:c.-296C>T, and *BRCA2*:c.-218G>A, were also analyzed in our earlier paper (Santana dos Santos et al., 2017). Although the cell lines used in the two studies were different (MDA-MB-231 in Santana dos Santos et al., 2017 and MCF7 and MDA-MB-468 here), the trends are the same in five out of six analyses. The difference for *BRCA2*:c.-296C>T, which causes a significant decrease in MDA-MB-231 and MCF7 cells, but not MDA-MB-468 cells, may again be indicative of differential gene expression in BC cell lines (Kao et al., 2009). Overall, however, the consistency of results performed in two separate laboratories underscores the robustness of the assay system.

Some variants were associated with a decrease in promoter activity, whereas others were associated with an increase. As TFs can

TABLE 5 Classification of prioritized variants

Gene	Genomic location (hg19)	HGVS c. nomenclature	Luciferase result	Combined interpretation of frequency data & multifactorial analysis	Highest MAF (population, database)	Prior probability of pathogenicity	Segregation Bayes score (# families)	Tumor histopathology likelihood ratio (# tumors)	Combined odds for causality	Posterior probability of pathogenicity[c]
BRCA1	g.41277676A>T	c.-408T>A	No effect	Uncertain		0.02				
BRCA1	g.41277648C>T	c.-380G>A	No effect	Uncertain		0.02		1.67 (1)	1.67	NA
BRCA1	g.41277646G>T	c.-378C>A	No effect	Uncertain	0.0015 (African, 1,000 Genomes)	0.02				
BRCA1	g.41277583del	c.-315del	Decrease	Uncertain		0.02				
BRCA1	g.41277555G>A	c.-287C>T	Increase	Uncertain		0.02		0.64 (1)	0.64	NA
BRCA1	g.41277541C>T	c.-273G>A	No effect	Not pathogenic[a]	0.0159 (African, 1,000 Genomes)	0.02				
BRCA1	g.41277532A>C	c.-264T>G	No effect	Uncertain		0.02		0.51 (1)	0.51	NA
BRCA1	g.41277488G>T	c.-220C>A	No effect	Uncertain		0.02				
BRCA1	g.41277460A>G	c.-192T>C	Decrease	Not pathogenic[a]	0.0159 (African, 1,000 Genomes)	0.02				
BRCA2	g.32889437G>A	c.-407G>A	No effect	Not pathogenic[b]	0.0080 (Prague, this study)	0.02		0.55 (6)	0.55	NA
BRCA2	g.32889449C>T	c.-395C>T	No effect	Uncertain		0.02				
BRCA2	g.32889548C>T	c.-296C>T	Decrease	Not pathogenic[b]	0.0080 (Prague, this study)	0.02	3.07 (1)	1.91 (8)	5.87	0.1069
BRCA2	g.32889564delG	c.-280del	No effect	Uncertain		0.02		0.69 (1)	0.69	NA
BRCA2	g.32889576C>G	c.-268C>G	No effect	Uncertain		0.02				
BRCA2	g.32889626G>A	c.-218G>A	No effect	Likely not pathogenic		0.02	0.52 (1)	0.72 (1)	0.38	0.0076
BRCA2	g.32889644C>T	c.-200C>T	No effect	Likely not pathogenic		0.02		0.37 (1)	0.37	0.0075
BRCA2	g.32889647A>C	c.-197A>C	No effect	Not pathogenic[b]	0.0014 (African, FLOSSIES)	0.02		1.08 (1)	1.08	NA
BRCA2	g.32889669C>T	c.-175C>T	No effect	Not pathogenic[a]	0.0197 (African, FLOSSIES)	0.02				
BRCA2	g.32889711T>G	c.-133T>G	No effect	Uncertain		0.02				
BRCA2	g.32889757T>G	c.-87T>G	No effect	Uncertain		0.02				
BRCA2	g.32889762G>C	c.-82G>C	No effect	Uncertain		0.02				

NA, not applicable: multifactorial classification not assigned as the combined odds of causality were insufficient (≥0.5 and ≤2) to derive a posterior probability of pathogenicity (Vallee et al., 2016).

[a] Not pathogenic based on frequency > 1% in an outbred sampleset.

[b] Variant allele assigned a low prior probability of pathogenicity of 0.02 assuming conservatively that 2/100 of such variants might be associated with a high risk of cancer and allele frequency ≥0.001 and < 0.01 in outbred sample set.

[c] Posterior probabilities used to assign IARC 5-tier class as described in Plon et al., 2008.

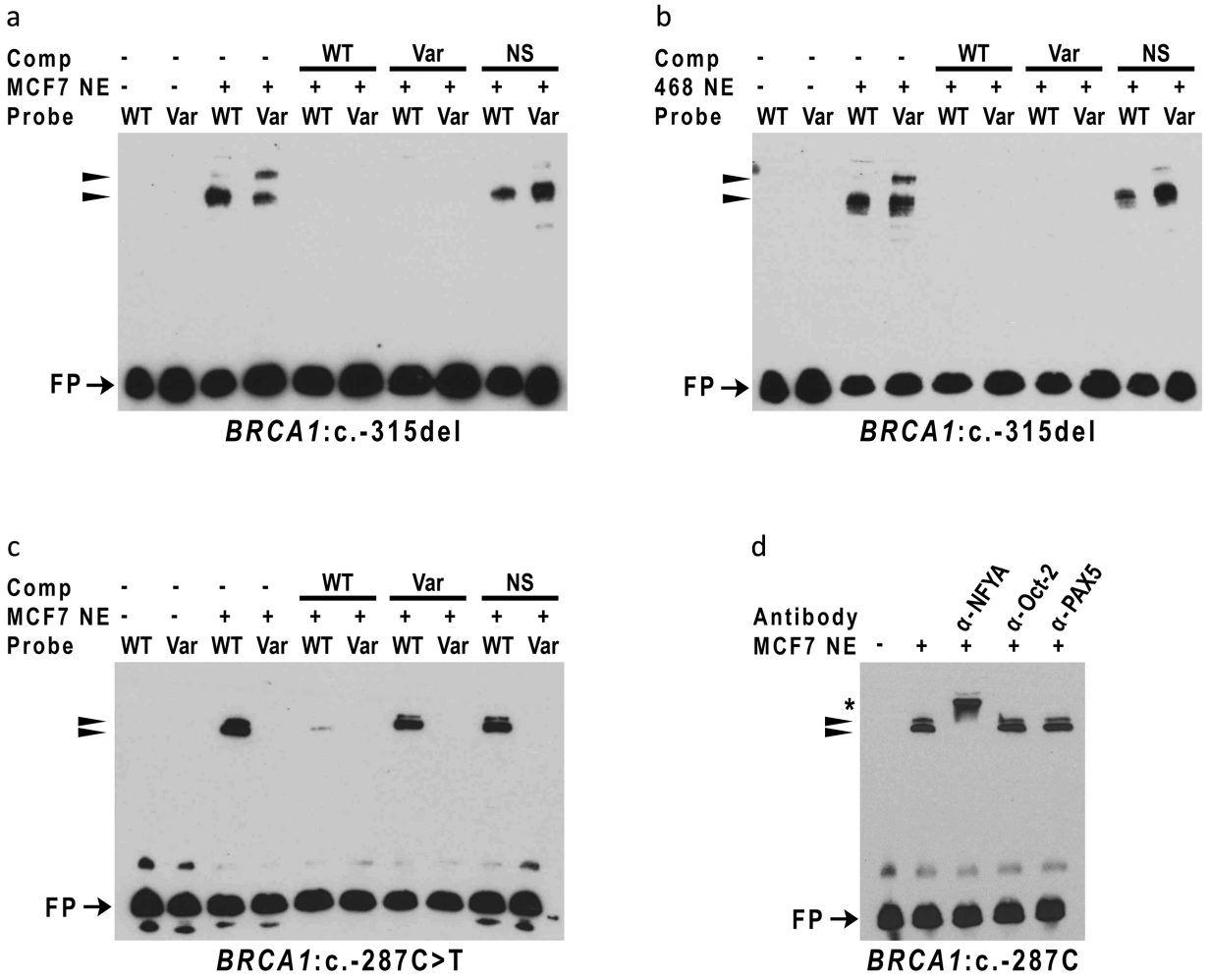

FIGURE 5 Variant sequences in the *BRCA1* 5′ region alter specific DNA:protein complex formation. Competition electrophoretic mobility shift assay (EMSAs) were performed using 3′ biotinylated double-stranded DNA probes containing sequences from the *BRCA1* 5′ region surrounding the B1:c.-315del (a and b) and B1:c.-287C>T (c) variants. DNA probes containing the wild-type (WT) or variant (Var) sequence were incubated with nuclear extracts from MCF7 cells (MCF7 NE) or MDA-MB 468 cells (468 NE) in the presence (+) or absence (–) of unlabeled WT, Var, or nonspecific (NS) competitor (Comp) DNA. Free unbound probe (FP) and specific DNA:protein complexes (arrowheads) are indicated. Supershift experiments (d) were performed with the *BRCA1*:c.-287C (WT) probe and antibodies to NFYA, Oct-2 (POU2F2) and PAX5. The supershifted NFYA complex is indicated by asterisk (*)

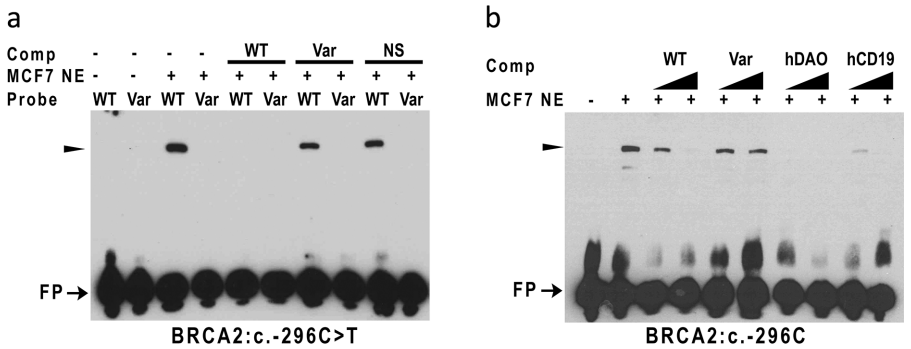

FIGURE 6 Variants in the 5′ region of *BRCA2* alter specific DNA:protein complex formation. Competition electrophoretic mobility shift assay (EMSAs; a) were performed using 3′ biotinylated double-stranded (ds) DNA probes containing sequences from the *BRCA2* 5′ region surrounding the BRCA2:c.-296C>T variant. DNA probes containing the wild-type (WT) or variant (Var) sequence were incubated with nuclear extracts from MCF7 cells (MCF7 NE) in the presence (+) or absence (–) of unlabeled WT, Var, or nonspecific (NS) competitor (Comp) DNA. Cross-competition EMSAs (b) contained *BRCA2* WT sequences and increasing concentrations of ds competitor DNA containing unlabeled WT, Var, or PAX5 binding sites from the *hCD19* gene and D-amino acid oxidase gene (*hDAO*). Free unbound probe (FP) and specific DNA:protein complexes (arrowheads) are indicated

function as activators or repressors, a variant-associated change in TF binding can result in either a decrease or an increase in promoter (or other regulatory element) activity. Differences in the quanta and direction of promoter activity have been reported previously (e.g., Fraile-Bethencourt et al., 2018; Santana dos Santos et al., 2017) and have also been shown to differ between cell lines potentially reflecting the availability of TFs or cofactors (e.g., Zn).

Three of the variants, *BRCA1*:c.-315del, *BRCA1*: c.-287C>T, and *BRCA2*:c.-296C>T, altered protein binding. ENCODE ChIP-seq data from BC cell lines indicate candidate proteins that are bound to the genomic regions containing these variants (Figure 2 and Supporting Information Figure S1). These include E2F1, CEBPB, GATA3, Max, ELF1, GABP, and FOXA1 for *BRCA1* and E2F1, MYC, ELF1, GABP, Max, and PML for *BRCA2*. Interestingly, a number of these factors have previously been implicated in BC.

In addition, ENCODE ChIP-seq data from cell lines derived from tissues other than breast indicate that the variants that affect protein binding are located within consensus motifs for specific TFs associated with these regions (Tables 2 and 3; Supporting Information Figure S1). *BRCA1*:c.-287C>T overlaps with the consensus binding motif for CCAAT Box binding factors, *BRCA1*:c.-315del is located in a consensus motif for CREB/ATF proteins, although the deletion does not modify this motif, and *BRCA2*:c.-296C>T is located within the consensus motif for PAX5. IT analysis also predicts that all these variants alter TF binding (Table 4 and Supporting Information Table S4). We show that *BRCA1*:c.-287C>T disrupts the binding of NFYA to the *BRCA1* promoter region. Furthermore, we present evidence that *BRCA2*:c.-296C>T disrupts the binding of PAX5. *BRCA1*:c.-315del lies in the so-called positive regulator region that has been shown to bind GABPα, CREB, and AP-1 proteins (Atlas et al., 2000; Atlas, Stramwasser, & Mueller, 2001; Graves, Zhou, MacDonald, Mueller, & Roskelley, 2007; Suen & Goss, 1999; Thakur & Croce, 1999). Although these proteins are generally considered activators of transcription, repression of promoter activity by *BRCA1*:c.-315del suggests the recruitment of an additional transcriptional repressor or corepressor to this region. IT analysis predicts creation of a binding site for POU2F2, a known repressor; however, we found no evidence to suggest that this variant increased POU2F2 binding in the cell line used, although it is possible that changes may be observable in other cell lines. Biochemical studies, including mass spectrometry, will be required to validate and discover other alterations in TF binding.

One variant, *BRCA1*:c.-287C>T, increased promoter activity and decreased protein:DNA interactions. This increase in promoter activity was unanticipated because this variant is within a consensus motif for the CCAAT box binding proteins, NFYA and NFYB, and mutation of this CCAAT box has previously been shown to reduce *BRCA1* promoter activity in MCF7 cells (Bindra et al., 2005; Xu, Chambers, & Solomon, 1997). This variant also decreases promoter activity in MDA-MB-231 cells (Santana dos Santos et al., 2017). Here, we show that the *BRCA1*:c.-287C>T variant reduces NFYA binding. Importantly, NFY proteins can function as transcriptional activators or repressors depending on recruitment of corepressors or coactivators (Peng & Jahroudi, 2002; Peng et al., 2007) and recruitment of TFs to neighboring sequences (Zhu et al., 2012) indicating possible mechanisms for divergent activities of NFY proteins at this site.

BRCA1:c.-192T>C, which lies in the 5′UTR, decreased reporter activity but did not bind any proteins from MCF7 nuclear extracts in EMSA analysis. Possibly, EMSA binding conditions are not optimal for binding of factors to this sequence or alternatively, this reduction in promoter activity could be by posttranscriptional mechanisms as seen for *BRCA2*:c.-26G>A (Gochhait et al., 2007).

Using existing prediction models developed for high risk variants, population frequency and clinical information classified 27 variants as "Not Pathogenic" or "likely Not Pathogenic." This included two *BRCA1* and six *BRCA2* variants with functional assay data available, six with no statistically significant effect on promoter activity, and two that decreased promoter activity in vitro. These two variants, *BRCA1*:*c.*-192T>C and *BRCA2*:*c.*-296C>T, were observed in population subgroup controls; notably *BRCA1*:*c.*-192T>C was observed at a frequency of >1%, which is considered stand-alone evidence against pathogenicity (defined as high risk of cancer) for *BRCA1/2* variation. This suggests that promoter region variants, irrespective of bioinformatic prediction or functional assay results, are unlikely to be associated with a high risk of cancer. This is consistent with current evidence from ENIGMA studies (de la Hoya et al., 2016), which suggest that an allele resulting in only ~20–30% expression of *BRCA1* transcript/s encoding functional transcripts is not associated with high risk of BC. The low impact of these variants on risk is likely to reflect the complex interplay of TFs and DNA elements, and possible redundancy in the system. For example, a variant in one TF binding site within a cluster may be buffered by other binding sites and thus insufficient on its own to reduce gene expression markedly (Lu & Rogan, 2018).

Given that moderate- and low-risk variants often occur in >1% of the population, and that the remaining 13 variants had insufficient evidence available to assess clinical significance, we cannot exclude the possibility that *BRCA1/2* promoter region variants, in particular those with proven functional effect, may be associated with a moderate or low risk of cancer. This indicates an urgent need to further develop prediction models to accommodate criteria for moderate- or low-risk variants by extending the *BRCA1/2*-specific criteria developed by ENIGMA (https://www.enigmaconsortium.org/), or even the generic variant classification criteria developed by the American College of Medical Genetics for Mendelian disorders (Richards et al., 2015).

This study has evaluated the significance of single nucleotide variants and small indels mapping to the 5′ noncoding region of *BRCA1* and *BRCA2* using bioinformatic, biological, and biochemical analyses in combination with consideration of clinical data that inform qualitative and quantitative variant classification. We present data to suggest that a subset of these variants have functional effects on gene regulation. We also present evidence that variants mapping to and affecting the function of *BRCA* promoters are not likely to be associated with a high risk of cancer. We propose that studies of differing design, such as very large-scale case-control sequencing studies able to detect rare variation, will be required to address if a low to moderate risk of cancer may be associated with *BRCA1/2* regulatory region variation that has not been captured to date by genome-wide association genotyping platforms. We believe that the bioinformatic and functional analysis

presented will be important to define the design and interpretation of such future sequencing studies. We also believe that this study highlights the challenges associated with classifying variants with respect to low or moderate disease risk, and the need to be cautious in the clinical use of information on individual variants that is likely to be one of many factors contributing to disease risk.

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DISCLOSURE STATEMENT

B.C.S is an employee of and P.K.R is co-founder of CytoGnomix, which has developed algorithms and software for interpretation of variants within transcription factor binding sites.

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cancers

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Article

Multigene Panel Germline Testing of 1333 Czech Patients with Ovarian Cancer

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Abstract: Ovarian cancer (OC) is the deadliest gynecologic malignancy with a substantial proportion of hereditary cases and a frequent association with breast cancer (BC). Genetic testing facilitates treatment and preventive strategies reducing OC mortality in mutation carriers. However, the prevalence of germline mutations varies among populations and many rarely mutated OC predisposition genes remain to be identified. We aimed to analyze 219 genes in 1333 Czech OC patients and 2278 population-matched controls using next-generation sequencing. We revealed germline mutations in 18 OC/BC predisposition genes in 32.0% of patients and in 2.5% of controls. Mutations in *BRCA1/BRCA2*, *RAD51C/RAD51D*, *BARD1*, and mismatch repair genes conferred high OC risk (OR > 5). Mutations in

BRIP1 and *NBN* were associated with moderate risk (both OR = 3.5). *BRCA1/2* mutations dominated in almost all clinicopathological subgroups including sporadic borderline tumors of ovary (BTO). Analysis of remaining 201 genes revealed somatic mosaics in *PPM1D* and germline mutations in *SHPRH* and *NAT1* associating with a high/moderate OC risk significantly; however, further studies are warranted to delineate their contribution to OC development in other populations. Our findings demonstrate the high proportion of patients with hereditary OC in Slavic population justifying genetic testing in all patients with OC, including BTO.

Keywords: ovarian cancer; next-generation sequencing; predisposition genes; cancer risk; mutation

1. Introduction

Ovarian cancer (OC) is the most severe gynecologic malignancy with stable incidence and mortality. The most frequent OC types (85–95%) are epithelial tumors, which are high-grade (HG) serous in 70% of cases [1,2]. Because of the nonspecific symptoms and a lack of presymptomatic screening modalities, most women are diagnosed with an advanced disease, having a dismal 25% 5-year survival rate [3].

The overall OC lifetime risk oscillates around 2% in the general female population in developed countries. Central and Eastern Europe, including the Czech Republic, represented a region with the highest OC incidence (11.9 ASRW per 100,000 females) and mortality (6.0 ASRW per 100,000 females) worldwide in 2018 (http://gco.iarc.fr). In the Czech Republic alone, annual OC incidence and mortality in 2018 reached 9.5 and 6.7 ASRW per 100,000 females, respectively.

Genetic predisposition for OC is unusually high and is reported in up to 25% of cases [4–6]. The most frequent germline mutations affect the *BRCA1* and *BRCA2* genes, conferring 24% and 8.4% OC lifetime risks, respectively [7]. The *BRCA1* and *BRCA2* mutation carriers frequently but not exclusively develop HG serous OC [8]. Carriers of mutations in these major OC predisposition genes have also very high risk of breast cancer (BC) development. A high OC risk has also been associated with germline mutations in *RAD51C*, *RAD51D*, Lynch syndrome genes, and *STK11*; a moderate OC risk with *BRIP1* [9–13]. Risks associated with germline mutations in genes with anticipated BC and/or OC predisposition (incl. *ATM*, *BARD1*, *CDH1*, *CHEK2*, *NBN*, *PALB2*, *PTEN*, and *TP53*) and in other candidate genes remain to be determined [14–17]. The identification of presymptomatic women at high risk who can benefit from risk-reducing salpingo-oophorectomy (RRSO) is of critical importance, as demonstrated by the reduced OC mortality in *BRCA1* and *BRCA2* mutation carriers undergoing preventive surgery [18].

In this report, we aim to establish an association of germline mutations with OC in the Czech patients belonging to the Slavic population that has not been systematically analyzed for OC predisposition. Seven Czech genetic laboratories participated in the analysis of 1333 Czech OC patients by the identical procedure using CZECANCA panel (CZEch CAncer paNel for Clinical Application) targeting 219 genes [19]. Prevalence of variants in genes affected in OC patients was assessed in 2278 population-matched controls. This analysis enabled us to comprehensively determine mutations frequency and clinicopathological characteristics of OC in carriers of mutations in genes with known OC predisposition but also to analyze contribution of population-specific variants in other candidate genes to OC predisposition.

2. Results

2.1. Description of Study Population

Altogether, samples obtained from 1333 OC patients diagnosed at seven centers were analyzed by the identical panel NGS using the CZECANCA panel targeting 219 cancer-predisposition and candidate genes and were evaluated centrally by the identical bioinformatics pipeline. From 1333

analyzed OC patients, 1045 (78.4%) women were diagnosed with OC only and 288 (21.6%) women with double primary tumors, including BC (210 patients; 15.8%) or other tumors (78 patients; 5.9%). The median age at OC diagnosis was 53.7 years (range 15–86 years). Almost half (47.6%) of the patients had a negative family cancer history. From 1120 OC patients with known histology, 728 (65.0%) women developed serous adenocarcinoma with prevailing HG tumors. Sixty percent of cases represented patients with advanced disease (stages III–IV). The clinicopathological characteristics are provided in Table S1.

2.2. Mutations in 18 Known/Anticipated Hereditary BC/OC Genes

We primarily focused on mutations in 18 BC/OC genes listed in the NCCN Guidelines for Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic (Version 1.2020; 4 December 2019). We identified 441 mutations in 427/1333 (32.0%) OC patients and 58/2278 (2.5%) mutation carriers among population-matched controls (PMC) in 18 known/anticipated BC/OC genes (Figure 1, Table 1, and Table S2). Thirteen multiple mutation carriers (Figure 1) identified among patients only (characterized in Table S3) were excluded from the subsequent analyses.

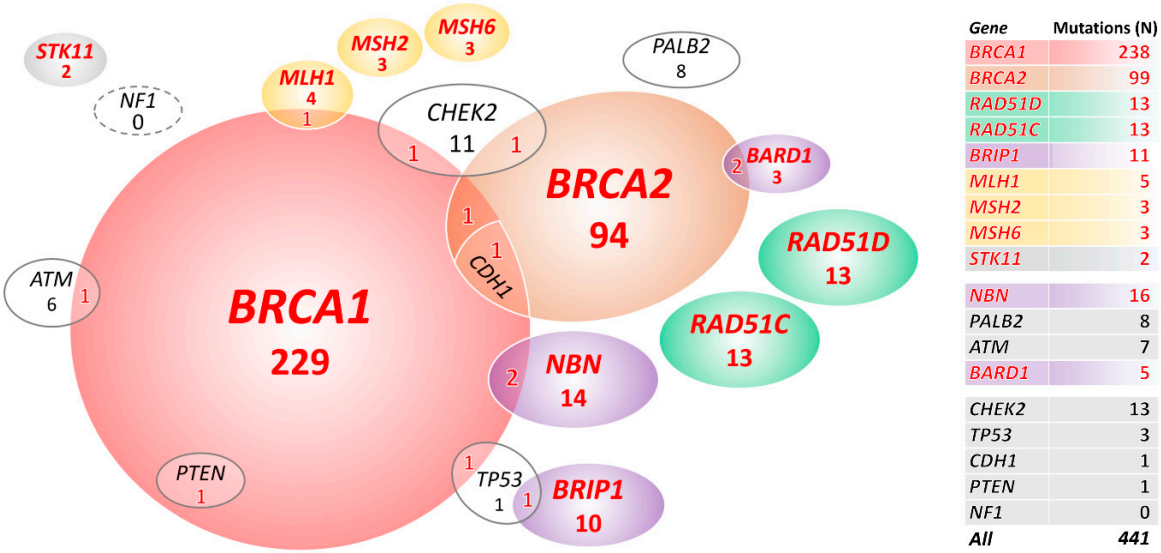

Figure 1. Overall, 427 mutation carriers of 441 mutations in 18 known/anticipated breast cancer (BC)/ovarian cancer (OC) predisposition genes. In total, 399 carriers in genes significantly associated with OC in our study are highlighted in red letters. *STK11* is highlighted as rarely mutated but established OC predisposition gene.

Carriers of germline mutations in 10 genes (including Lynch syndrome genes analyzed as a group together) had significantly increased OC risk (Table 1 in bold). We found the prevailing *BRCA1* or *BRCA2* germline alterations in 323/1320 (24.5%) patients and in 12/2278 (0.5%) PMC. Further, 65/1320 (4.9%) OC patients carried a mutation in 8 other genes significantly associated with OC risk in our study (including 2 carriers of mutations in *STK11*, an established high-risk OC gene that did not reach significant association in our study due to low frequency of mutation carriers in patients; Figure 1). We found only 19/2278 (0.8%) carriers of mutations in these 8 genes in PMC.

Table 1. Mutation frequencies in 1320 ovarian cancer cases and in 2278 population-matched controls (PMC).

Gene	1320 OC Patients [a] N Mutations (%)	2278 PMC N mutations (%)	OR (95% CI); p [a]
Increased OC risk [b]			
BRCA1 [c]	**229 (17.35)**	**5 (0.22)**	**95.2 (40.1–295.2); 1.83×10^{-97}**
BRCA2 [c]	**94 (7.12)**	**7 (0.31)**	**24.9 (11.6–63.6); 1.16×10^{-33}**
RAD51D	**13 (0.98)**	**2 (0.09)**	**11.3 (2.6–103.4); 9.66×10^{-5}**
RAD51C	**13 (0.98)**	**4 (0.18)**	**5.7 (1.7–23.8); 0.001**
BRIP1 [c]	**10 (0.76)**	**5 (0.22)**	**3.5 (1.1–13); 0.03**
MLH1 [c]	**4 (0.3)**	**1 (0.04)**	6.9 (0.7–340.4); 0.06 [d]
MSH2	**3 (0.23)**	**0**	**0.049 [d]**
MSH6	**3 (0.23)**	**0**	**0.049 [d]**
STK11	2 (0.15)	0	0.13
Potentially increase or insufficient evidence OC risk [b]			
NBN [c]	**14 (1.06)**	**7 (0.31)**	**3.5 (1.3–10.2); 0.006**
PALB2	8 (0.61)	9 (0.40)	1.5 (0.5–4.5); 0.45
ATM [c]	6 (0.45)	8 (0.35)	1.3 (0.4–4.3); 0.78
BARD1 [c]	**3 (0.23)**	**0**	**0.049**
No increased risk of OC [b]			
CHEK2 [c]	11 (0.83)	8 (0.35)	2.4 (0.9–6.8); 0.06
TP53[c]	1 (0.08)	2 (0.09)	0.9 (0–16.6); 1
CDH1[c]	0	0	-
PTEN [c]	0	0	-
NF1	0	0	-

[a] Prevalence of mutations in all 1333 patients (including 13 multiple mutation carriers) is provided in Table S2. [b] Gene classification according to the NCCN guidelines version 2020.1. [c] Excluding 13 multiple mutation carriers described in Figure 1 and Table S3. [d] When analyzed Lynch syndrome genes collectively: OR = 22.63 (95% CI 3.4–958.5); $p = 1.95 \times 10^{-05}$.

The copy number variation (CNV) analysis in 18 OC/BC genes revealed 37 large genomic rearrangements in 37/1333 (2.8%) patients. They affected seven genes (23×*BRCA1*, 4×*BRIP1*, 4×*CHEK2*, 2×*MLH1*, 2×*STK11*, 1×*PALB2*, and 1×*CDH1*) and accounted for 8.4% (37/441) of all pathogenic mutations in these genes. Except 1 whole gene duplication of *MSH6* (classified as VUS), we found no CNV in analyzed controls in these 18 genes.

2.3. Clinical and Histopathological Characteristics of Mutation Carriers

Subsequently, we described the clinicopathological characteristics of the mutation carriers in 10 genes associated with OC risk (Figure 2 and Table S4). Multiple mutation carriers (Table S3) were excluded from this analysis.

2.3.1. Age at OC Diagnosis

The highest mutation frequency was found in patients diagnosed with OC at 40–49 and 50–59 years (37.4% and 40.7%, respectively) and the lowest in patients diagnosed before the age of 30 (8.3%; Figure 2A). Interestingly, the mutation frequency in the group of the oldest patients (≥70 years) was twice higher than in the youngest (<30 years) patients' subgroup ($p = 0.013$ for difference).

This difference was primarily caused by *BRCA1/BRCA2* mutations (3.6% vs. 18.1% in patients <30 vs. ≥70 years), as the frequency of *non-BRCA* genes mutations was similar (4.8% vs. 4.3%). The median age at diagnosis was significantly different in *BRCA1* (51.0 years; range 23–78) and *BRCA2* (58.4 years; range 27–78) mutation carriers ($p = 8.5\times10^{-10}$), respectively. The median age at diagnosis in other genes with at least 10 identified mutation carriers increased gradually from *RAD51C* (52.2 years; range 25–69) to *NBN* (54.5 years; range 18–76), *RAD51D* (56.0 years; range 36–69), and *BRIP1* (58.0 years; range 30–71). We observed a younger median age at diagnosis in carriers of mutations in Lynch syndrome genes 46.0 years (range 35–73).

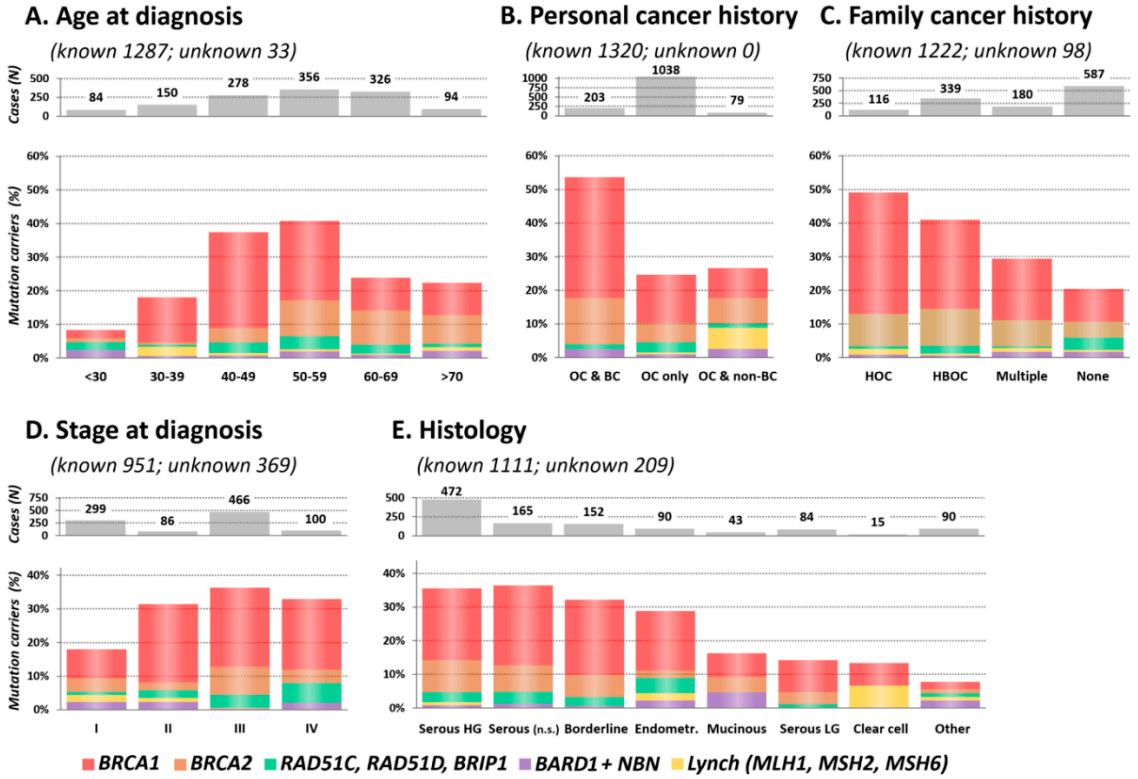

Figure 2. Proportion of mutation carriers in clinicopathological subgroups, including (**A**) Age at OC diagnosis; (**B**) Personal cancer history; (**C**) Family cancer history; (**D**) Stage at diagnosis; (**E**) Histology in 1320 OC patients.

2.3.2. Personal and Family Cancer History

The highest proportion of mutations (109/203; 53.7%) was detected in double primary OC and BC patients, while in patients diagnosed with OC only and double primary OC and non-BC cancer, it reached 256/1038 (24.7%) and 21/79 (26.6%), respectively (Figure 2B). The frequency of mutations in patients from hereditary OC families (HOC) was 49.1% (57/116; Figure 2C). Decreasing proportion of mutation carriers in other family cancer history categories (41.0% in HBOC and 29.4% in multiple cancer) was dominantly caused by decreasing *BRCA1* mutation prevalence. Nevertheless, in 587 OC patients without a positive family cancer history, we still identified 120 (20.4%) carriers of pathogenic mutations.

2.3.3. Stage and Histology

Almost 60% of patients were diagnosed at FIGO stage III or IV (Figure 2D). In contrast, 6/8 informative Lynch syndrome gene mutation carriers were diagnosed with stage I tumors.

The mutation rate stratified OC into two histological clusters. The high mutation rate subgroup included 879 patients with HG/unspecified serous, borderline, and endometrioid tumors with 303 (34.5%) carriers, while the low mutation rate subgroup included 232 patients with low-grade (LG)

serous, mucinous, clear cell, and other tumors with 28 (12.1%) carriers. *BRCA1/2* mutations in HG serous carcinomas were more than twice as frequent (146/472; 30.9%) as in LG serous ones (11/84; 13.1%). Interestingly, the distribution of *BRIP1/RAD51C/RAD51D* mutations among histological types was similar to that of *BRCA1/2*. The lowest proportion of mutations (7/90; 7.8%) was found in rare histological cancer types (herein denominated as "Other").

2.4. Mutations in Additional 201 Analyzed Genes

Finally, we reviewed the presence of germline variants in additional 201 genes targeted by the CZECANCA panel [19]. This analysis revealed 230 mutations in 89 genes in 208 (15.6%) patients (Table S5). Of these, 149 (11.2%) patients carried mutations in "additional" genes exclusively while 59 (4.4%) patients carried a mutation in "additional" genes alongside a mutation in one of the 10 OC risk genes. Mutations in these "additional" genes were rare and their prevalence was significantly higher in patients over controls in only four genes (Table 2). However, only mutations in *PPM1D* were significantly associated with OC risk ($p = 0.003$) following Bonferroni correction and exclusion of carriers of mutations in OC predisposition genes. All *PPM1D* mutations were mosaic with MAF = 14%–60% and MAF = 17%–19% in patients and controls, respectively. It should be noted that blood for genetic testing was sampled after the application of chemotherapy in all *PPM1D* positive patients (in average at 38 months after treatment; ranged 4 months–7.1 years). Seven out of 15 *PPM1D* mutation carriers harbored an additional mutation in another DNA repair gene (3×*BRCA2*, 1×*PALB2*, 1×*EXO1*, and 1×*PMS1*). MAF of *PPM1D* mutations correlated neither with age at OC diagnosis nor with the time from the last chemotherapy (Table S6). Mutations in *PPM1D* and *SHPRH* were significantly associated only with age > 60 years ($p = 0.001$), whereas frequency of *NAT1* mutations in particular categories was similar (Table S7). Uncorrected p values were marginally significant also for germline variants in *MMP8* and *FANCG* in OC patients when carriers of mutations in 10 BC/OC predisposition genes significantly associating with OC risk in our study were excluded (Table 2).

Table 2. Additional 201 analyzed genes significantly associated with OC risk in the group of all OC patients and in a subgroup of 934 patients without mutations in 10 established OC predisposition genes.

Gene	Patients N Mutations (%)	2278 PMC N Mutations (%)	OR (95% CI); p (Bonferroni Corrected *p*)
All 1333 OC patients			
PPM1D	16 (1.20)	2 (0.09)	13.82 (3.24–124.22); 7.4×10^{-6} (0.001)
NAT1	13 (0.98)	5 (0.22)	4.48 (1.49–16.07); 0.003 (n.s.)
SHPRH	5 (0.38)	1 (0.04)	8.57 (0.96–404.83); 0.028 (n.s.)
934 OC patients without mutations in 10 genes significantly associated with OC in our study			
PPM1D	12 (1.28)	2 (0.09)	14.80 (3.28–136.67); 1.7×10^{-5} (0.003)
NAT1	8 (0.86)	5 (0.22)	3.96 (1.13–15.30); 0.026 (n.s.)
MMP8	6 (0.64)	4 (0.18)	3.67 (0.87–17.74); 0.041 (n.s.)
FANCG	5 (0.53)	2 (0.09)	6.12 (1.00–64.45); 0.025 (n.s.)

n.s., nonsignificant.

3. Discussion

The analysis of 1333 Czech OC patients and 2278 population-matched controls provides the most comprehensive view of the genetic architecture of OC predisposition in the Slavic population. From 18 OC/BC predisposition genes listed in current NCCN breast/ovarian familial cancer guidelines, mutations in 10 genes were significantly associated with OC risk in our population being present in 399/1333 (29.9%) OC patients and 31/2278 (1.4%) PMC (Figure 1). Mutations in eight remaining genes were extremely rare (*CDH1*, *PTEN*, *STK11*, and *TP53*) or absent (*CDKN2A* and *NF1*) or did not significantly differ in frequency among cases and controls (*ATM*, *PALB2*, and *CHEK2*). Mutations in *BRCA1/2*, *RAD51C/D*, and Lynch syndrome genes were associated with a high OC risk, while mutations in *BRIP1* were associated with a moderate OC risk in our study (Table 1), in concordance with previous

reports [9,10,20,21]. The *BRCA1* and *BRCA2* mutations, present in 84.0% of all mutation carriers, were by far the most frequent alterations found in 17.9% and 7.4% of our patients, respectively. Mutations in other eight genes leaded by *RAD51C/RAD51D/BRIP1* affected additional 5.0% of patients, as shown also by others recently [5,6,22]. Germline mutations in Lynch syndrome genes together associated with high OC risk. Mutations in *MLH1* prevailed similarly as in Lynch syndrome patients diagnosed with colorectal cancer [23].

In contrast to previous studies, our results suggest increased OC risk in carriers of *NBN* and *BARD1* mutations [12,24]. We did not find significant increase of OC risk for carriers of mutations in *ATM* and *PALB2*, as noticed previously [12,24,25]. However, further analyses considering very large population-matched studies or studies considering families of mutation carriers can better disclose moderate risk associations, as shown for *PALB2* mutations recently [26].

Overrepresentation of mutations in the *CHEK2* gene in OC patients in this study was marginally nonsignificant in contrast to our previous report where we identified moderately increased OC risk for *CHEK2* mutation carriers [27]. However, last four *CHEK2* coding exons were not targeted in our gene panel omitting possible deleterious *CHEK2* alterations identified in our previous study in which last four coding exons were analyzed separately in both cases and controls. Mutations in *NF1* were absent and were extremely rare in *CDH1* and *PTEN*, just like *STK11* mutations found in a patient with nonepithelial OC, a characteristic Peutz–Jeghers syndrome manifestation [9]. Altogether, the high overall frequency of mutations in OC predisposition genes in our study is in agreement with some previous studies [4–6,28] and may contribute to a high OC incidence in our population.

Multigene testing revealed 13 carriers of multiple pathogenic mutations (1.0% of patients). Similar frequency of individuals with this multilocus inherited neoplasia alleles syndrome (MINAS) [29] was shown also in previous analyses of OC patients [30,31].

We analyzed available phenotype characteristics in 1320 OC patients with one pathogenic mutation at the most in 10 genes associated with OC risk in our study (Figure 2). While the highest prevalence of *BRCA1/2* mutation carriers was in patients diagnosed with double primary OC and BC, mutations in *RAD51C/RAD51D/BRIP1* prevailed in patients diagnosed with OC only (Figure 2B); nevertheless, their distribution among histological subtypes was similar to that in *BRCA1/2* mutation carriers (Figure 2E). In contrast to Castera et al. who found mutations in *RAD51C/RAD51D/BRIP1* dominantly in French OC patients with a positive family OC history [32], we identified mutations in these genes in 1/116 (0.9%) and 22/587 (3.7%) carriers in HOC patients and in patients with a negative family cancer history, respectively. Further, we have noticed a surprisingly high frequency of OC-predisposing mutations in older patients. Their prevalence in patients ≥ 60 years was 23.6%, whereas Harter et al. found in this age group 18.9% mutation carriers even though frequency of mutation carriers in patients <60 years in both studies was comparable (32.6% and 33.2%, respectively) [28]. *BRCA1* mutations dominated in patients <60 years over *BRCA2* mutations, while in patients ≥ 60 years, their frequencies were comparable. Moreover, we revealed 29 *BRCA1/2* mutation carriers (13.9% of patients) in 208 OC patients diagnosed at ≥60 years with no family cancer history, while Morgan and colleagues detected only two (4.3%) *BRCA1/2* mutations in 46 sporadic OC patients ≥ 60 years [33]. Even in the oldest subgroup of our OC patients diagnosed at ≥70 years, the frequency of *BRCA1/2* mutation carriers exceeded 18%, while in other studies, *BRCA1/2* mutations' frequency in this age category was below 10% [34,35]. This high frequency of *BRCA1/2* mutations in our patients ≥70 years contrasted with a low frequency in women diagnosed at <30 years (18.1% vs. 3.6%; $p = 0.003$; Figure 2A). The difference was even more apparent in "sporadic" OC cases (with no family cancer history), where *BRCA1/2* mutations were found in 6 out of 45 (13.3%) women ≥70 years but in none of 52 cases diagnosed at <30 years. It should be emphasized that although rare histological OC types were more frequent in the subgroup of 52 patients diagnosed with sporadic OC at <30 years, 32 (65.3%) of 49 informative cases developed invasive epithelial OC.

Mutations in OC predisposition genes significantly prevailed in subgroups with high-grade/nonspecified serous, borderline, and endometrioid tumors over subgroup with low-grade serous,

mucinous, clear cell, or other rare histologic types (Figure 2E). Surprisingly, the overall mutation frequency in patients with borderline tumors was comparable with that of in HG serous OC (32.2% and 36.7%, respectively; Figure 2E). Thus, we compared mutation frequency in patients with no family cancer history diagnosed with these histological tumor types, and we found that although the mutation frequency in sporadic borderline tumors was half in comparison to sporadic HG serous (Figure 3), it still largely exceeded 10% in both hereditary and sporadic cases, justifying the genetic testing of borderline tumors. The large proportion of borderline tumors with positive family cancer history in our study suggested that this OC subtypes belong to a possible manifestation of a cancer predisposition. However, our observation needs to be confirmed in other populations as current reports about borderline tumors in *BRCA1/2* mutation carriers are limited.

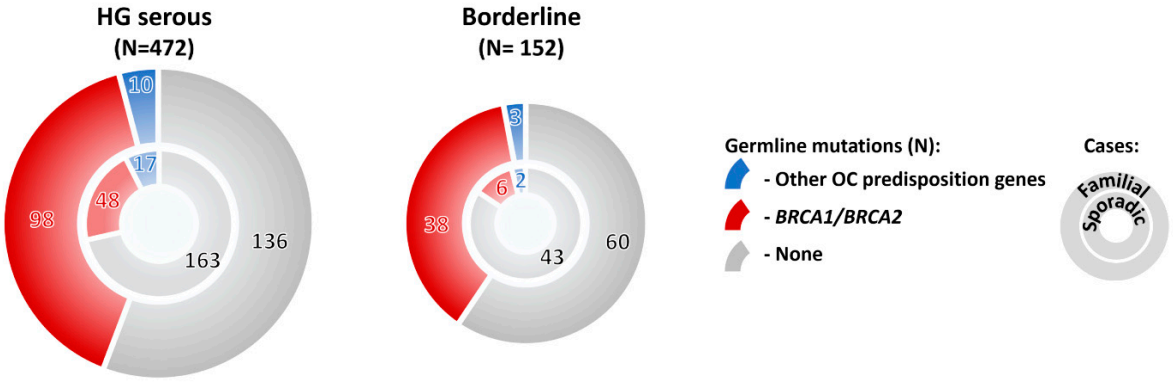

Figure 3. Frequency of mutations in 10 BC/OC predisposition genes significantly associated with OC in our study in OC patients with high-grade (HG) serous and borderline tumors, respectively. The patients were subdivided into subgroups with positive (familial cases) and negative (sporadic cases) family cancer history, respectively.

The multigene panel enabled us to identify other candidate genes associating with increased OC risk. We noticed many rare truncating variants episodically affecting various genes and clustering into *PPM1D*, *NAT1*, and *SHPRH* in OC patients. The *PPM1D* gene, coding for WIP1 phosphatase, was the only candidate associated with OC risk following multiple testing correction. Similarly to the previous studies describing its mosaic variants in OC patients [36–38], we also found mosaic gain-of-function mutations resulting in increased WIP1 phosphatase activity [38]. All *PPM1D* mutations in our patients were identified in postchemotherapy treatment blood samples suggesting their somatic origin [39]. Germline mutations in *NAT1* have not been analyzed for OC predisposition so far. However, several polymorphisms in *NAT1* (coding for arylamine N-acetyltransferase 1 engaged in carcinogen metabolism and detoxification) were shown to modify the risk of various cancers [40,41]. The *SHPRH* gene codes for E3 ubiquitin-protein ligase targeting PCNA upon DNA damage [42]. Contribution of *SHPRH* germline variants to OC risk remains elusive. Overall, low mutation frequencies found in gene candidates in our study precluded its precise OC risk estimations and will require large, multiethnic, case-control studies, segregation analyses in affected families, and functional analyses. Alongside variants clustering to a few candidate genes, we identified rare mutations in a gene family coding for Fanconi anemia (FA) proteins involved in the repair of DNA interstrand crosslinks [43]. Several FA genes belong to established OC predisposition genes, including *BRCA1* (*FANCS*), *BRCA2 (FANCD1*), *RAD51C (FANCO)*, *PALB2 (FANCN)*, and *BRIP1 (FANCJ)*. Except these, we found rare mutations in other FA genes (*FANCG*, *FANCD2*, and *FANCA*) in 11 (0.83%) of 1333 OC patients compared to 5 in 2278 PMC (0.2%), with cumulative OR = 3.78 (95% CI 1.21–13.91; $p = 0.02$). Interestingly, these rare mutations were detected almost exclusively in patients without mutations in other OC predisposition genes.

The strengths of this study include an identical NGS analysis and bioinformatics pipeline in all patients, a careful curation of clinical data, and an ethnically homogeneous set of patients and controls representing the largest sample set from the region of Central and Eastern Europe. Despite

that, the number of individuals still did not allow the precise OC risk calculations in rarely mutated genes. Although all OC cases in the Czech Republic are eligible for genetic testing, OC patients with positive family cancer history and earlier-onset individuals were enriched in our study, especially in a small subgroup enrolled before 2015 (in the Center A only).

Whether the high prevalence of clinically important germline mutations in OC patients justifies population-wide screening is a vivid matter of debate [44–48]. We emphasize that we found *BRCA1/2* mutations in 14.5% of OC patients with no family cancer history who would currently not be revealed presymptomatically without population screening. We assume that careful application of germline testing in all OC patients and their relatives would reduce OC burden in our population. Moreover, the mutations in *BRCA1/2* [49,50] and other OC predisposition genes [51,52] represent valuable predictive biomarkers improving OC chemotherapy.

4. Materials and Methods

Analyzed patients (N = 1333) were enrolled in 2010–2018 and included all OC cases regardless of familial cancer history or OC histology subtypes. As knowledge about germline mutations' frequency in women diagnosed with BTO is limited, we included these histological subtypes to our study. Clinicopathological data were obtained during genetic counselling or retrieved from the patients' records. OC patients with a positive cancer family history were stratified into (i) hereditary ovarian cancer (HOC) families with OC and other nonbreast cancer (BC) in the family history; (ii) hereditary breast and/or ovarian cancer (HBOC) families with BC and OC or other cancer in the family history, and (iii) multiple cancer families with non-OC and non-BC in the family history. Index patients were tested in seven centers: (A) First Faculty of Medicine, Charles University, Prague (N = 637); (B) Masaryk Memorial Cancer Institute, Brno (N = 357); (C) Gennet, Prague (N = 273); (D) AGEL Laboratories, Novy Jicin (N = 34); (E) GHC Genetics (N = 12); (F) Pronatal (N = 11), and (G) University Hospital Olomouc (N = 9).

Population-matched controls (PMC; N = 2278) included 616 noncancer controls collected in centers A (N = 344), B (N = 150), and D (N = 122), and 1662 unselected controls provided by the National Center for Medical Genomics (http://ncmg.cz). The noncancer controls were volunteers (78 males and 538 females) aged ≥ 60 years without a personal or family cancer history (in first-degree relatives). The unselected controls (1170 males and 492 females; median age 57 years, range 18–88 years) were unrelated individuals analyzed by whole-exome sequencing (WES) for various noncancer conditions.

All patients and controls were Caucasians of a Czech origin. Written informed consent was obtained from all patients and controls. The study was approved by the Ethics Committee of the General University Hospital in Prague; ethics approval number was 92/14. The study was performed in accordance with the Declaration of Helsinki.

4.1. Next-Generation Sequencing

Germline blood-derived DNA was analyzed by the CZECANCA (CZEch CAncer paNel for Clinical Application; custom-made SeqCap EZ choice panel; Roche) panel NGS targeting 219 genes on MiSeq (Illumina), as described in details previously [19]. Sequencing reads were aligned by Novoalign v2.08.03 to the human reference genome (hg19). Variants were identified using GATK and Pindel, CNVs using CNV score [19]. The entire diagnostic pipeline was successfully tested using European Molecular Genetics Quality Network schemes (EMQN) and validated as we have described recently [19].

4.2. Variant Classification

We first analyzed 18 genes considered clinically relevant to the HBOC syndrome (MIM #604370) by NCCN, namely, *ATM, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MSH2, MSH6, NBN, PALB2, PTEN, RAD50, RAD51C, RAD51D, STK11,* and *TP53*. Germline variants (with frequency ≤ 0.01 and ≤0.05 in 1000 Genomes project and noncancer PMC, respectively) were classified into three

groups: i) pathogenic/likely pathogenic, ii) variants of unknown significance (VUS), and iii) likely benign/benign, based on recommendations from the ENIGMA consortium (https://enigmaconsortium.org). All nonsense/frameshift/splicing (± 1–2 bp) mutations/CNVs were considered pathogenic/likely pathogenic unless classified as other in the ClinVar database; whole gene duplications were considered VUS. The other types of mutations were considered pathogenic/likely pathogenic only if classified as such in ClinVar by at least two submitters. TP53 variants were classified using the IARC TP53 database (http://p53.iarc.fr/), *CHEK2* VUS using a recently published functional assay [27].

Subsequently, we analyzed variants in another 201 genes targeted by the CZECANCA panel. Nonsense/frameshift/splicing (± 1–2 bp) mutations/CNVs (except whole gene duplications) with frequency ≤0.01 and ≤0.05 in 1000 Genomes project and in noncancer PMC, respectively, were considered pathogenic.

All pathogenic/likely pathogenic mutations in patients and noncancer PMC were confirmed by Sanger sequencing and CNVs by MLPA (if available) or by qPCR (protocol available on request), and they were submitted to ClinVar under the submission ID SUB5822876.

4.3. Statistical Analysis

The odds ratio (OR) for particular gene was calculated using Fisher's exact test, and p values <0.05 were considered significant. The multiple mutation carriers were excluded from the OR calculations. For the identification of other OC candidate genes, the Bonferroni correction was employed. The associations between mutation status and clinicopathological characteristics were estimated using Fisher's exact test, and p values <0.05 were considered significant.

5. Conclusions

Our study demonstrated that nearly one in three OC patients carries a pathogenic mutation in genes significantly associated with OC. The mutation frequency exceeded 10% in all clinicopathological subgroups, regardless of the age at diagnosis, clinical or histopathological characteristics, with an exception of women diagnosed with OC before the age of 30 or with rare histological OC subtypes. Importantly, we found that the high mutation prevalence included borderline tumors justifying genetic testing of all OC patients, including women diagnosed with borderline tumors. Surprisingly, *BRCA1/2* mutations were not associated with sporadic OC in very young women (≤30 years). Besides the established OC predisposition genes, *NBN* and *BARD1* were significantly associated with a moderate OC risk; however, further studies will be required to specify the associated OC risk and to identify the value of the detected genetic mutations in terms of disease prognosis and therapy prediction. Hence, analyses of rarely mutated BC/OC predisposition genes that failed to increase OC risk in our study are further warranted to evaluate their association with OC in future larger dataset and/or in frame of international consortia. These should include also other candidate alterations with predictive and/or prognostic potential.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/4/956/s1, Table S1: the clinicopathological characteristics of 1333 ovarian cancer patients, Table S2: mutation frequencies in ovarian cancer cases and population-matched controls, Table S3: clinical and pathological characteristics in multiple mutation carriers, Table S4: clinicopathological characteristics of mutation carriers in HBOC genes listed in NCCN guidelines (mutation carriers in a group of 1320 ovarian cancer patients (after exclusion of 13 multiple mutation carriers shown in Table S3) (10 genes significantly associated with OC risk in our study are highlighted), Table S5: mutations in 201 additional analyzed genes with associated OC risk (significantly associated genes ($p < 0.05$) are highlighted), Table S6: characteristics of 15 *PPM1D* mutation carriers, and Table S7: clinicopathological characteristics of mutation carriers in *NAT1*, *PPM1D*, and *SHPRH* significantly associated with OC risk.

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Identification of Germline Mutations in Melanoma Patients with Early Onset, Double Primary Tumors, or Family Cancer History by NGS Analysis of 217 Genes

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Abstract: Cutaneous melanoma is the deadliest skin malignity with a rising prevalence worldwide. Patients carrying germline mutations in melanoma-susceptibility genes face an increased risk of melanoma and other cancers. To assess the spectrum of germline variants, we analyzed 264 Czech melanoma patients indicated for testing due to early melanoma (at <25 years) or the presence of multiple primary melanoma/melanoma and other cancer in their personal and/or family history. All patients were analyzed by panel next-generation sequencing targeting 217 genes in four groups: high-to-moderate melanoma risk genes, low melanoma risk genes, cancer syndrome genes, and other genes with an uncertain melanoma risk. Population frequencies were assessed in 1479 population-matched controls. Selected *POT1* and *CHEK2* variants were characterized by functional assays. Mutations in clinically relevant genes were significantly more frequent in melanoma patients than in controls (31/264; 11.7% vs. 58/1479; 3.9%; $p = 2.0 \times 10^{-6}$). A total of 9 patients (3.4%) carried mutations in high-to-moderate melanoma risk genes (*CDKN2A*, *POT1*, *ACD*) and 22 (8.3%) patients in other cancer syndrome genes (*NBN*, *BRCA1/2*, *CHEK2*, *ATM*, *WRN*, *RB1*). Mutations in high-to-moderate melanoma risk genes (OR = 52.2; 95%CI 6.6–413.1; $p = 3.2 \times 10^{-7}$) and in other cancer syndrome genes (OR = 2.3; 95%CI 1.4–3.8; $p = 0.003$) were significantly associated with melanoma risk.

We found an increased potential to carry these mutations (OR = 2.9; 95%CI 1.2–6.8) in patients with double primary melanoma, melanoma and other primary cancer, but not in patients with early age at onset. The analysis revealed affected genes in Czech melanoma patients and identified individuals who may benefit from genetic testing and future surveillance management of mutation carriers.

Keywords: melanoma; familial melanoma; hereditary cancer predisposition; germline mutations; panel sequencing; NGS

1. Introduction

With 287,723 newly diagnosed cases and 60,712 fatalities in 2018, cutaneous melanoma remains the deadliest skin malignity globally. The highest standardized melanoma incidence occurs in Australia and New Zealand; however, European and US patients account for more than 75% of new melanoma cases annually [1]. The GLOBOCAN cancer registry ranks the Czech Republic as 18th among 185 countries in the world in terms of age-standardized melanoma incidence rates (between the USA and Canada) [2].

The risk of melanoma is largely modified by factors influencing individual sensitivity to UV radiation and sunlight exposure, and sunburns during childhood in particular are a major behavioral risk factor [3]. Other individual host factors include the amount, type, and arrangement of cutaneous melanin, the presence of multiple atypical moles (the most frequent precancerous melanoma lesions), and a family history of melanoma [4].

The hereditary component of melanoma development has been assessed in a large prospective study of twins from Nordic countries revealing melanoma heritability with a familial cancer risk of 19.6% and 6.1% for monozygotic and dizygotic twins, respectively, compared with 1.2% for the overall population [5]. The proportion of familial melanoma cases is approximately 5–10%; however, pathogenic germline mutation carriers have been identified in only a minority of the analyzed familial melanoma cases [6].

The major melanoma-susceptibility gene is *CDKN2A*, coding for two alternatively transcribed mRNAs translated into the cyclin-dependent kinase inhibitor $p16^{INK4}$ and the tumor suppressor $p14^{ARF}$ participating in p53 activation, respectively [7]. Germline *CDKN2A* mutations have been found in about 20–40% of melanoma-prone families (with ≥3 melanoma cases), but in only 0.2–3% of non-familial melanoma cases [8,9]. Other high-risk but extremely rare germline mutations affect cyclin-dependent kinase 4 (*CDK4*) and BRCA1-associated protein 1 (*BAP1*) genes [10,11]. Germline *CDK4* mutations cluster in exon 2, coding for a domain interacting with $p16^{INK4}$ [12]. The BAP1 protein codes for deubiquitinase, counteracting BRCA1-BARD1 ubiquitin ligase activity [13]. Hereditary *BAP1* mutations predispose people to hypopigmented skin melanoma, uveal melanoma, mesothelioma, renal cell carcinoma, and other cancers [13]. Other potential high- to moderate-risk genes include *ACD* (also known as *TPP1*), *POT1*, and *TER2IP* coding for shelterin proteins forming a telomere-protecting complex [14]. Rare promoter mutations in telomerase (*TERT* gene) coding for an enzyme-maintaining telomere length have been found in familial melanoma [15]. An increased melanoma risk has been documented in carriers of germline mutations causing other cancer syndromes, including hereditary breast and ovarian cancer (*BRCA1/BRCA2*), retinoblastoma (*RB1*), or xeroderma pigmentosum (*XPs*) [16]. The low-risk group includes variants in genes coding for proteins involved in melanogenesis (MC1R, MITF, OCA2, SLC45A2, TYR, TYRP1) and other processes (ASIP, CASP8, MTAP, OBFC1), revealed dominantly by genome-wide association studies (GWAS) [17–19]. The identification of individuals carrying germline mutations in melanoma-predisposition genes enables their tailored surveillance with an early detection of melanoma and other associated tumors, and with genetic counselling for their relatives.

The Czech national cancer registry has recorded nearly doubled melanoma incidence during the past 25 years (from 7.55 cases per 100,000 inhabitants in 1994 to 13.47 in 2018), and melanoma has become the most rapidly growing malignant tumor among children and teenagers [20,21]. However, an analysis of genetic factors contributing to its development has not been performed in the Czech Republic to date.

Our study aimed primarily to characterize the spectrum and prevalence of germline mutations influencing melanoma risk. We have analyzed 264 high-risk Czech melanoma patients by panel next generation sequencing (NGS) targeting 217 genes that included eight high-to-moderate melanoma risk genes, 26 low melanoma risk genes, 37 other cancer-predisposing genes and 146 genes altered in melanoma but not associated with increased familial risk. Another task of our study was to identify melanoma patients who may benefit from genetic testing by comparing clinicopathological data from the carriers and non-carriers of germline mutations.

2. Materials and Methods

2.1. Study Population

We analyzed genomic DNA obtained from the peripheral blood of 264 unrelated melanoma patients indicated for a genetic analysis by medical geneticists based on individual or family criteria (Table 1). All patients were Caucasians of a Czech origin and provided written informed consent with the analysis approved by Ethics Committee of the General University Hospital in Prague (No.: 56/15 Grant VES 2016 AZV 1.LFUK from 2015/06/18). The patients included a subgroup of 129 individuals (97 females/32 males) indicated at the General University Hospital in Prague and 135 individuals (96 females/39 males) indicated at the Masaryk Memorial Cancer Institute in Brno. Known clinicopathological characteristics are provided in Supplementary Table S1.

Table 1. Characteristic of subgroups combining personal cancer history (rows) and family cancer history (FCH; columns) criteria in 264 melanoma (M.) patients enrolled in the study.

Criteria	Posit. FCH incl. M.	Posit. FCH incl. Other Cancers	Negative FCH	Unknown FCH	Patients; N (%)	Mean Age; yrs (Range)
Multiple primary M. & other cancer	0	4	0	2	6 (2.3)	45.0 (38–58)
Multiple primary M.	5	8	3	1	17 (6.4)	37.3 (24–75)
M. & other cancer	9	45	9	8	71 (26.9)	47.3 (14–83)
M. only, dg at < 25 yrs	5	17	11	3	36 (13.6)	20.0 (9–24)
M. only, dg at ≥ 25 yrs	41	62	24	7	134 (50.8)	37.1 (25–69)
Patients; N (% of all)	60 (22.7)	136 (51.5)	47 (17.8)	21 (8.0)	264 (100)	37.7 (9–83)
Mean age; yrs (range)	38.9 (9–69)	37.8 (14–83)	33.0 (15–66)	44.2 (14–75)	-	-

The control population included germline variants in targeted genes obtained from whole exome sequencing (WES) performed for various non-cancer conditions in 1479 unselected, adult, anonymized, ethnically matched controls (1014 males, mean age 55.5 years, range 18–88 years and 465 females, mean age 56.8 years, range 18–84). These anonymized genotypes of population-matched controls were provided by the National Center for Medical Genomics (http://ncmg.cz).

2.2. CZMELAC Sequence Capture Panel

The CZMELAC panel (CZech MELAnoma panel for Cancer predisposition) targeted 217 genes including (i) high-to-moderate and (ii) low melanoma risk genes, (iii) hereditary cancer syndrome genes with an uncertain melanoma risk, (iv) genes associated with "melanoma" in the Phenopedia database with at least two entries (assessed June 16, 2016; Table 2) [6,9,14,16,22–25].

The primary gene target for probe coverage was represented by all coding exons, including 10 bases from adjacent intronic regions, and it was designed using the NimbleDesign software (Roche) as

described previously [26,27]. The final CZMELAC panel target reached 563,471 bases. Because of the strict design conditions, some repeats and homologous regions were left untargeted (Supplementary Table S2).

Table 2. Analyzed genes in CZEMELAC (CZech MELAnoma panel for Cancer predisposition) panel. Detailed information, including full names of analyzed genes, is provided in Supplementary Table S2.

High-to-moderate melanoma risk genes	*ACD, BAP1, CDK4, CDKN2A, MITF, POT1, TERF2IP, TERT*
Low melanoma risk genes	*AGR3, ARNT, ASIP, CASP8, CCND1, CDKN2B, CLPTM1L, FTO, HERC2, IRF4, MC1R, MGMT, MTAP, MX2, OBFC1, OCA2, PARP1, PLA2G6, SETDB1, SLC24A4, SLC45A2, TERF1, TERF2, TINF2, TYR, TYRP1*
Hereditary cancer syndrome genes with uncertain melanoma risk	*APC, ATM, BARD1, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, FH, CHEK2, KIT, MET, MSH2, MSH3, MSH6, NBN, NF1, NF2, PALB2, PMS2, POLD1, POLE, PTEN, RAD51C, RAD51D, RB1, RET, SDHA, SDHB, SDHC, SDHD, SMAD4, STK11, TP53, VHL, WRN, WT1*
Genes with unknown impact on hereditary melanoma development	*ABLIM1, APEX1, ATRN, AURKA, BBC3, BLM, BRAF, BRMS1, CASP10, CBL, CCAR2, CCNH, CDK10, CDK7, CDKN1A, CDKN1B, CDKN1C, CEBPA, COX8A, CTLA4, CTNNB1, CYP11A1, CYP17A1, CYP19A1, CYP1A1, CYP1A2, CYP3A5, DAB2IP, DCAF4, DDB1, DDB2, EDNRB, EGF, EGFR, EIF1AX, EPCAM, ERBB2, ERBB4, ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, ERCC6, ERCC8, EXOC2, EZH2, FANCC, FANCL, FANCM, FAS, FASLG, FGFR2, FGFR4, FLCN, FLT1, FOXP3, GATA2, GATA4, GC, GNA11, GNAQ, GPC3, GSTM1, GSTM3, GSTP1, GSTT1, H2AFY, HRAS, IDH1, IDH2, IFIH1, IFNA1, IFNG, IL10, IL2RA, IL4, IL6, IL8, ING4, KAT6A, KIAA1967, KMT2A, KRAS, LRIG1, MAP2K1, MDM2, MLH1, MLH3, MMP1, MMP3, MUTYH, MYH7B, NCOA6, NFKB1, NFKBIE, NOD2, NOTCH3, NRAS, PAX5, PDGFRA, PIGU, PIK3CA, PIK3R1, PIK3R4, PMAIP1, PMS1, POLH, POMC, PPM1D, PPP6C, PRF1, PTGS2, PTCH1, PTPN11, PTPN22, RAC1, RAD23A, RAD23B, RASEF, RECQL, RECQL4, RHOBTB2, RUNX1, SBDS, SF3B1, SH2B3, SLX4, SMARCB1, SNX31, STAG2, STK19, SUZ12, TACC1, TERC, TLR3, TRPM1, TSC1, TSC2, VDR, XAB2, XPA, XPC, XRCC1, XRCC3, ZNF365*

2.3. Targeted NGS Analysis

Genomic DNA was isolated from peripheral blood and 200–500 ng was used to prepare the NGS library. DNA was diluted in low TE buffer [10 mM Tris-HCl (pH 8.0) with 0.1 mM EDTA] and sheared by ultrasound (Covaris E220; Covaris, Chicago, IL, USA) to approximately 200 bp fragments checked using Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The subsequent end-repair, A-tailing, and ligation of adapters were performed using the KAPA HTP Library Preparation kit (Roche, Basel, Switzerland) according to the manufacturer with in-house prepared adapters. The processed fragments were size-selected (targeting 250–450 bp fragments) and primed with barcodes (identical to Illumina TruSeq HT index i7 and i5) by ligation-mediated PCR (LM-PCR), using in-house prepared double-indexing primers, to distinguish individual samples in subsequent pooling. The size and quality of fragments after the dual size selection and LM-PCR were controlled using Agilent High Sensitivity DNA Kit. Thirty individual samples (33 ng each) were pooled for enrichment and hybridized for 72 h with the CZMELAC panel probes (SeqCap EZ Choice Library; Roche, Basel, Switzerland). The enriched targeted sequences were amplified by post-capture PCR to create the final sequencing library. The enrichment was controlled using qPCR (NimbleGen SeqCap EZ Library SR User's Guide). The final 15 µM library was sequenced on MiSeq using MiSeq Reagent Kit v. 3 (150 cycles; Illumina, San Diego, CA, USA) targeting 100× mean coverage per sample.

2.4. Bioinformatics

The CZMELAC panel sequencing data generated in FASTQ files were analyzed as described previously [27]. Novoalign was used for mapping FASTQ files to hg19 reference. The variant-call format (VCF) files were processed by the GATK pipeline (https://software.broadinstitute.org/gatk/) from BAM files. The VCF files were annotated using SnpEff. We identified medium-size indels (insertions or duplications >35bp) using Pindel (http://gmt.genome.wustl.edu/packages/pindel/) and copy number variations (CNV) using CNVkit (https://pypi.python.org/pypi/CNVkit), using the settings that we described in detail recently [26,27].

2.5. Variant Filtration and Prioritization

The primary list of annotated sequences was filtered in successive steps that included the elimination of: (i) low quality variants (quality < 150); (ii) out of bait variants (intergenic/deep intronic/UTR variants); (iii) intronic variants out of canonical splicing sites (±1–2 nucleotides in introns); (iv) variants with a minor allele frequency (MAF) > 0.003 in any of the ExAC/ESP6500/1000Genomes databases; (v) variants with MAF > 0.001 (n > 2) in 1479 population-matched controls; (vi) synonymous variants; (vii) variants referred to as benign or likely benign (B/LB) in ClinVar; (viii) variants located in the repeat masking track from the UCSC Genome Browser; (ix) frameshift/stop-gain variants in the last exon. Filtration steps ii-ix were not applied if the found variants were referred to as pathogenic/likely pathogenic (P/LP) in ClinVar or "deleterious" in our functional analyses. The dataset of the control population was filtered identically. The final set of P/LP variants included only frameshift, stop-gain, frameshifting CNV, canonical splicing, ClinVar P/LP variants, and variants classified as "deleterious" by our functional analyses. All P/LP variants (variants with very strong and strong evidence of pathogenicity according to the ACMG guidelines [28] denoted throughout this text also as "mutations") were in melanoma patients manually inspected in IGV and, when uncertain, confirmed by Sanger sequencing. The CNV P/LP variants were confirmed by multiplex ligation-dependent probe amplification (MLPA; for *CHEK2*) or by quantitative PCR (for *SLC45A2* and *TRPM1*; Supplementary Figure S1).

2.6. Analysis of Splicing Alterations

All RNA samples obtained from peripheral blood or from expanded leukocytes (with/without nonsense-mediated decay inhibitor) were analyzed for splicing alterations using targeted RNA NGS with the CZMELAC panel, as described recently [29].

2.7. Statistical Analysis

The differences between the analyzed groups and subgroups were calculated by χ^2 or Fischer exact tests.

2.8. Functional Assays for Selected Germline Variants

2.8.1. CHEK2 Functional Analysis

A functional analysis of *CHEK2* VUS was performed as described recently [30]. Human RPE1-CHEK2-knock-out cells were transfected with wild-type or mutant EGFP-CHK2 and the level of KAP1-S473 phosphorylation was determined by immunofluorescence microscopy using ScanR station (Olympus, Tokyo, Japan).

2.8.2. POT1 Functional Analysis

Cell lines and plasmids. MCF-7 and HEK293 cells (generously provided by Rene Medema, NKI, Amsterdam) were grown in DMEM containing 6% FBS, penicillin (100 U/mL) and streptomycin (0.1 mg/mL). The cells were regularly tested for mycoplasma contamination using the MycoAlert kit (Lonza, Basel, Switzerland). A DNA fragment corresponding to human POT1 was PCR-amplified from pLPC-myc-hPOT1 (Addgene, ID:12387, Watertown, MA, USA) and inserted in frame into the XhoI/XmaI sites of pEGFP-C3. Plasmid pCDNA-3xFLAG-NLS-TPP1 was obtained from Addgene (ID: 53585, Watertown, MA, USA). Cells were transfected with plasmid DNA using polyethylenimine 40K (Polysciences, Warrington, PA, USA).

Immunofluorescence microscopy. To evaluate the localization of POT1 at telomeres, MCF-7 cells grown on coverslips were transfected with EGFP-POT1 or EGFP-POT1-P116L and analyzed by immunofluorescence microscopy. Cells were pre-extracted with 0.5% Triton-X 100 in ice-cold PBS for 5 min and fixed with 4% PFA for 15 min in room temperature (RT). Cells were blocked in 1% BSA for 30 min. Coverslips were incubated with TRF2 antibody (clone B-5, Santa Cruz, Dallas, TX, USA) for

2 h in RT, washed 3× in PBS, incubated with secondary antibody for 1h in RT. After washing in PBS and DAPI, coverslips were mounted with Vectashield and images were acquired using the confocal microscope Leica (Wetzlar, Germany) TCS SP8 equipped with a 63x/1.40 objective.

Immuno-precipitation. The ability of POT1 to interact with the shelterin complex was evaluated by immuno-precipitation. HEK293 cells were co-transfected with FLAG-TPP1 and EGFP, EGFP-POT1 or EGFP-POT1-P116L. Cells were extracted in IP buffer (50 mM Tris pH 8.0, 120 mM NaCl, 1% Tween-20, 0.1% NP-40, 1.0% glycerol, 2 mM EDTA, 3 mM EGTA, 10 mM $MgCl_2$, protease inhibitors (Roche, Basel, Switzerland) and EtBr (50 µg/mL)) and sonicated 3 × 20 sec. Clarified cell extracts were incubated with GFP-Trap beads (Chromotek, Planegg, Germany) for 1 h. After washing 4× with IP buffer, bound proteins were eluted with Laemli buffer and separated by SDS-PAGE.

Telomeric DNA binding assay. POT1 binding to telomeric DNA was tested in vitro as described [31,32]. HEK293 cells transfected with EGFP, EGFP-POT1 or EGFP-POT1-P116L were extracted in IP buffer, sonicated and centrifuged for 20 min at 4 °C. Cell extracts were precleared with streptavidin sepharose beads for 1 h. Supernatants were then incubated with 2 µg of biotinylated telomeric DNA (ssG: biotin-TATATA(TTAGGG)8) or (tel5: biotin-GCAAGCTTTACCGATACA GC(TTAGGG)5) [31,32], or control DNA (ssC: biotin-TATATA(CCCTAA)8), for 12 h and Streptavidin beads were added for 1 h before washing with IP buffer. Bound proteins were eluted with Laemli buffer and analyzed by Western blotting (WB) using antibody against GFP (clone 7.1, Roche, Basel, Switzerland).

3. Results

3.1. Germline Variants in Analyzed Genes

The overall mean coverage for all samples reached 116.7× with a good coverage uniformity across 217 analyzed genes (mean percent of target bases with coverage 20×, 50×, and 100× was 99.3%, 96.9%, and 79.2%, respectively). Panel NGS in 264 patients yielded 16,359 unique germline variants. Five hundred and sixteen of them remained after the application of variant filtration rules (described in the Methods section). Variants of uncertain significance (VUS) represented a majority (87%) of them and were excluded from further analyses as clinically inconclusive at the moment. The final 83 pathogenic/likely pathogenic (P/LP) germline variants (66 unique) in 71/264 (26.8%) melanoma patients were detected in 42/217 targeted genes (Supplementary Table S3) and included five copy number variants (CNV; two in *CHEK2* and *SLC45A2*, respectively, and one in *TRPM1*; Supplementary Figure S1). Using the identical prioritization procedure, we identified 225 P/LP variants in 204/1479 (13.8%) controls in 82/217 targeted genes, including two CNV (both in the *CHEK2* gene). Overall, 43/264 (16.3%) patients (Table 3) and 87/1479 (5.9%) controls carried a mutation in a gene previously associated with melanoma or other cancer.

Table 3. Germline P/LP (pathogenic/likely pathogenic) variants in melanoma patients.

(a)	Gene: Coding Sequence (Protein) Change - Concomitant Mutation	Mel Site (Age) (b)	Other Tumors in Proband (Age)	Family Cancer History Tumor Type (N) (c)
	High-to-moderate risk genes			
F	*CDKN2A*: c.16_20del5 (p.G6Qfs*7)	TR (38)	none	BC (1), Leu (1), Mel (1), other 3 relatives with unknown tumors
F	*CDKN2A*: c.71G>C (p.R24P)	TR (24)	Mel (35)	CRC (1), Mel (1), UrC(1)
F	*CDKN2A*: c.71G>C (p.R24P)	TR (28)	Mel (38)	Mel (2)
F	*CDKN2A*: c.95_112del (p.L32_L37del)	LE (28)	GC (48)	BC (2), CRC(1), GC (1), LC (1), Mel (2)
M	*CDKN2A*: c.334C>G (p.R112G)	HE (43)	none	Mel (1), PaC(1)
F	*CDKN2A*: c.457+4_457+5delAG (p.Y129Hfs*11)	TR (29)	Mel (34)	BT (1)
F	*POT1*: c.347C>T (p.P116L); - *CHEK2*: c.909-2028_1095+330del5395 (p.M304Lfs*15)	UE (41)	Mel (41,42,44); BC (47)	RC (1)
F	*POT1*: c.703-1G>C (p.V235Gfs*22)	n.a. (37)	TC (34); BT (47)	BC (1), CRC (1), LC(1), SgT (1), TC (1)
M	*ACD*: c.755delA (p.D255Afs*9)	UE (39)	none	negative

Table 3. *Cont.*

[a]	Gene: Coding Sequence (Protein) Change - Concomitant Mutation	Mel Site (Age) [b]	Other Tumors in Proband (Age)	Family Cancer History Tumor Type (N) [c]
Low-risk genes				
F	*OCA2*: c.1211C>T (p.T404M); - *KAT6A*: c.1138G>T (p.E380*)	n.a. (29)	none	Mel(1)
M	*OCA2*: c.1327G>A (p.V443I)	TR (15)	none	negative
F	*OCA2*: c.1327G>A (p.V443I)	TR (43)	none	BC (3), CRC (3), PaC (1)
F	*OCA2*: c.1327G>A (p.V443I)	LE (52)	Ly (38); SkC (49)	Leu (1), Unknown (1)
M	*OCA2*: c.2037G>C (p.W679C)	n.a. (50)	none	negative
M	*OCA2*: c.2037G>C (p.W679C)	n.a. (68)	SkC (68)	n.a.
M	*TYRP1*: c.1054_1057del4 (p.N353Vfs*31); - *TRPM1*: Δe2-7 (p.?)	TR (36)	none	Mel (2)
M	*SLC45A2*: Δe1-2 (p.?); - *GSTM3*: c.393C>A (p.Y131*)	EY (25)	none	n.a.
F	*SLC45A2*: Δe1-4 (p.?)	TR (42)	BC (41)	PrC (1)
M	*TYR*: c.650G>A (p.R217Q)	TR (37)	none	negative
F	*TYR*: c.1037-7T>A (p.?); - *FANCC*: c.455dupA (p.N152Kfs*9)	HE (66)	BC (52); CRC (66)	BC (2), HCC (1),
F	*TINF2*: c.796C>T (p.R266*)	UE (48)	none	CRC (2), GbC (1), Mel (1), PrC (2), RC (1), Sarcoma (1)
Hereditary cancer syndrome genes				
F	*NBN*: c.657_661del5 (p.K219Nfs*16)	TR (24)	none	BC (1), BT (1), Mel (1)
F	*NBN*: c.657_661del5 (p.K219Nfs*16)	EY (25)	none	negative
M	*NBN*: c.657_661del5 (p.K219Nfs*16)	TR (37)	none	n.a.
F	*NBN*: c.657_661del5 (p.K219Nfs*16)	HE (45)	Mel (68); OC (56)	n.a.
F	*NBN*: c.657_661del5 (p.K219Nfs*16)	TR (65)	OC (67)	negative
M	*NBN*: c.1126delG (p.D376Ifs*2)	n.a. (47)	none	LC (2), Mel (1),
F	*NBN*: c.1723G>T (p.E575*); - *NFKBIE*: c.165_169dup5 (p.E57Afs*51)	LE (9)	none	Mel (1)
M	*BRCA2*: c.475G>A (p.V159M)	UE (45)	RC (46)	HL (1)
F	*BRCA2*: c.1389_1390delAG (p.V464Gfs*3)	LE (47)	BC (59,59)	GC (2)
F	*BRCA2*: c.5682C>G (p.Y1894*)	n.a. (67)	BT (59); BC (56)	3 sisters with gynecological tumors, LC (1), retinoblastoma (1)
M	*BRCA2*: c.7007G>A (p.R2336H); - *IFIH1*: c.2464C>T (p.R822*)	HE (22)	none	BT (1), PrC (2), TC (1)
M	*BRCA2*: c.8168_8172ins4 (p.Y2726Mfs*10); - *TYRP1*: c.1254C>A (p.Y418*)	n.a. (40)	Mel (36); NHL (38)	LC (2)
F	*BRCA1*: c.68_69delAG (p.E23Vfs*17)	TR (47)	UrC (56); OC (57)	n.a.
F	*BRCA1*: c.1687C>T (p.Q563*)	EY (54)	BC (46)	OC (1)
F	*BRCA1*: c.4214delT (p.I1405Kfs*10); - *ATM*: c.7630-2A>C (p.?); - *MUTYH* c.1187G>A (p.G396D)	LE (46)	OC (46); BC (49)	BC (3), OC (2)
F	*BRCA1*: c.5266dupC (p.Q1756Pfs*74)	TR (53)	BC (54)	negative
M	*CHEK2*:c.909-2028_1095+330del5395 (p.M304Lfs*15)	UE (28)	none	CRC(1), Ly (1), Mel (1), MMT (1)
M	*CHEK2*: c.846+4_846+7del4 (p.D265-H282del)	TR (38)	none	BC (1), CRC (2)
F	*ATM*: c.381delA (p.V128*) - *WRN*: c.1105C>T (p.R369*)	TR (41)	Mel (50)	BC (2), PaC (1)
F	*ATM*: c.5932G>T (p.E1978*)	TR (35)	none	LC (1), UrC (1)
F	*RAD51D*: c.405+2T>C (p.?); - *CHEK2*: c.917G>C (p.G306A)	TR (26)	none	CC (1)
F	*RB1*: c.608-1G>T (p.?)	TR (32)	BC (45)	GbC (1), LC (1)

[a] gender: M—male; F—female. [b] Melanoma localization: EY—eye; HE—head; LE—lower extremity; TR—trunk; UE—upper extremity. [c] BC—breast cancer; BT—brain tumor; CC—cervix cancer; CRC—colorectal cancer; GC—gastric cancer; GbC—gallbladder cancer, HCC—hepatocellular cancer; (n)HL—(non)Hodgkin lymphoma; LC—lung cancer; Leu—leukemia; Ly—lymphoma; Mel—melanoma; MMT—malignant mesenchymal tumor; OC—ovarian cancer; PaC—pancreatic cancer; PrC—prostate cancer; RC—renal cancer; SgT—salivary gland tumor; SkC—skin cancer; TC—thyroid cancer; UrC—urinary cancer. The reference numbers for genes listed in this table are provided in Supplementary Table S1.

3.1.1. Mutations in High-to-Moderate Melanoma Risk Genes

The highest prevalence in a subgroup of high-to-moderate melanoma risk genes was found in *CDKN2A* (NM_000077). Disease-causing variants identified in six patients included ClinVar P/LP missense variants c.71G>C (p.R24P; in two patients) and c.334C>G (p.R112G), frameshift c.16_20delGGGAG (p.G6Qfs*7), in-frame c.95_112del18 (p.L32_L37del; shortening C-terminal part of

ankyrin 1 domain and adjacent β-hairpin loop), and the novel splicing alteration c.457+4_457+5delAG, resulting in the activation of an aberrant splicing site (r.384_457del74) and a frameshift (p.Y129Hfs*11; Figure 1).

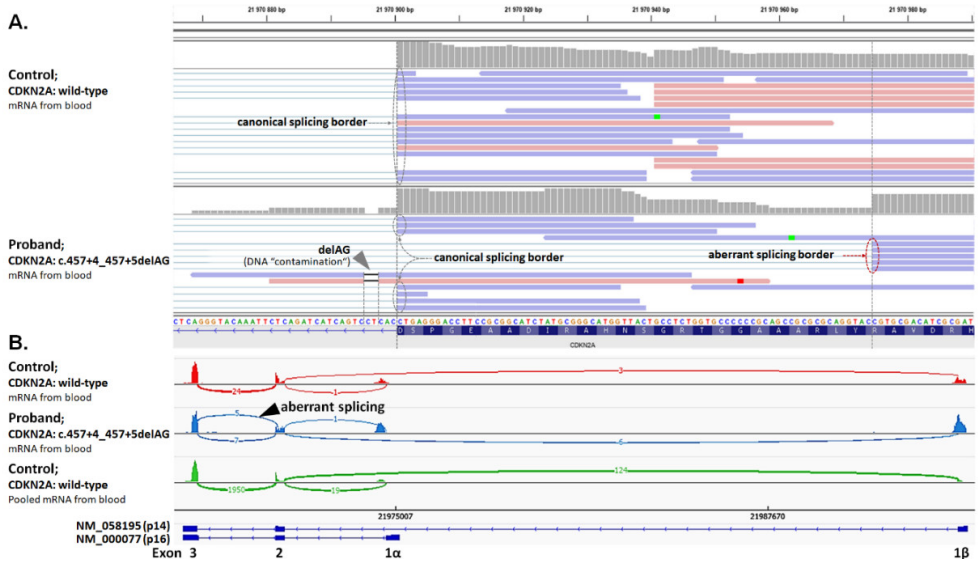

Figure 1. Characterization of splicing aberrations in *CDKN2A*. (**A**) NGS analysis of RNA isolated from blood lymphocytes identified aberrant splicing in a proband carrying the c.457+4_457+5delAG variant (visible as two reads originated from DNA "contamination"; grey arrowhead). The variant causes the elimination of the canonical splice site and activation of the cryptic splice site within exon 2, resulting in the deletion of 74 nts (r.384_457del74) and premature protein termination (p.Y129Hfs*11). (**B**) The sashimi plot shows the presence of aberrant splicing in 5/12 reads in a proband's sample, absent in 24 reads of a control with wild-type *CDKN2A*, and another 1950 reads of 100 pooled controls.

Two germline mutations were also found in *POT1* (NM_015450). The c.703-1G>C mutation found in a proband with melanoma, dysplastic nevi, and thyroid cancer (Figure 2A) affected the canonical acceptor splice site of intron 10 resulting in exon 10 skipping at the mRNA level (r.703_869del167) and a frameshift (p.V235Gfs*22; Figure 2B). The rare missense variant c.347C>T changed the conserved amino acid p.P116L [33] in a patient with superficial spreading melanoma and breast cancer carrying also a germline deletion of 5395bp affecting exons 9 and 10 of the *CHEK2* gene (NM_007194) (Figure 2C). To dissect the functional consequences of the *POT1* missense variant inherited from the maternal branch of the family, we performed a functional analysis. First, we immuno-precipitated wild-type EGFP-POT1 or mutant EGFP-POT1-P116L from transiently transfected cells and found that both variants bound comparable levels of TPP1 (alias ACD) protein which mediates the binding of POT1 to the shelterin complex (Figure 2D). Confocal microscopy revealed that EGFP-POT1-P116L colocalized with TRF2, suggesting that it can assemble into the shelterin complex and correctly localize to telomeres (Figure 2E). Since the p.P116L mutation resides within the oligosaccharide/oligonucleotide-binding (OB1) domain [34], we hypothesized that it may impair the binding of POT1 to ssDNA. Indeed, we found that only the wild-type POT1 (but not POT1-P116L) mutant bound to the biotinylated telomeric G strand efficiently (Figure 2F). We concluded that although the p.P116L isoform can localize to telomeric dsDNA through its interaction with ACD, it fails to bind telomeric ssDNA, which makes it a functionally deleterious mutation contributing to melanoma risk.

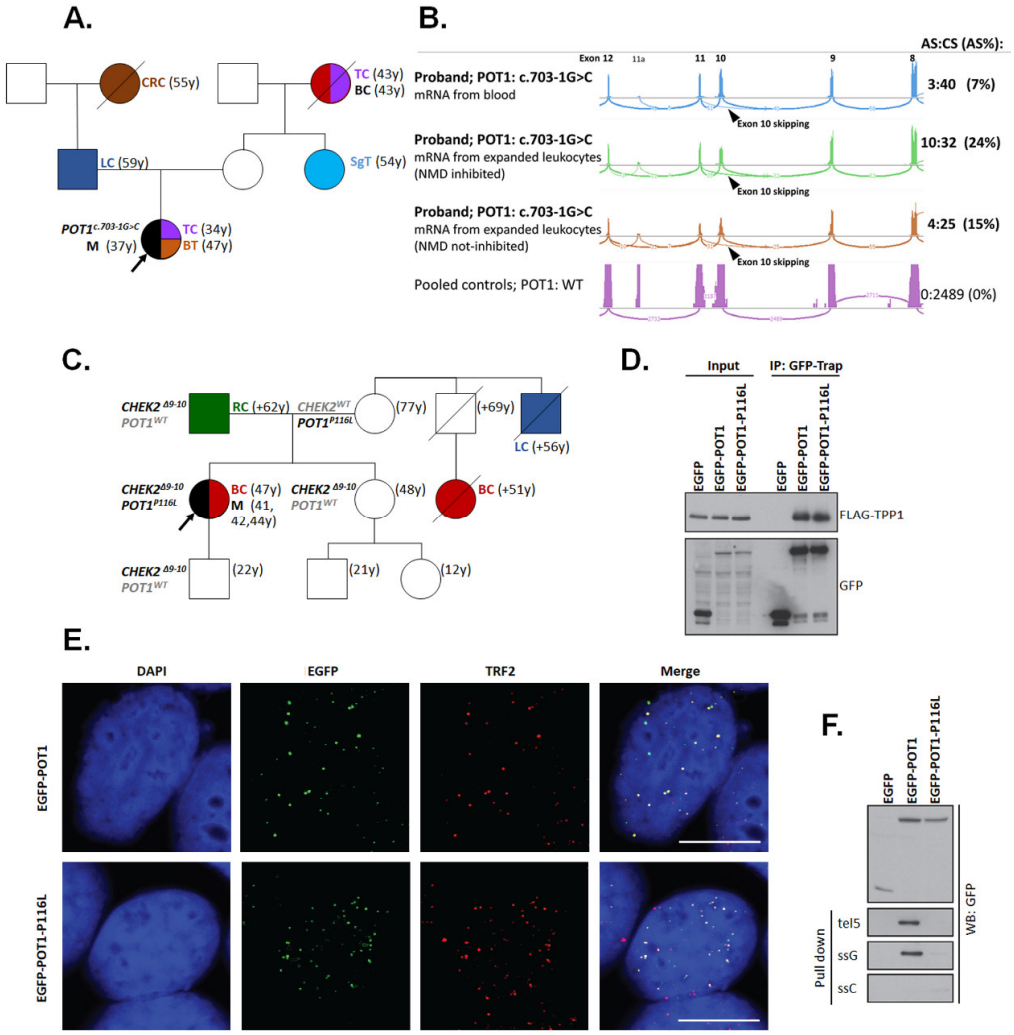

Figure 2. Characterization of *POT1* germline variants. (**A**) Family of a patient carrying c.703-1G>C. (**B**) The variant causes aberrant splicing (AS) with exon 10 skipping (r.703_869del167; arrowhead; resulting in a frameshift at the protein level: p.V235Gfs*22) that was never observed in an analysis of wild-type POT1 samples (compared in blue and purple sashimi plots). However, AS mRNA is mostly subjected to nonsense-mediated decay (NMD). The number of NGS reads of non-degraded AS products in comparison with reads from canonical splicing (CS) products increased upon the cultivation of the patient's lymphocytes with puromycin (an NMD inhibitor; compared as green and brown plots). (**C**) Segregation of germline mutations in a family with missense p.P116L *POT1* and CNV *CHEK2* (c.909-2028_1095+330del5395) germline mutations. (**D**–**F**) Functional characterization was performed for the p.P116L *POT1* mutation. (**D**) POT1-P116L interacts with shelterin components. Extracts from cells transfected with FLAG-TPP1 (alias ACD) and EGFP, EGFP-POT1 or EGFP-POT1-P116L were immuno-precipitated using GFP-Trap. Bound proteins were analyzed with EGFP and FLAG antibodies. (**E**) POT1-P116L is able to localize to telomeres. Cells transfected with EGFP-POT1 or EGFP-POT1-P116L were fixed and stained with TRF2 antibody and analyzed using confocal microscopy. A representative image of a single plane is shown. Bar indicates 10 μm. (**F**) POT1-P116L mutant does not bind telomeric ssDNA. Extracts from cells transfected with EGFP, EGFP-POT1 or EGFP-POT1-P116L were incubated with biotinylated oligonucleotides corresponding to telomeric ssDNA (tel5 and ssG) or control DNA (ssC) and pulled down with streptavidin beads. The bound proteins were analyzed by immunoblotting using anti-GFP antibody. Abbreviations: BC—breast cancer; BT—brain tumor; CRC—colorectal cancer; LC—lung cancer; M—melanoma; RC—renal cancer; SgT—salivary gland tumor; TC—thyroid cancer.

One patient carried the c.755delA (p.D255Afs*9) mutation in *ACD* (NM_001082486), another shelterin complex gene associated with high melanoma risk [35]. This mutation results in the truncation of the POT1-binding domain of the ACD protein. Another *ACD* mutation, c.617dupT (p.H206Qfs*26), was the only P/LP variant from the category of high-to-moderate risk genes found in the control group. Although we did not find mutations in *TERT*, *BAP1*, or *CDK4*, germline mutations in the high-to-moderate risk category were present in 3.4% of patients (Table 4).

Table 4. Frequency of pathogenic/likely pathogenic (P/LP) germline variants in melanoma-susceptibility subgroups classified according to the risk of hereditary/familial melanoma risk. Eleven carriers of more than one P/LP variant were excluded from the analysis.

Melanoma Susceptibility Class	P/LP Variants; N (%)		OR (95%CI); *p*
	264 Patients	1479 Controls	
Multiple Mutation Carriers INCLUDED *			
High-to-moderate risk melanoma genes	9 (3.4)	1 (0.1)	52.2 (6.6–413.1); 3.2×10^{-7}
Low-risk melanoma genes	12 (4.5)	35 (2.4)	1.9 (1.0–3.8); 0.06
Hereditary cancer syndrome genes	22 (8.3)	57 (3.9)	2.3 (1.4–3.8); 0.003
Genes with unknown familial melanoma risk	28 (10.6)	132 (8.9)	1.2 (0.8–1.8); 0.4
Multiple Mutation Carriers EXCLUDED			
High-to-moderate risk melanoma genes	8 (3.2)	1 (0.1)	48.1 (6.4–2116.9); 1.5×10^{-6}
Low-risk melanoma genes	8 (3.2)	35 (2.4)	1.3 (0.5–3.0); 0.51
Hereditary cancer syndrome genes	16 (6.3)	57 (3.9)	1.7 (0.9–3.0); 0.09
Genes with unknown familial melanoma risk	28 (10.6)	132 (8.9)	1.2 (0.8–1.8); 0.4

* If carriers of concomitant mutations pertained to more than one risk group, they were assigned to a group with a higher risk as shown in Table 3: High-risk melanoma genes > Hereditary cancer syndrome genes > Low-risk melanoma genes > Genes with unknown familial melanoma risk.

3.1.2. Mutations in Low-Risk Melanoma Genes

The low-risk melanoma gene group revealed 12 carriers of mutations in 5 genes (Table 3; another *TYRP1* carrier also had a pathogenic *BRCA2* mutation). Hereditary melanoma risk was not increased in carriers of low-risk gene mutations (Table 4); however, we found a higher frequency in patients vs. controls for mutations in *TYRP1* (0.8 vs. 0%; $p = 0.02$) and *OCA2* (2.3 vs. 0.5%; OR = 4.3; 95%CI 1.2–14.2; $p = 0.01$); Supplementary Table S4.

3.1.3. Mutations in Genes Associated with Hereditary Cancer Syndromes

Altogether, 22/264 (8.3%) patients (Table 3) and 57/1479 controls (3.9%) carried a P/LP variant in genes associated with hereditary cancer syndromes. Overrepresentation of mutations in patients indicated an increased melanoma risk in carriers of mutations in hereditary cancer syndrome genes (OR = 2.27; 95%CI = 1.36–3.78; $p = 0.003$); however, melanoma risk lost its significance after the exclusion of six patients carrying other concomitant mutations (Table 4). The mutations in *NBN* (OR = 10.0; 95%CI 2.5–47.0; $p = 3.2 \times 10^{-4}$) and *BRCA2* (OR = 9.5; 95%CI 1.8–61.4; $p = 0.003$) were the most frequent and significantly associated with hereditary melanoma. The frequencies of germline mutations in *CHEK2* gene (Supplementary Figures S1 and S2), *BRCA1*, and *MUTYH* were three times higher in patients over controls but marginally insignificant (all $p = 0.051$; Supplementary Table S4).

3.1.4. Mutations in Other Genes with Unknown Familial Melanoma Risk

Mutations in 23 other genes with unknown familial melanoma risk were found in 28/264 (10.7%) patients and in a similar proportion of controls (132/1479; 8.9%). Neither the genes individually (Supplementary Table S5) nor the entire group of these genes (Table 4) were associated with a significant increase in melanoma risk.

3.2. Clinicopathological Characteristics of Melanoma Patients Carrying Germline Mutations

A total of 11 carriers of more than one P/LP variant were excluded from the comparison of clinicopathological characteristics performed in the remaining 60 carriers of P/LP variants and 193 non-carriers (Figure 3A).

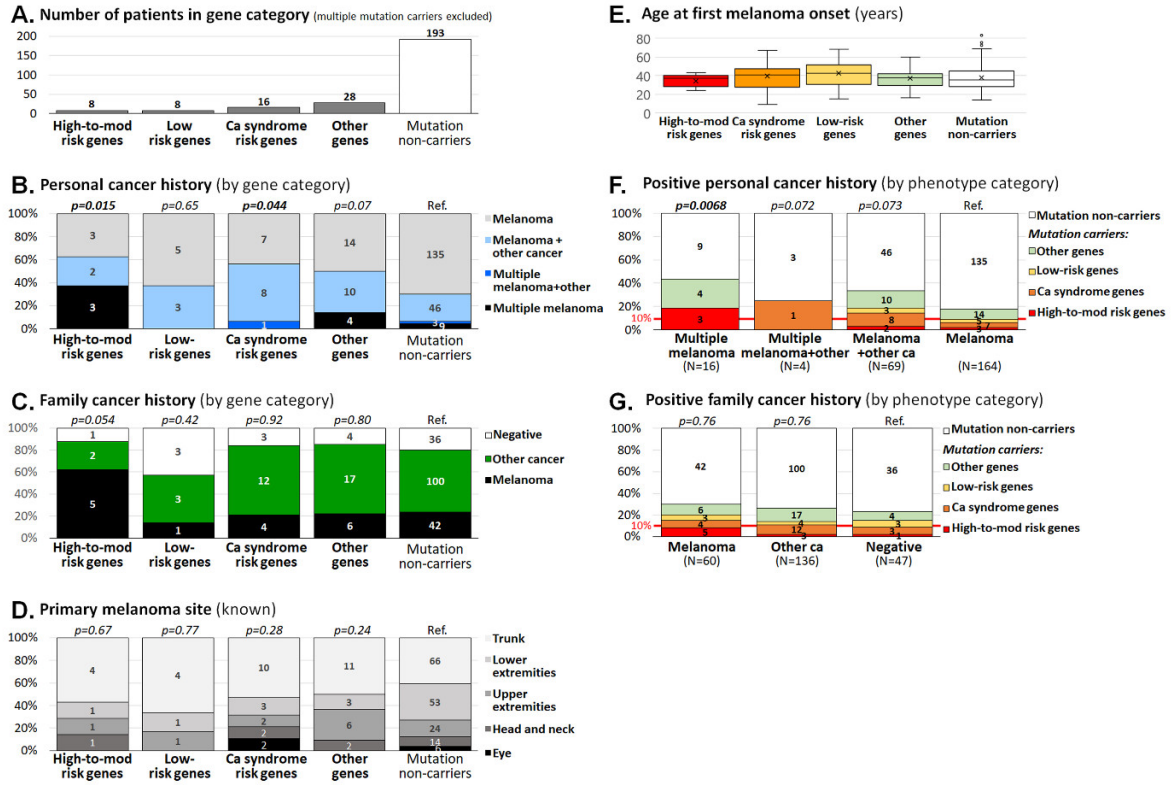

Figure 3. Clinicopathological characteristics of melanoma patients based on the presence of germline mutations. Panel **A** overviews the number of melanoma patients in the gene categories displayed in panels **B** to **E**. The *p*-values express significance of the differences in distribution of variables between particular category of mutation carriers and non-carriers (considered as the reference). Panel **F** and **G** display proportion of mutation carriers in analyzed gene categories in individuals with positive personal cancer history (**F**); excluding 11 multiple mutation carriers) and in individuals with known positive family cancer history (**G**); excluding 21 individuals with unknown family cancer history). Differences in proportions of carriers and non-carriers (*p*-values) in particular subgroups were calculated in patients with positive personal history (**F**) against patients with melanoma only (Ref.) and in patients with positive family cancer history (**G**) against patients with negative cancer history (Ref.).

Classification according to the presence of mutations in melanoma susceptibility classes (shown in Table 4) revealed an increased frequency of patients with multiple melanoma or double primary tumors among the carriers of mutations in high-to-moderate melanoma risk genes (5/8; 63% patients) and in cancer syndrome genes (9/16; 56% of patients), respectively, when compared with non-carriers (58/193; 30% of patients; Figure 3B). On the other hand, no difference was found in the presence of melanoma or other cancers in patients' relatives, anatomical localization of melanoma, or age at melanoma onset (Figure 3C–E). The importance of personal cancer history for the potential to carry a mutation was confirmed when we calculated the proportion of patients with germline mutations in particular personal cancer history categories (Figure 3F). We noticed a significantly increased proportion of mutation carriers among patients with multiple melanoma (7/16; 44% of patients), compared with patients with single melanoma (29/164; 18% patients; $p = 0.021$). It is noteworthy that 14/89 (16%) patients with more than one tumor in personal history (i.e., patients with multiple melanoma, multiple

melanoma plus other cancer, and melanoma plus other cancer) carried a mutation in a clinically relevant gene (a high-to-moderate risk melanoma gene or a cancer syndrome gene), compared with 10/164 carriers (6%) among patients with single melanoma only ($p = 0.023$). Thus, tumor multiplicity (not restricted to melanoma multiplicity) in probands increased the risk that they will carry a mutation (OR = 2.9; 95%CI 1.2–6.8). A positive family cancer history did not increase the risk of being a mutation carrier (Figure 3G); however, the prevalence of mutations in patients with a positive family cancer history (24/196 carriers, 12%) surpassed the 10% threshold justifying genetic testing in this group (in contrast to 4/47; 8.5% positively tested patients without family cancer history; $p = 0.6$).

Altogether, 7/11 double mutation carriers (excluded from the analysis of clinicopathological data) carried at least one mutation in high-risk melanoma (*POT1*/*CHEK2*) or syndromic (*ATM*/*WRN*, *BRCA1*, *BRCA2* (2x), *CHEK2*/*RAD51D*, *NBN*) genes (Table 3). Melanoma or tumor multiplicity in personal cancer history was present in four (36%) of these patients and all of them had a positive family cancer history, indicating that personal or family cancer history positivity was also more frequent among double mutation carriers.

4. Discussion

Our analysis demonstrated that 31/264 (11.7%) high-risk Czech melanoma patients (compared with 35/1479 or 2.3% controls) carried a mutation in some of the clinically important high-to-moderate melanoma risk genes (9 patients; 3.4%) or other cancer syndrome-associated genes (22 patients; 8.3%). As expected, *CDKN2A* was the most frequently mutated gene in the high-to-moderate risk gene group (in six analyzed patients; 2.3%). Four out of six *CDKN2A* mutation carriers developed >1 melanoma (3 patients) or other cancer (1 patient); all six carriers had a positive family cancer history and five of them had at least one relative with melanoma. The progressively rising probability of *CDKN2A* mutation prevalence with an increasing number of affected relatives with melanoma was described by Goldstein and colleagues in their study analyzing families of a European descent with at least three melanoma patients [36]. The frequency of *CDKN2A* mutation carriers rose from <40% for patients with three relatives with melanoma to >90% for those with more than six relatives with melanoma. In line with this observation, we have noticed three *CDKN2A* mutation carriers among 50 patients with one melanoma relative (6%) and two *CDKN2A* carriers among 10 patients with two melanoma relatives (20%). Goldstein et al. also observed an increased prevalence of pancreatic cancer patients in families with *CDKN2A* mutations (found in one p.R112G mutation carrier in our study). Germline mutations in high-risk melanoma susceptibility genes convey an increased risk of other cancers modifying genetic counselling in mutation carriers [24]. The spectrum of tumors in relatives diagnosed with cancer in the families of six *CDKN2A* mutation carriers included melanoma (7×), breast cancer (3×), rectal cancer (2×), and gastric, pancreatic, lung, and endometrial cancer, brain tumor, and leukemia (one each).

The three remaining patients with germline mutations in high-to-moderate melanoma risk genes carried a P/LP variant in genes coding for shelterin complex proteins. The protection of telomeres protein 1 (POT1) is essential for the control of telomere length by inhibiting telomerase [32]. In addition, POT1 prevents hyper-resection at telomeric ends by inhibiting ATR [37]. The function of POT1 at telomeres is determined by its interaction with the telomeric single-stranded 5′-TTAGGG-3′ repeats and with the TRF1/2 subunits of the shelterin complex through TPP1 (ACD) protein. Interaction with telomeric G-strand DNA is mediated by the two N-terminal OB domains of POT1, whereas the C-terminal part of POT1 interacts with TPP1 (ACD) [38]. Previous in silico and functional studies identified unstable binding and defective interaction with ssDNA for the p.R117C missense variant [33,39]. We found the adjacent p.P116L variant, described previously in a patient with sporadic cardiac sarcoma [33], in a patient with multiple melanoma and breast cancer, who also carried a large pathogenic *CHEK2* deletion. A functional analysis of the P116L isoform demonstrating its normal interaction with TPP1 (ACD) protein but impaired ssDNA binding led us to conclude that p.P116L is a functionally defective mutation. Germline *POT1* mutations have been initially described as increasing the risk of melanoma, but later studies indicate a broader cancer spectrum associated with these

mutations. Notably, *POT1* mutations have recently been associated with familial non-medullary thyroid cancer [40–42]. A duplicity of thyroid cancer with melanoma has been identified in a patient with a newly characterized splicing *POT1* mutation (thyroid cancer was present in the patient's untested mother's mother). In a single melanoma patient with a negative family cancer history, we identified a mutation in the *ACD* gene truncating the C-terminal proportion of the protein containing POT1- and TINF2-interacting domains required for the localization of ACD protein into the shelterin complex. Overall, high-to-moderate risk germline mutations affecting shelterin complex genes were found in three (1.1%) analyzed patients in our study. We also detected another shelterin gene truncating mutation affecting the *TINF2* gene that we included in the low-risk gene category; however, another *TINF2* truncation has recently been described to segregate with multiple thyroid cancer and melanoma in one family [43]. A higher prevalence of mutations in *ACD*, *TERF2IP*, and *POT1* was identified in 12/132 (9.1%) high-risk *CDKN2A/CDK4/TERT/BAP1* wild-type European and Australian patients with multiple melanoma (≥3) [44]. A higher prevalence of germline mutations in *BAP1* (not identified in our patients) and *POT1* was also reported in a recent study by Pastorino and colleagues who identified seven carriers (2.6%) of mutations in each of these two genes among 273 Italian melanoma patients [45]. The enrollment of 22 melanoma patients with atypical Spitz nevi with relatives developing *BAP1*-related tumors can explain an increased prevalence of *BAP1* mutation carriers in this Italian study. Germline *BAP1* mutations were rarely identified in Czech patients so far, dominantly in probands with uveal melanoma or Spitz nevi [46,47].

The highest prevalence of germline mutations in our melanoma patients was found in the *NBN* gene (in 7/264 patients; 2.7%), coding for nibrin, a protein contributing to a MRN complex formation, sensing for DNA double strand breaks. We found the most frequent, Slavic founder germ-line hypomorphic variant c.657del5 in five patients [48]. Two of them also developed ovarian cancer, which was associated with *NBN* germline mutations in our population [49]. An increased melanoma prevalence among *NBN* c.657del5 mutation carriers was reported from Poland (with a frequency comparable to our patients) and southern Germany (with lower prevalence) [50–52]. Two of our melanoma patients (diagnosed with melanoma at 9 and 47 years, respectively; both with a melanoma-positive family cancer history) carried other rare *NBN* truncations. Gass and colleagues [53] reported a female carrier of the c.698_701del4 germline mutation developing melanoma, squamous cell carcinoma, and breast cancer with a sister suffering from melanoma and other relatives affected by various cancer types, indicating that other *NBN* truncations increase melanoma risk. Analyses of *NBN* in other cancers demonstrated a highly variable population-specific prevalence of its germline mutations. Current NCCN guidelines report an association of *NBN* mutations with an increased breast cancer risk (https://www.nccn.org/professionals/physician_gls/pdf/genetics_bop.pdf), but further studies of unselected cancer patients with carefully population-matched controls are required to determine cancer risk associated with other cancer types, including melanoma. The prevalence of *NBN* mutations but also *BRCA2* mutations was significantly (nine-fold) higher in patients than in controls. P/LP variants in *BRCA1* and *CHEK2* were less enriched in patients over controls and statistically insignificant ($p = 0.051$; Supplementary Table S5). The role of germline mutations in the breast-ovarian cancer predisposition genes *BRCA1* and *BRCA2* in the risk of familial melanoma development is still a matter of debate [54] and the exact melanoma risk increase (if any) in mutation carriers is uncertain. The same could be said of *CHEK2* as documented in a recent meta-analysis evaluating the association of germline *CHEK2* mutations with melanoma [55]. Large studies utilizing large gene panels to analyze patients with unselected melanoma or, even better, unselected cancer, will be required to dissect the risk of melanoma associated with hereditary cancer syndrome genes. However, we would like to emphasize that 4/9 *BRCA1* or *BRCA2* pathogenic mutation carriers and all *CHEK2* P/LP variant carriers would not be eligible for germline genetic testing according to the current guidelines, despite the fact that all other mutation carriers (except for one patient with the founder c.5266dupC *BRCA1* mutation) had a positive family cancer history and four also developed secondary tumors alongside solitary or

multiple melanoma (Table 3). The genetic counselling was offered to all carriers of mutations in high and moderate cancer risk genes.

An analysis of clinicopathological characteristics shows not only that multiple primary melanoma patients carry an increased risk of mutations in melanoma-predisposition genes, but also that the presence of melanoma and other non-melanoma cancer in the proband increased the potential to carry a clinically meaningful mutation in a melanoma predisposition or hereditary cancer syndrome gene.

We are aware of some limitations of our study. Most melanoma patients analyzed in our study were referred to the analysis by medical geneticists. This fact explains the enrichment of patient population in early-onset, multiple cancer, and family cancer-positive cases and incomplete clinicopathological data that lack phenotypic characteristics (eye and hair color, skin phototype according to Fitzpatrick, total number of nevi, the presence of clinically atypical nevi, freckle density, iris pigmentation), lifetime history of sunburns, and specific melanoma characteristics (histological subtype, Breslow thickness, clinical staging) in most of the patients. We are also aware that the gene selection in our CZMELAC panel would omit potentially clinically important gene(s). However, we would like to emphasize that we aimed to evaluate the importance of known melanoma/other cancer predisposition genes and candidate genes for clinical purposes in our melanoma patients rather than to identify genes that have not been associated with hereditary melanoma so far. Furthermore, only P/LP mutations were considered for subsequent statistical analyses. We excluded all VUS (except those in *CHEK2* and *POT1* that we functionally classified as deleterious) as currently clinically inconclusive, being aware that some of them may represent potentially important variants in both patient and control datasets. The presence of VUS substantially hampers the clinical utility of NGS diagnostics. Classifications of VUS frequently require demanding and time-consuming functional analyses that are beyond the expertise available in most of diagnostic laboratories. Therefore, VUS classifications, which are critically important for appropriate clinical interpretations of variants in cancer predisposition genes, are an opportunity for a collaborative effort of international consortia bringing together experts from various disciplines, who may provide substantial capacity for in vitro testing of VUS characterized by the co-operating laboratories.

In conclusion, we comprehensively assessed the prevalence of germline variants affecting currently known or candidate melanoma-predisposition genes in Czech melanoma patients and in the general population. Our analysis demonstrated that high-to-moderate risk genes, including genes coding for shelterin complex proteins, should be targeted in the multicancer panel NGS analysis. An analysis of clinicopathological characteristics indicated that patients eligible for such an analysis should not be restricted to multiple primary melanoma patients or patients with a positive familial melanoma cancer history, but they should also include melanoma patients with other primary cancer and melanoma patients with a positive family cancer history.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9059/8/10/404/s1, Table S1: Clinicopathological characteristics of analyzed melanoma patients, Table S2: List of 217 targeted genes in CZMELAC panel, Table S3: List of 83 P/LP variants found in melanoma patients (column H) and 225 P/LP variants identified in controls, Table S4: Frequency of pathogenic/likely pathogenic germline mutations in 89 out of 217 analyzed genes identified in 264 high-risk melanoma patients or in 1479 population-matched controls, Table S5: Found germline P/LP variants in genes with unknown association to familial melanoma, Figure S1: Intragenic deletions and duplications from technical control samples with known alterations and in samples from analyzed patients. Figure S2: New *CHEK2* germline variants (p.T133A and p.Y297D) identified in two melanoma patients were functionally classified as neutral in RPE1-CHEK2-KO cell-based assay.

Author Contributions: Conceptualization: Z.K., L.M.; methodology: Z.K., L.M., P.K.; software: P.Z., L.S., V.S.; validation: L.S., S.J., J.K., M.C., P.K.; formal analysis: L.S., S.J., R.S., M.V.; investigation: L.S., S.J., R.S., J.K., M.C., Z.V., M.J., J.S.; resources: E.M., M.V., O.K., P.D., V.S., L.F., L.M.; data curation: E.M., M.V., O.K., L.F.; writing—original draft preparation: L.S., Z.K.; writing—review and editing: all authors; visualization: Z.K., P.Z., P.K.; funding acquisition: P.D., L.M., Z.K., P.K. All authors have read and agreed to the published version of the manuscript.

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Identification of deleterious germline *CHEK2* mutations and their association with breast and ovarian cancer

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Germline mutations in checkpoint kinase 2 (*CHEK2*), a multiple cancer-predisposing gene, increase breast cancer (BC) risk; however, risk estimates differ substantially in published studies. We analyzed germline *CHEK2* variants in 1,928 high-risk Czech breast/ovarian cancer (BC/OC) patients and 3,360 population-matched controls (PMCs). For a functional classification of VUS, we developed a complementation assay in human nontransformed RPE1-*CHEK2*-knockout cells quantifying CHK2-specific phosphorylation of endogenous protein KAP1. We identified 10 truncations in 46 (2.39%) patients and in 11 (0.33%) PMC ($p = 1.1 \times 10^{-14}$). Two types of large intragenic rearrangements (LGR) were found in 20/46 mutation carriers. Truncations significantly increased unilateral BC risk (OR = 7.94; 95%CI 3.90–17.47; $p = 1.1 \times 10^{-14}$) and were more frequent in patients with bilateral BC (4/149; 2.68%; $p = 0.003$), double primary BC/OC (3/79; 3.80%; $p = 0.004$), male BC (3/48; 6.25%; $p = 8.6 \times 10^{-4}$), but not with OC (3/354; 0.85%; $p = 0.14$). Additionally, we found 26 missense VUS in 88 (4.56%) patients and 131 (3.90%) PMC ($p = 0.22$). Using our functional assay, 11 variants identified in 15 (0.78%) patients and 6 (0.18%) PMC were scored deleterious ($p = 0.002$). Frequencies of functionally intermediate and neutral variants did not differ between patients

Key words: breast cancer, ovarian cancer, germline mutations, CHEK2, VUS, KAP1, functional assay

Abbreviations: BC: breast cancer; CHEK2: checkpoint kinase 2; CI: confidence interval; CNV: copy number variant(s); CPG: cancer-predisposing genes; CZECANCA: CZEch CAncer paNel for Clinical Application; DHPLC: denaturing high performance liquid chromatography; EGFP: enhanced green fluorescent protein; ExAC: exome aggregation consortium; HRMA: high-resolution melting analysis; IGV: integrative genomics viewer; KAP1: KRAB-associated protein 1; KO: knockout; LGR: large genomic rearrangement(s); MLPA: multiplex ligation-dependent probe amplification; NFE: non-Finnish European; NGS: next-generation sequencing; OC: ovarian cancer; OR: odds ratio; PMC: population-matched controls; RPE1: retinal pigmented epithelial cells; SNV: single nucleotide variant

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and PMC. Functionally deleterious *CHEK2* missense variants significantly increased BC risk (OR = 3.90; 95%CI 1.24–13.35; $p = 0.009$) and marginally OC risk (OR = 4.77; 95%CI 0.77–22.47; $p = 0.047$); however, carriers low frequency will require evaluation in larger studies. Our study highlights importance of LGR detection for *CHEK2* analysis, careful consideration of ethnicity in both cases and controls for risk estimates, and demonstrates promising potential of newly developed human nontransformed cell line assay for functional *CHEK2* VUS classification.

What's new?

The tumor suppressor gene checkpoint kinase 2 (*CHEK2*) encodes a protein that serves an important role in DNA repair. However, *CHEK2* is also vulnerable to mutations that potentially impact breast cancer risk. Using a functional cell-based assay, the authors of the present study show that truncating and missense *CHEK2* variants are associated with risk of both breast and ovarian cancer. One-third of truncating mutations involved large genomic rearrangements. In addition, *CHEK2* mutations predisposed women to specific breast cancer types, and *CHEK2* mutation carriers with a family history of cancer were at increased risk of developing second primary cancers.

Introduction

Approximately 10% of breast cancer (BC) and 20% of ovarian cancer (OC) cases arise as a hereditary disease in patients carrying a pathogenic mutation in BC/OC-predisposing genes.[1,2] The clinical utility of pathogenic mutations in major BC/OC genes (*BRCA1* and *BRCA2*) is well established but it remains less certain for a growing group of cancer-predisposing genes (CPG) whose germline mutations confer a moderate cancer risk (*ATM*, *CHEK2*, *PALB2*).[3] This problem is becoming even more critical with the introduction of multigene panel next-generation sequencing (NGS) into the routine genetic analysis of high-risk BC/OC individuals.[4]

Germline *CHEK2* mutations have been linked with susceptibility to several malignancies including BC.[5] The *CHEK2* gene codes for serine/threonine CHK2 kinase involved in DNA damage response (DDR). Activated by a DNA lesion, ATM kinase catalyzes CHK2 T68 phosphorylation promoting CHK2 homodimerization through its forkhead-associated domains and kinase domain autophosphorylation.[6,7] Activated CHK2 phosphorylates multiple proteins involved in DNA repair and DDR, including BRCA1/BRCA2 and p53.[8,9] Another CHK2 substrate is KRAB-associated protein 1 (KAP1, alias TIF1β, TRIM28) a universal corepressor required for transcriptional repression mediated by the KRAB protein superfamily. CHK2-mediated KAP1 S473 phosphorylation reduces its transcription repression resulting in wide effects on gene expression.[10] Although the role of the ATM–CHK2–p53 pathway in the DNA damage-induced cell cycle checkpoint is redundant, CHK2 participates in p53-dependent cell death.[11–14]

The association of germline *CHEK2* variants with BC was assessed early in studies genotyping European founder mutations including the truncating mutation c.1100delC and the missense variant c.470T>C (p.I157T).[5] Subsequent meta-analyses demonstrated that while c.1100delC represents a moderate-risk variant for unselected (OR = 2.7; 95% confidence interval [CI] 2.1–3.4), early onset (OR = 2.6; 95%CI 1.3–5.5) and familial BC (OR = 4.8; 95% CI 3.3–7.2),[15] p.I157T is a low-risk variant with OR <1.5 for all BC subgroups.[16] Other founder variants include the spliceogenic mutation c.444+1G>A (IVS2+1G>A) and a large genomic rearrangement (LGR) with exon 9–10 deletion (c.909-2028_1095+330del5395) identified in Slavic populations,[17] and the Ashkenazi Jewish founder missense mutation c.1283C>T (p.S428F).[18]

Only few early studies analyzed the entire *CHEK2* coding sequence and revealed that c.1100delC and p.I157T represent only a fraction of *CHEK2* variants in BC patients.[19–22] Recent panel NGS analyses in large cohorts have shown that the *CHEK2* mutation rate is one of the highest among non-*BRCA1/BRCA2* genes in BC in individuals of Ashkenazi Jewish or European ancestry.[23–26] However, the classification of most missense variants remains uncertain,[27] their assessment is problematic,[4] and nearly one-third of *CHEK2* variants are reported discordantly.[28]

In contrast to BC, the association of *CHEK2* germline variants with OC risk is disputable. While several case–control studies have not significantly associated the c.1100delC mutation with OC development,[29,30] recent panel NGS analyses in 4,439 and 6,001 OC samples from the US identified *CHEK2* as the third most frequently affected susceptibility gene.[31,32]

In our study, we identified germline *CHEK2* variants in 1,928 high-risk BC/OC patients and 3,360 population-matched controls (PMCs). Subsequently, we have developed a cell-based assay utilizing a human RPE1 cell line model with endogenous *CHEK2* knockout to functionally classify the identified variants of unknown significance (VUS). This strategy enabled us to identify deleterious germline *CHEK2* mutations, to evaluate cancer risk in their carriers and to describe the clinical and histopathological characteristics of breast tumors in mutation carriers.

Methods

Detailed information is provided in Supporting Information Methods.

Subjects

The patient group included 1,928 BC/OC patients (herein denoted as *all patients*) referred by clinical geneticists for a CPG-mutation analysis performed at the Laboratory of Oncogenetics, First Faculty of Medicine, Charles University, in 1997–2017. Overall, 424/1,928 patients carried a mutation in other (i.e., non-*CHEK2*) cancer-predisposing gene for BC (*BRCA1*, *BRCA2*, *PALB2*, *TP53*) or OC (*BRCA1*, *BRCA2*, *RAD51C*, *RAD51D*, *MLH1*, *MSH2*, *MSH6*) and were denoted herein as *other CPG-mutated*. Remaining 1,504/1,928 patients were negative for mutations in aforementioned genes (herein denoted as *other CPG-wt*). All participants signed an informed consent approved by the local ethical committee. Clinical and histopathological data (Supporting Information Table S1) were obtained during genetic counseling or retrieved from the patients' records.

The set of 3,360 adult PMCs comprised 720 samples of noncancer individuals, 369 samples of adult blood donors, 609 noncancer controls aged >60 years without cancer in first-degree relatives and 1,662 individuals analyzed by exome sequencing at the National Center for Medical Genomics (http://ncmg.cz). In total, PMC set included 1,593 female (with median age 66 years, range 20–98 years) and 1,767 male (with median age 60 years, range 18–94 years) controls. All patients and controls were Caucasians, of the Czech origin.

Mutation analyses

Until 2015, mutation analyses of the entire *CHEK2* coding sequence in BC patients were performed by a high-resolution melting analysis (HRMA) of all coding exons. LGRs were analyzed by a multiplex ligation-dependent probe amplification (MLPA), as described previously.[33] All OC patients' samples, samples from BC patients enrolled since 2015, and samples from all identified *CHEK2* variant carriers were analyzed by a CZECANCA panel (CZEch CAncer paNel for Clinical Application; custom-made SeqCap EZ choice panel, Roche) targeting 219 genes with MiSeq (Illumina) NGS as described recently.[34] The coverage uniformity enabled to evaluate CNVs at 100× average coverage. *CHEK2* variants identified in patients were also sequenced at the mRNA (cDNA) level to determine a potential impact on splicing. NGS-analysis performed in 2,271/3,360 (67.6%) PMC samples (609 noncancer controls and 1,662 NCMG controls) included SNV/indels and CNV analyses. In remaining 1,089/3,360 (32.4%) PMC samples (720 noncancer individuals and 369 blood donors), entire *CHEK2* coding sequence was analyzed by HRMA, similarly as in patients and mutation-specific PCR/HRMA was used for identification of two *CHEK2* LGRs identified in our population (see Supporting Information Methods for details). The consequences of the identified missense variants were predicted by *in silico* tools: Align-GVGD, MutationTaster, CADD, SIFT, PolyPhen-2, Spidex and GERP.

Cell lines

To generate RPE1-*CHEK2*-KO cells, hTERT-RPE1 cells were transfected with a *CHEK2*-CRISPR/Cas9-KO plasmid (Santa Cruz Biotechnology, Santa Cruz, CA; sc-400,438) and a *CHEK2*-HDR plasmid (1:1) and selected by puromycin (7.5 μg/ml) for 3 weeks. The integration of an HDR cassette into the *CHEK2* locus was confirmed by sequencing and a loss of CHK2 expression by immunoblotting (all used antibodies are described in Supporting Information Methods). To remove the HDR cassette, cells were transfected with Cre vector (Santa Cruz, sc-418,923) and RFP-negative cells were selected by flow cytometry. For stable complementation of CHK2, RPE1-*CHEK2*-KO cells were transfected with a linearized pcDNA4-EGFP-*CHEK2* plasmid, selected with zeocin for 3 weeks and single clones were expanded. Plasmid DNA was transfected using polyethylenimine HCl MAX (MW 40000, Polysciences, Warrington, PA) at a 1:5 ratio and growth media were changed after 3 hr. Silencer Select siRNA oligonucleotides (5 nM, Ambion) were transfected using RNAiMAX (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Plasmids

CHEK2 mutants were generated using QuickChange II Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA). Wild-type or mutated *CHEK2* was amplified by PCR and cloned in frame into pcDNA4-EGFP or pGEX-6P-1 plasmids using a Gibson assembly kit (NEB). All mutants were verified by Sanger sequencing. A DNA fragment corresponding to the GVKRSRSGEGEV peptide (containing S473) from human KAP1 was ligated in frame into a pGEX-6P-1 plasmid. Alternatively, a fragment corresponding to T2A-EGFP was ligated into the XbaI site of pcDNA4, and subsequently a fragment corresponding to wild-type or mutant FLAG-*CHEK2* was cloned into *Hind*III/*Xho*I sites resulting in a plasmid for bicistronic expression of FLAG-CHK2 and EGFP.

Immunofluorescence microscopy, cell-based assay for the detection of CHK2 activity

RPE1-*CHEK2*-KO cells transfected with an empty EGFP plasmid, wild-type or mutant EGFP-*CHEK2* were seeded on glass coverslips and fixed by 4% paraformaldehyde 48 hr after transfection. Cells were permeabilized by 0.2% Triton X-100 in PBS for 20 min and blocked with 3% BSA in PBS at room temperature. The coverslips were incubated with the KAP1-pS473 antibody for 1 hr at room temperature, three times washed with PBS and incubated with the goat-antimouse Alexa568 antibody and DAPI. After the PBS washing, the coverslips were mounted using Vectashield H-1000 and imaged using a Scan^R microscope (Olympus, Waltham, MA) equipped with an ORCA-285 camera and a 40×/1.3 NA objective. The total intensity of the KAP1-pS473 signal per nucleus was determined in cells expressing low levels of GFP. Three independent experiments were performed and >300 cells were quantified per condition in each experiment. The KAP1-pS473 signal in cells expressing only EGFP typically reached <10% of the signal in cells expressing wild-type CHK2 and was subtracted as a background. The KAP1-pS473 signal measured in cells expressing mutant CHK2 was normalized to wild-type CHK2-expressing cells. The activities of the analyzed

variants were classified as normal, intermediate or deleterious based on mean pS473 reaching >50%, 25–50% and <25% of wild-type CHK2, respectively.

In vitro kinase assays

Escherichia coli BL21 transformed with wild-type or mutant pGEX-6P-1-*CHEK2* plasmids were induced at A_{600} = 0.6 by 0.2 mM IPTG and grown for 5 hr at 37°C. The bacteria were lysed in ice-cold PBS supplemented with 0.1% TX-100 and 1 mM PMSF and sonicated 2 × 30 sec. Cleared lysates were incubated with Glutathione Sepharose 4 Fast Flow beads (GE Healthcare, Chicago, IL) for 5 hr at 4°C. Bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris pH 8.0 and mixed with 30% glycerol. Protein concentration was determined by a BCA assay (Pierce, Puyallup, WA). Purified CHK2 was incubated in a kinase buffer (10 mM HEPES pH 7.4, 2.5 mM β-glycerolphosphate, 2 mM EDTA, 1 mM EGTA, 4 mM $MgCl_2$, 100 μM ATP) with GST-KAP1 substrate (2 μg) for 20 min at 30°C and its phosphorylation was detected by immunoblotting using KAP1-pS473 antibody. Alternatively, wild-type or mutant EGFP-CHK2 was immunoprecipitated from transfected HEK293 cells using GFP-Trap (Chromotek, Munich, Germany), treated with λ-phosphatase (200 U/reaction, Santa Cruz). Beads were washed three times with PBS and incubated for 20 min at 30°C with GST-KAP1 in the kinase buffer supplemented with PhosSTOP inhibitor (Roche, Basel, Switzerland). Alternatively, CHK2 kinase activity was measured in crude bacterial lysates *in vitro* using Omnia kinase assay kit (Life Technologies) as described previously.[19]

Statistical analysis

The patients were stratified according to (*i*) functional classes of germline *CHEK2* variants (deleterious, intermediate, neutral), (*ii*) the presence of a mutation in other (i.e., non-*CHEK2*) CPG and (*iii*) cancer and histopathological characteristics. Associations between the *CHEK2* mutation status and cancer diagnoses were analyzed using 3,360 PMC. The strength of the associations was estimated by the odds ratio (OR) in Fisher's exact test and p values <0.05 were considered significant.

Results

Germline CHEK2 variants are more frequent in cancer patients than in PMC

We analyzed germline *CHEK2* variants in 1,928 high-risk Czech BC/OC patients and 3,360 PMCs. We identified 36 distinct nonsynonymous variants (Table 1) in 131/1,928 (6.79%) patients and 142/3,360 (4.23%) PMC ($p = 7.4 \times 10^{-5}$).

Ten different frame-shift and splicing mutations ("All truncations" in Table 1) were found in 46 patients (2.39%) and 11 PMC (0.33%; $p = 1.3 \times 10^{-11}$). The most prevalent alterations were LGRs, present in 20 (1.04%) patients and four PMC (0.12%). LGRs included a recurrent exon 9–10 (5,395 bp) deletion and a novel exon 8 (5,601 bp) deletion. The c.1100delC mutation was found in seven (0.36%) patients and three PMC (0.09%). We identified three spliceogenic variants altering the mRNA sequence: c.444+1G>A, recurrent, population-specific c.846+4_846+7delAGTA (resulting in in-frame exon 7 skipping), and c.1260-8A>G (splice acceptor-shift with 7b exonization; Supporting Information Fig. S1). Variants reported as pathogenic in the ClinVar database, causing a frame-shift or truncating the kinase domain were considered pathogenic. Five of 46 patients with a truncating *CHEK2* mutation (four with female BC and one with double primary BC/OC) carried an additional pathogenic mutation in *BRCA1* or *BRCA2* (but not in another CPG). These patients were assigned into a group of 424 other CPG-mutation carriers.

Twenty-six distinct missense variants were found in 88 (4.56%) patients and 131 (3.90%) PMC (p = 0.22; Table 1). The most frequent variant was p.I157T with comparable prevalence in patients (58 carriers; 3.01%) and PMC (104 carriers; 3.10%; p = 0.93). Functional consequences of the detected missense variants predicted *in silico* yielded contradictory results (Supporting Information Table S2). While MutationTaster, CADD, and GERP predicted all SNVs as deleterious (except a maximum of 3/26 scored as neutral), the remaining four prediction tools, Align-GVGD, SIFT, PolyPhen2 and Spidex, were 100% and ≥75% concordant for 4/26 and 16/26 variants, respectively. Since the clinical significance of the detected SNVs was described as uncertain or conflicting in the ClinVar database (Table 1), we subjected them to subsequent functional analyses.

Functional assays identified deleterious CHEK2 missense variants

To evaluate the enzymatic activity of the identified CHK2 protein variants, we developed a cell-based assay quantifying KAP1-S473 phosphorylation in nontransformed human RPE1 cells. First, we verified the specificity of a monoclonal antibody against phosphorylated KAP1-S473 by immunoblotting and immunofluorescence microscopy (Supporting Information Fig. S2A). Next, we used the CRISPR/Cas9 technology to inactivate *CHEK2* in RPE1 cells (RPE1-*CHEK2*-KO; Fig. 1*a*, Supporting Information Figure S2B). A complete loss of CHK2 as well as RNAi-mediated CHK2 depletion impaired KAP1-S473 phosphorylation in RPE1 cells after ionizing radiation exposure. In contrast, CHK2 loss did not affect the phosphorylation of KAP1 at S824, an established ATM kinase site (Fig. 1*a*). A similar effect was also observed after treating the cells with neocarzinostatin and etoposide (Supporting Information Fig. S2C), suggesting that CHK2 phosphorylates KAP1 at S473 after the induction of DNA damage in general. A stable expression of EGFP-CHK2 in RPE1-*CHEK2*-KO cells rescued the phosphorylation of KAP1 at S473 after exposure to ionizing radiation, further confirming that CHK2 phosphorylates KAP1 after genotoxic stress (Fig. 1*b*). Finally, we transiently expressed the wild-type or mutant CHK2 isoforms in RPE1-*CHEK2*-KO cells and quantified the level of KAP1-S473 phosphorylation by immunofluorescence microscopy (Fig. 2*a*). We supplemented this cell-based model with a semiquantitative measurement of KAP1-pS473 in a cell-free *in vitro* assay using purified CHK2 and GST-KAP1 peptide as a substrate (Fig. 2*b*).

Table 1. The prevalence of *CHEK2* germline variants

Variant; cDNA (reference: NM_007194.3)	Variant; protein	rs number	ClinVar class	Unilateral FBC (*n* = 1,298)	Bilateral FBC (*n* = 149)	MBC (*n* = 48)	BC and OC (*n* = 79)	OC only (*n* = 354)	All patients (*n* = 1,928)	PMC (*n* = 3,360)
TRUNCATING mutations ([a]frame-shift; [b]in-frame)										
c.100–101delCA[a]	p.Q34Vfs*42	NA	NA	–	–	–	1	–	1	–
c.277delT[a]	p.W93Gfs*17	rs786203458	5	3	2	–	–	–	5	–
c.283C>T[a]	p.R95*	rs587781269	5	–	–	–	–	–	–	1
c.366delA[a]	p.E122Dfs*8	rs1555927302	5	1	–	–	–	–	1	–
c.444+1G>A[a]	p.E149Ifs*6	rs121908698	5	4	1	–	–	–	5	2
c.846+4_846+7delAGTA[b]	p.D265_H282del	rs764884641	3	7	–	–	–	–	7	–
c.846+1888_908+987del5601[a]	p.P283Dfs*8	NA	NA	2	–	–	–	–	2	–
c.909-2028_1095+330del5395[a]	p.M304Lfs*16	NA	5	11	1	3	1	2	18	4
c.1100delC[a]	p.T367Mfs*15	rs555607708	5	5	–	–	1	1	7	3
c.1260-8A>G[a]	p.L421Ifs*4	rs863224747	3	1	–	–	–	–	1	1
All truncations (%)				33 (2.54)[1]	4 (2.68)	3 (6.25)	3 (3.80)	3 (0.85)	46 (2.39)[1]	11 (0.33)
p-value (Fisher exact test)				9.4×10^{-11}	0.003	8.6×10^{-4}	0.004	0.14	1.3×10^{-11}	Ref.
Missense *CHEK2* mutations classified as DELETERIOUS										
c.190G>A	p.E64K	rs141568342	3–4	3	–	–	–	1	4	2
c.503C>T	p.T168I	rs730881684	3	–	–	1	1	–	2	–
c.520C>G	p.L174V	rs876659400	3	1	–	–	–	–	1	–
c.917G>C	p.G306A	rs587780192	3–4	1	–	–	–	–	1	2
c.980A>G	p.Y327C	rs587780194	3	1	–	–	–	–	1	–
c.1037G>A	p.R346H	rs730881688	3	1	–	–	–	–	1	–
c.1180G>A	p.E394K	rs587780169	3	1	–	–	–	–	1	–
c.1183G>C	p.V395 L	rs587780170	3	–	–	–	–	1	1	–
c.1270T>C	p.Y424H	rs139366548	3	–	1	–	–	–	1	–
c.1274C>T	p.P425L	rs1555913537	3	1	–	–	–	–	1	–
c.1421G>A	p.R474H	rs121908706	3	–	–	–	–	1	1	2
All deleterious missense variants (%)				9 (0.69)	1 (0.67)	1 (2.08)	1 (1.27)	3 (0.85)	15 (0.78)	6 (0.18)
p-value (Fisher exact test)				0.009	0.26	0.09	0.15	0.047	0.002	Ref.
Missense *CHEK2* variants classified as INTERMEDIATE										
c.470T>C	p.I157T	rs17879961	3–5	38	6	2	3	9	58	104
c.688G>T	p.A230S	rs748636216	3	–	–	–	–	–	–	1
c.715G>A	p.E239K	rs121908702	3	–	–	–	–	–	–	2
c.1067C>T	p.S356L	rs121908703	3	–	–	–	–	–	–	1
c.1217G>A	p.R406H	rs200649225	2–3	–	–	–	–	–	–	1
All intermediate missense variants (%)				38 (2.93)[2]	6 (4.03)[2]	2 (4.17)	3 (3.80)	9 (2.54)	58 (3.01)[2]	109 (3.24)
p-value (Fisher exact test)				0.64	0.63	0.67	0.74	0.63	0.68	Ref.

(Continues)

Table 1. The prevalence of *CHEK2* germline variants (Continued)

Variant; cDNA (reference: NM_007194.3)	Variant; protein	rs number	ClinVar class	Unilateral FBC (*n* = 1,298)	Bilateral FBC (*n* = 149)	MBC (*n* = 48)	BC and OC (*n* = 79)	OC only (*n* = 354)	All patients (*n* = 1,928)	PMC (*n* = 3,360)
Missense *CHEK2* variants classified as NEUTRAL										
c.7C>T	p.R3W	rs199708878	3	–	–	1	–	–	1	–
c.538C>T	p.R180C	rs77130927	1–3	1	–	–	–	–	1	3
c.539G>A	p.R180H	rs137853009	3	1	–	–	–	–	1	1
c.541C>T	p.R181C	rs137853010	3	–	–	–	–	–	–	3
c.542G>A	p.R181H	rs121908701	3	1	–	1	–	–	2	–
c.1091T>C	p.I364T	rs774179198	3	1	–	–	–	–	1	–
c.1309A>G	p.K437E	rs764238637	3	1	–	–	–	–	1	–
c.1312G>T	p.D438Y	rs200050883	3	3	–	–	–	1	4	2
c.1427C>T	p.T476M	rs142763740	3–4	2	–	–	–	1	3	3
c.1525C>T	p.P509S	rs587780179	3	1	–	–	–	1	2	4
All neutral missense variants (%)				11 (0.85)	–	2 (4.17)	–	3 (0.85)	16 (0.83)	16 (0.48)
p-value (Fisher exact test)				0.14		0.03		0.42	0.14	Ref.
All CHEK2 missense variants (%)				58 (4.47)	7 (4.70)	5 (10.42)	4 (5.06)	14 (3.95)[3]	88 (4.56)[3]	131 (3.90)
p-value (Fisher exact test)				0.38	0.52	0.04	0.55	0.96	0.22	Ref.

The prevalence of individual variants (divided into subgroups of truncating mutations and missense variants classified according to the results of an RPE1-*CHEK2*-KO cell-based analysis as deleterious, intermediate and neutral; Fig. 2*a*). It is displayed for all patients, their subgroups (unilateral female BC [FBC], bilateral FBC, male BC [MBC], double primary BC and OC and OC only) and population-matched controls (PMC; used as the reference). NA, not available.

[1]Include a FBC compound heterozygote of c.277delT and c.444+1G>A.

[2]Include two p.I157T homozygotes (with unilateral and bilateral FBC all diagnosed at <50 years, respectively).

[3]Four other compound heterozygotes in patients group were carriers of p.D265_H282del+p.D438Y, c.5601del+p.I157T, c.1100delC+p.I157T and p.E64K+p.I157T. The NM_007194.3 CHEK2 transcription variant A was used as the reference.

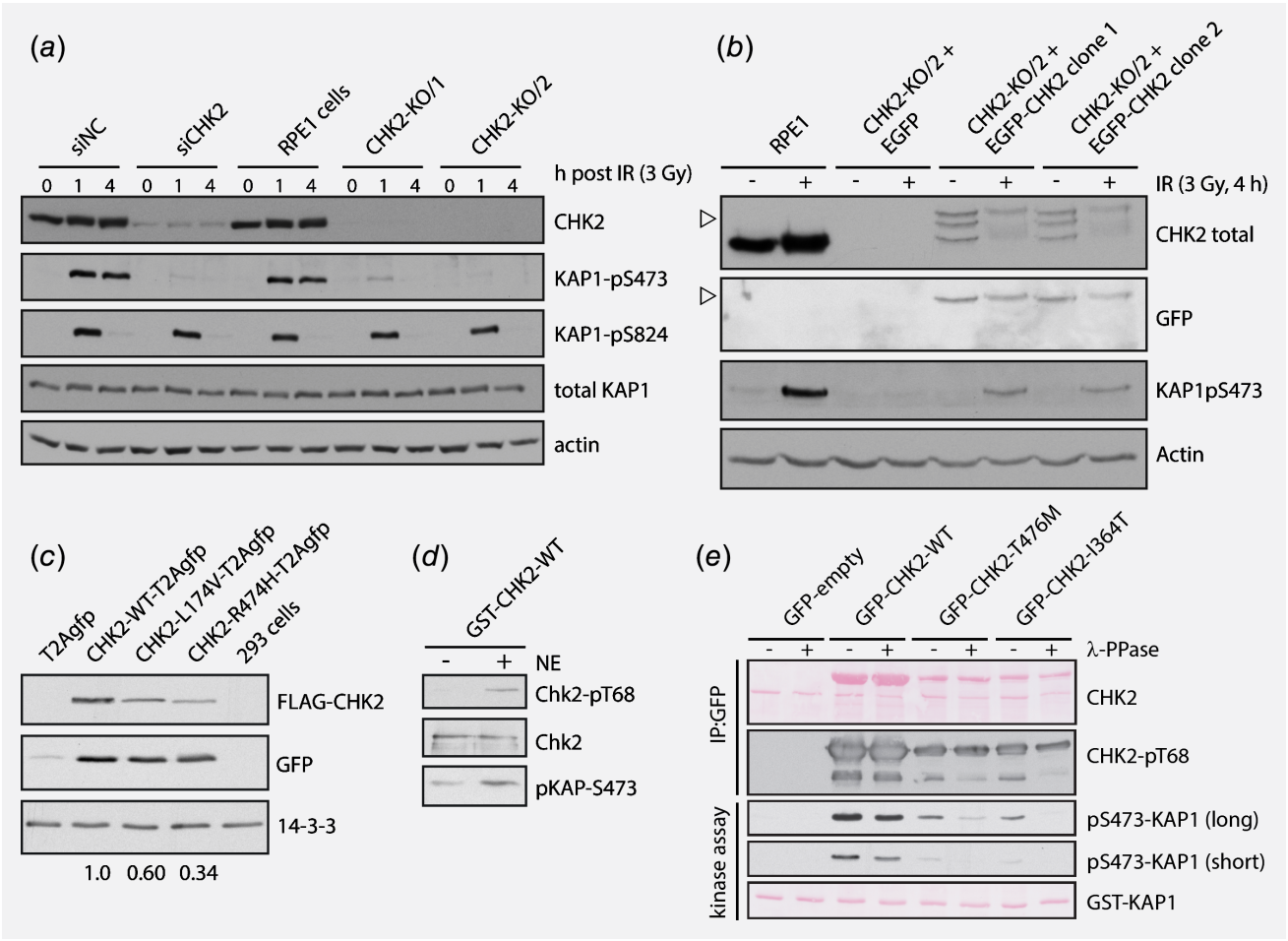

Figure 1. Characterization of a model system for functional analysis of *CHEK2* variants in RPE1-*CHEK2*-KO cells. Comparison of CHK2 depletion and knockout (*a*). RPE1 cells were transfected with control (siNC) or CHK2 siRNA (siCHK2) and assayed in parallel with parental RPE1 (RPE1 cells) and two clones of RPE1-*CHEK2*-KO cells (KO/1 and KO/2, respectively). Cells were harvested 0, 1 or 4 hr after exposure to IR (3 Gy) and analyzed by immunoblotting. Rescue of the *CHEK2* knockout (*b*). RPE1-*CHEK2*-KO cells were transfected with EGFP or EGFP-*CHEK2*-WT plasmids, selected with zeocin and exposed or not to IR. Parental RPE1 cells are shown for comparison. Arrowheads indicate the position of EGFP-CHK2. Impact of *CHEK2* mutations on protein stability (*c*). HEK293 cells were mock-treated or transfected with plasmids (1 μg) coding T2Agfp, CHK2-WT-T2Agfp, CHK2-L174V-T2Agfp or CHK2-R474H-T2Agfp and whole cell lysates were harvested after 20 hr. Numbers indicate the level of FLAG-CHK2 normalized to the level of GFP. Impact of CHK2 phosphorylation on its activity *in vitro* (*d*). Wild-type CHK2 purified from bacteria was incubated or not with nuclear extract from HCT116 cells (NE) in the presence of ATP at 30°C. After the addition of PBS, CHK2 was purified again using glutathione beads. Eluted CHK2 was incubated with KAP1 substrate and phosphorylation was assayed by KAP1-pS473 antibody. Impact of CHK2 phosphorylation on its activity (*e*). HEK293 cells were transfected with plasmids coding EGFP, EGFP-CHK2-WT, EGFP-CHK2-T476M, EGFP-CHK2-I364T. After 48 hr proteins were immunoprecipitated by GFP-Trap and treated or not with λ-phosphatase. Kinase activity was measured in the presence of phosphatase inhibitors and using GST-KAP1 as a substrate (shown in short and long exposition, respectively). [Color figure can be viewed at wileyonlinelibrary.com]

The results of the KAP1 cell-based analysis were in full agreement with KAP1 *in vitro* assays for 13 out of 26 tested missense variants, deleterious mutations (p.D265_H282del and c.1100delC) and wild-type CHK2 (Fig. 2, Supporting Information Table S2). Another five SNVs agreed partially between these two KAP1 assays (being intermediate in one and deleterious or neutral in the complementary assay). Eight variants were discrepant between cell based and *in vitro* KAP1 assays. As an example of discrepant results, the p.L174V variant showed only slightly decreased catalytic activity *in vitro*, but failed to phosphorylate KAP1 in cells. A comparison of the expression levels of CHK2-V174 and wild-type CHK2 both expressed from the bicistronic vector together with GFP (Fig. 1*c*) showed a suppressed expression of p.L174V to ~60% of wild-type CHK2, most probably reflecting impaired folding and/or reduced protein stability. Surprisingly, some variants with low *in vitro* activity were still able to phosphorylate KAP1 in human cells to a similar extent as wild-type CHK2. We hypothesized that the CHK2 kinase activity in human cells is influenced by its posttranslational modifications and, therefore, may differ from bacterially expressed CHK2. Indeed, preincubation of CHK2 purified from bacteria with nuclear extract led to CHK2-T68 phosphorylation. Subsequently, modified CHK2 showed higher ability to phosphorylate KAP1-S473 compared to

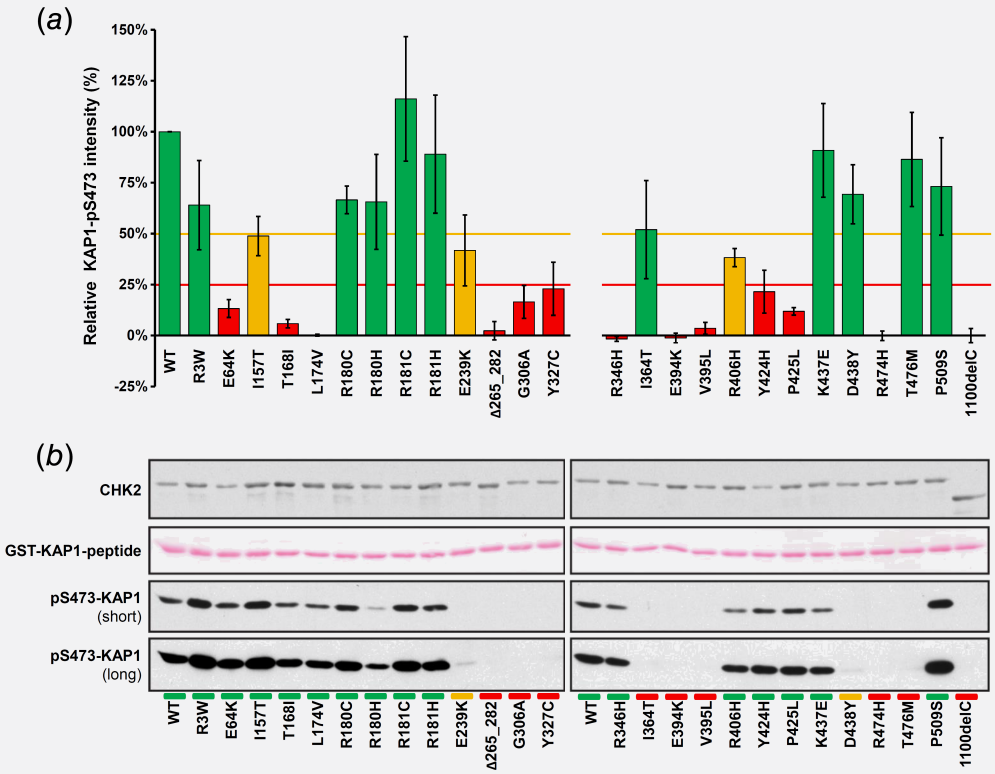

Figure 2. Functional classification of *CHEK2* germline variants was based on RPE1-*CHEK2*-KO cell-based assay. The chart describes relative levels of CHK2-dependent KAP1-S473 phosphorylation in RPE1-*CHEK2*-KO cells (*a*) for detected CHK2 variants. Variants were scored according to the WT (100%) and c.1100delC (0%) CHK2 kinase activity: ›50% as "neutral" (green), 25–50% as "intermediate" (yellow) and ‹25% as "deleterious" (red). Error bars represent standard deviations (SD). Immunoblotting of phosphorylated GST-purified KAP1-peptide at S473 by purified CHK2 isoforms *in vitro* (*b*) was used to complement the assay in RPE1 cells. The individual panels show amounts of particular CHK2 isoforms and GST-KAP1-peptide, and intensity of KAP1-pS473 staining after incubation with purified CHK2 (in short and long exposition, respectively). Colors bars represent classifications from *a*; Δ265_282 means p.D265_H282del. (See online version for color images). *Note*: Variants p.A230S and p.S356L found in PMC (exome samples; not shown in this figure) were functionally classified by the RPE1-CHEK2-KO cell-based assay as intermediate (Supporting Information Table S2). [Color figure can be viewed at wileyonlinelibrary.com]

unmodified CHK2 (Fig. 1*d*). Conversely, the phosphatase treatment of CHK2 immunoprecipitated from HEK293 cells suppressed the *in vitro* activity of p.T476M and p.I364T variants that originally scored well in the cell-based assay (Fig. 1*e*). Our results suggest that posttranslational modifications substantially modulate CHK2 kinase activity and thus the human cell-based assay may better reflect the real CHK2 kinase activity *in vivo*. We also functionally analyzed detected VUS using commercial Omnia kinase *in vitro* assay that fully or partially corresponded to a principally comparable KAP1 *in vitro* assay for 23/26 VUS (Supporting Information Table S2, Fig. S3); however, was unable to dissect VUS discordant between KAP1 assays. Therefore, results from our cell-based assay (Fig. 2*a*), that reflects *in vivo* behavior of analyzed CHK2 variants more appropriately, led us to use solely this assay for the final functional VUS classification (Table 1).

The cell-based assay revealed strongly reduced kinase capacity (<25% of wild-type CHK2) for 11/26 missense variants that were classified as deleterious (Fig. 2*a*). These variants were significantly enriched in patients over PMC (Table 1). A significantly reduced kinase activity was also observed in recurrent c. 846+4_846+7del-AGTA (in-frame exon 7 deletion; p.D265_H282del) eliminating the structurally important αC helix (residues 269–280) in the kinase domain.[7] The available pedigrees of patients with deleterious missense variants and c.846+4_846+7delAGTA are provided in the Supporting Information Figure S4. Five missense variants (p.I157T and four VUS identified only in PMC) were functionally classified as intermediate, with kinase activity at 25–50% of wild-type CHK2 in the cell-based assay. Ten missense variants with normal or mildly reduced catalytic activity (retaining >50% of wild-type CHK2) were considered neutral.

CHEK2 mutations are associated with BC and OC risk

We evaluated the association of *CHEK2* germline variants and cancer risk in diagnosis subgroups, considering all 1,928 patients and separately 1,504 patients without other CPG mutation. Regardless of the presence of other CPG mutations, truncating *CHEK2* variants significantly increased cancer risk in all analyzed subgroups except patients with OC only (Table 2). The most significant association was identified for group of 1,298 unilateral female BC patients that included 33 carriers (2.54%) of *CHEK2* truncations (OR = 7.94; 95%CI 3.90–17.47; $p = 9.4 \times 10^{-11}$). Truncations in *CHEK2* had the third highest mutation rate in this subgroup, preceded by *BRCA1* (153 carriers; 11.79%) and *BRCA2*

Table 2. Risk associated with germline *CHEK2* truncating and functionally classified missense variants (deleterious, intermediate and neutral) in all analyzed patients and in a subgroup of patients negatively tested for mutations in other cancer-predisposing genes against frequencies of *CHEK2* variants found in Czech population-matched controls PMC, Table 1

Group of patients *CHEK2* variant group	All patients			Other cancer-predisposing genes wt patients		
	Carriers; *N* (%)	OR (95%CI)	*p*-value	Carriers; *N* (%)	OR (95%CI)	*p*-value
Unilateral female BC (I)	*n* = 1,298			*n* = 1,065		
Truncations	33 (2.54)	**7.94 (3.90–17.47)**	**9.4×10^{-11}**	29 (2.72)	**8.52 (4.11–18.97)**	**1.2×10^{-10}**
Deleterious missense	9 (0.69)	**3.90 (1.24–13.35)**	**0.009**	8 (0.75)	**4.23 (1.28–14.82)**	**0.008**
Intermediate missense	38 (2.93)	0.90 (0.60–1.32)	0.64	34 (3.19)	0.98 (0.64–1.47)	0.99
Neutral missense	11 (0.84)	1.79 (0.75–4.11)	0.14	10 (0.94)	1.98 (0.80–4.66)	0.11
Bilateral female BC (II)	*n* = 149			*n* = 104		
Truncations	4 (2.68)	**8.39 (1.92–28.74)**	**0.003**	4 (3.85)	**12.15 (2.77–41.94)**	**8.1×10^{-4}**
Deleterious missense	1 (0.67)	3.77 (0.08–31.42)	0.26	1 (0.96)	5.42 (0.12–45.31)	0.19
Intermediate missense	6 (4.03)	1.25 (0.44–2.88)	0.63	5 (4.81)	1.51 (0.47–3.74)	0.39
Neutral missense	0 (0)	–	–	0 (0)	–	–
Male BC (III)	*n* = 48			*n* = 39		
Truncations	3 (6.25)	**20.21 (3.50–80.00)**	**8.6×10^{-4}**	3 (7.69)	**25.23 (4.34–101.34)**	**4.7×10^{-4}**
Deleterious missense	1 (2.08)	11.87 (0.25–100.83)	0.10	1 (2.56)	14.66 (0.31–125.29)	0.08
Intermediate missense	2 (4.17)	1.30 (0.15–5.07)	0.67	2 (5.13)	1.61 (0.19–6.39)	0.37
Neutral missense	2 (4.17)	**9.07 (0.98–40.41)**	**0.03**	2 (5.13)	**11.26 (1.21–50.79)**	**0.02**
BC and OC (IV)	*n* = 79			*n* = 40		
Truncations	3 (3.80)	**11.99 (2.11–46.6)**	**0.004**	2 (5.00)	**15.97 (1.67–77.08)**	**0.01**
Deleterious missense	1 (1.27)	7.15 (0.15–59.97)	0.15	0 (0)	–	–
Intermediate missense	3 (3.80)	1.18 (0.24–3.67)	0.74	1 (2.50)	0.76 (0.02–4.61)	0.99
Neutral missense	0 (0)	–	–	0 (0)	–	–
OC only (V)	*n* = 354			*n* = 256		
Truncations	3 (0.85)	2.60 (0.46–9.91)	0.14	3 (1.17)	3.61 (0.64–13.78)	0.07
Deleterious missense	3 (0.85)	**4.77 (0.77–22.47)**	**0.047**	3 (1.17)	**6.62 (1.07–31.22)**	**0.02**
Intermediate missense	9 (2.54)	0.78 (0.34–1.55)	0.63	8 (3.13)	0.96 (0.40–1.99)	0.99
Neutral missense	3 (0.84)	1.79 (0.33–6.28)	0.42	2 (0.78)	1.65 (0.18–7.06)	0.37
Any female BC (I + II + IV)	*n* = 1,526			*n* = 1,209		
Truncations	40 (2.62)	**8.19 (4.11–17.75)**	**4.1×10^{-12}**	35 (2.90)	**9.07 (4.49–19.87)**	**2.4×10^{-12}**
Deleterious missense	11 (0.72)	**4.06 (1.37–13.39)**	**0.006**	9 (0.74)	**4.19 (1.33–14.34)**	**0.006**
Intermediate missense	47 (3.08)	0.95 (0.66–1.35)	0.79	40 (3.31)	1.02 (0.69–1.49)	0.92
Neutral missense	11 (0.72)	1.52 (0.64–3.49)	0.30	10 (0.83)	1.74 (0.70–4.10)	0.18
Any OC (IV + V)	*n* = 433			*n* = 296		
Truncations	6 (1.39)	**4.28 (1.29–12.69)**	**0.009**	5 (1.69)	**5.23 (1.41–16.45)**	**0.007**
Deleterious missense	4 (0.92)	**5.21 (1.08–22.06)**	**0.02**	3 (1.01)	**5.72 (0.92–26.94)**	**0.03**
Intermediate missense	12 (2.77)	0.85 (0.42–1.56)	0.77	9 (3.04)	0.94 (0.41–1.87)	0.99
Neutral missense	3 (0.69)	1.46 (0.27–5.12)	0.47	2 (0.68)	1.42 (0.16–6.09)	0.65

The calculations were performed in individual diagnostic subgroups (Roman numerals I–V) and in aggregated groups of any female BC (subgroups I, II and IV) and any OC patients (subgroups IV and V). "Other CPG-wt" group consists of patients without germline mutations in genes predisposing for BC (*BRCA1*, *BRCA2*, *PALB2*, *TP53*) or OC (*BRCA1*, *BRCA2*, *RAD51C*, *RAD51D*, *MLH1*, *MSH2*, *MSH6*). Significant association of *CHEK2* variants with cancer risk is highlighted (in bold). Both aggregated subgroups (Any FBC and Any OC) include patients with double primary BC and OC (IV).

(56 carriers; 4.31%), and followed by *PALB2* (21 carriers; 1.62%) and *TP53* (3 carriers; 0.23%). We also observed a significantly higher prevalence of *CHEK2* truncations in small subgroups of patients with bilateral female BC (4/149; 2.68%; $p = 0.003$), male BC (3/48; 6.25%; $p = 8.6 \times 10^{-4}$) and with double primary BC/OC (3/79; 3.80% $p = 0.004$); however, the low number of patients and mutations limits relevance of calculated ORs. The analysis of two aggregated subgroups of "any female BC" and "any OC" patients (overlapping in patients diagnosed with double primary BC/OC; Table 2) reflected clinically relevant overall risk for BC and OC development in females with *CHEK2* truncations. We found significant associations with both cancer types, which was substantially higher and more significant for "any female BC" (OR = 8.19; 95%CI 4.11–17.75; $p = 4.1 \times 10^{-12}$) than for "any OC" (OR = 4.28; 95%CI 1.29–12.69; $p = 0.009$) subgroups in all patients as well as in patients after excluding those with mutations in other CPG (OR = 9.07; 95%CI 4.49–19.87; $p = 2.4 \times 10^{-12}$ and OR = 5.23; 95%CI 1.41–16.45; $p = 0.007$, respectively).

While the frequencies of functionally deleterious SNV were significantly more frequent in unilateral female BC, OC, any female BC and also any OC subgroups (Tables 1 and 2), the frequencies of functionally neutral or intermediate SNVs did not differ from PMC in any patient subgroup (except for neutral SNVs in a small subgroup of 48 male BC patients). Risks associated with functionally deleterious SNV were lower than risks associated with truncations, except that in OC patients. However, low number of functionally deleterious SNV carriers makes our findings only suggestive but not conclusive.

Twelve out of 54 *BRCA1/BRCA2*-negative *CHEK2* mutation carriers had a VUS in other genes, in which further modification of cancer risk cannot be ruled out (Supporting Information Table S3).

CHEK2 mutations predispose to specific BC types and multiple cancer development

We evaluated histopathological tumor characteristics in 1,209 *other CPG-wt* female BC patients. Breast tumors in *CHEK2* mutation carriers differed from noncarriers, tended to be more frequently of luminal A and less frequently of basal BC subtype, with lower grade and with nonsignificant tendency toward lower clinical stage (Fig. 3; Supporting Information Table S4). Histology, menopausal status and indication criteria for testing did not differ among *CHEK2* mutation carriers and noncarriers. Although the most frequent p.I157T variant did not affect BC risk, its carriers had a similar tendency for BC subtype distribution. Phenotypical characteristics of functionally deleterious missense and truncating *CHEK2* mutation carriers were similar (Supporting Information Table S5).

Second primary cancers (other than BC/OC; Supporting Information Table S3) were diagnosed in *CHEK2* mutation carriers more frequently (10/54; 18.5%) than in carriers of other CPG mutations (25/424; 5.9%; $p = 0.003$) or noncarriers (110/1,403; 7.8%; $p = 0.01$). All 10 *CHEK2* mutation carriers with second cancer (developing 13 tumors together including two cases each of colon, thyroid, renal, head/neck cancers or hematological malignancy, and one case each of lung, urinary bladder or endometrial cancer) had a positive family cancer history.

Discussion

The frequency of germline truncating and splice site *CHEK2* mutation carriers in our study strongly prevailed in all patients over PMC (2.39% *vs.* 0.33%; $p = 1.3 \times 10^{-11}$) but the frequencies of missense variants were comparable (4.56% *vs.* 3.90%; $p = 0.22$). Most missense variants, especially in moderate risk genes (including *CHEK2*) are interpreted as inconclusive VUS, lacking clearly defined risk estimates and representing a major drawback for multigene testing in diagnostic settings.[26,27] Only several reports have described a functional characterization of *CHEK2* VUS by *in vitro*[19,22] or yeast models.[35,36] The *in vitro* assays measure CHK2 kinase catalytic activity over artificial substrate but do not reflect changes in CHK2 intracellular targeting, stability and posttranslational modifications. Moreover, transient CHK2 overexpression can cause its autophosphorylation even in the absence of DNA damage, bypassing necessity for CHK2-T68 phosphorylation and participation of FHA domain on CHK2 activation *in vivo*.[37] Yeast analyses are based on functional complementation of *RAD53*-defective *Saccharomyces cerevisiae* cells by human CHK2 homolog. A growth rate of the yeast cells upon DNA damage correlates with functional competence of the analyzed *CHEK2* variant in this assay. In contrast, our newly developed RPE1-*CHEK2*-KO cell-based assay allowed us to quantify catalytic activity of analyzed *CHEK2* variants in nontransformed human cells in the presence of CHK2 natural upstream activators and downstream substrates.

Altogether, results of functional analysis for 18/26 (69%) of analyzed missense VUS were in full agreement or partially overlapped between our KAP1 cell-based and *in vitro* analyses. Remaining eight variants (p.E64K, p.T168I, p.L174V, p.R346H, p.I364T, p.Y424H, p.P425L, p.T476M) scored discordantly. In subsequent analyses of p.L174V, p.I364T and p.T476M variants, we demonstrated that discordance between results of cell-based and *in vitro* assays resulted from their fundamental differences (Figs. 1*c*–1*e*). Variant p.L174V only mildly decreased KAP1 phosphorylation *in vitro*, but failed to phosphorylate KAP1 in cells. Further analysis revealed that this variant impairs intracellular protein stability explaining its functional defect in cells. This rare FHA domain variant was described once in ClinVar. We identified p.L174V in BC patient diagnosed at 35 years carrying also a pathogenic *BRCA1* mutation (Supporting Information Fig. S4). Variant p.I364T showed low KAP1 phosphorylation *in vitro* but was able to phosphorylate KAP1 in cells. Subsequent analysis demonstrated that CHK2-T364 protein was phosphorylated at T68 when immunoprecipitated from cells and that removing this modification by λ-phosphatase treatment strongly reduced its catalytic activity (Figs. 1*d* and 1*e*) comparable to that in wild-type CHK2. Moreover, Chrisanthar *et al.* described normal dimerization and autophosphorylation, and only mildly reduced kinase activity for p.I364T, concluding a nonaffected

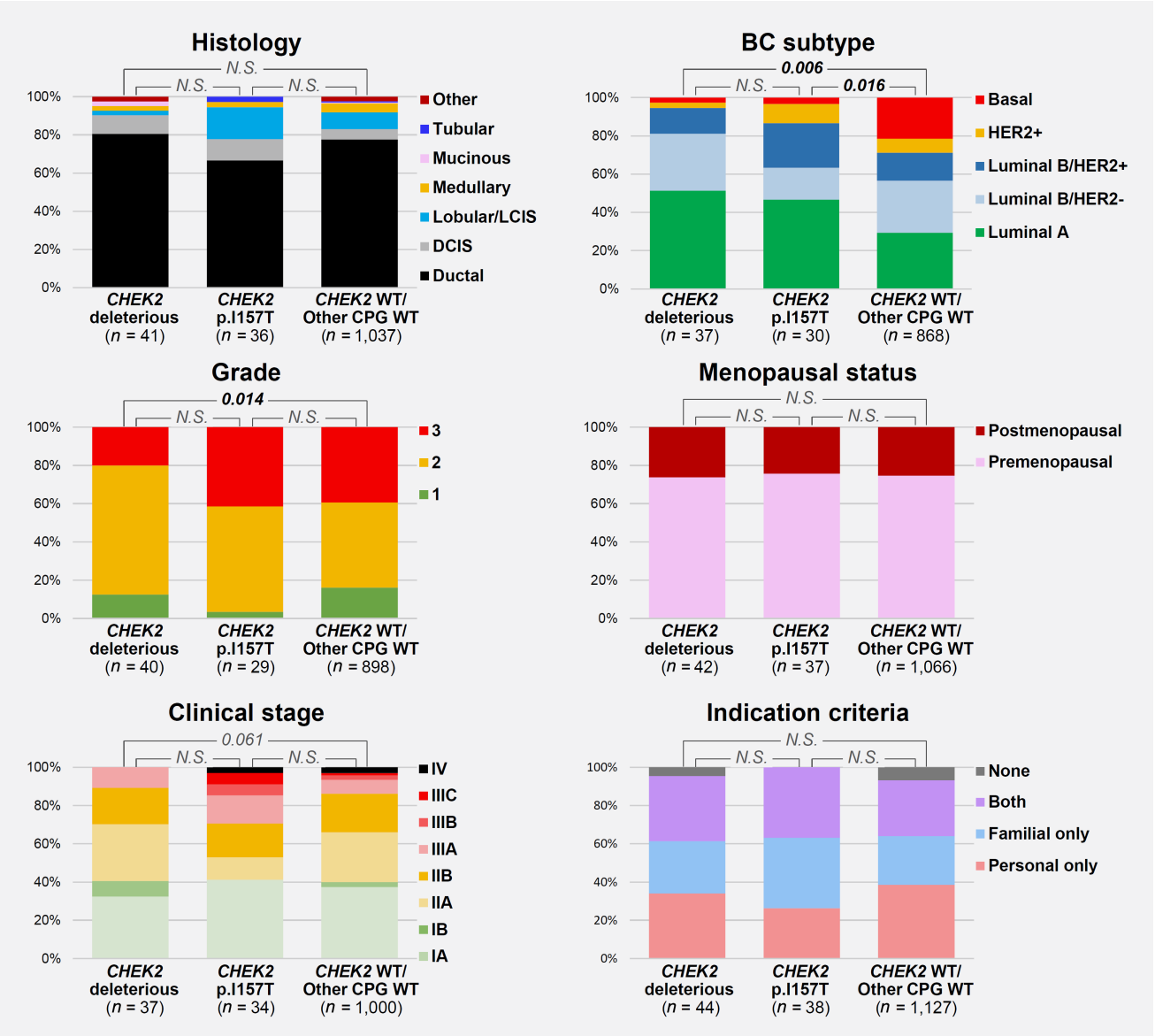

Figure 3. Clinical and histopathological characteristics of female BC patients. A subgroup of 1,209 other CPG-wt patients with any BC were stratified according to the presence of germline deleterious *CHEK2* mutation (truncating or pathogenic missense; $n = 44$), p.I157T ($n = 38$) and *CHEK2*-wt patients ($n = 1,127$), respectively. Significant differences between groups are highlighted in bold (N.S. denoted for not significant differences with $p < 0.1$). Numbers in parenthesis (n) characterize number of individuals with known values for particular characteristic. *Note*: "Other CPG-wt" group consists of patients without germline mutation in genes predisposing for BC (*BRCA1*, *BRCA2*, *PALB2*, *TP53*) or OC (*BRCA1*, *BRCA2*, *RAD51C*, *RAD51D*, *MLH1*, *MSH2*, *MSH6*). [Color figure can be viewed at wileyonlinelibrary.com]

kinase function;[38] Delimitsou *et al.* recently scored p.I364T by *S. cerevisiae* assay functionally intermediate (Supporting Information Table S2).[36] We identified this variant in premenopausal BC patient with no cancer diagnosed in first or second-degree relatives. The p.T476M variant behaved similarly as p.I364T, with T68 phosphorylation-dependent kinase activity (Fig. 1*e*). This variant was classified by Delimitsou intermediate, but previous analyses by Roeb *et al.*[35] and Desrichard *et al.*[19] (Supporting Information Table S2) scored p.T476M deleterious by yeast and *in vitro* assays, respectively. We found this variant in three patients and three PMC. Moreover, in concordance with our cell-based assay, the p.T476M was classified as likely benign by Myriad using history weighting algorithm.[39]

Another five discrepant variants were scored in our cell-based assay functionally deleterious. The p.E64K variant affecting SQ/TQ domain was previously analyzed by Wu *et al.*[40] who described its reduced autophosphorylation, CDC25C phosphorylation and severely impaired T68 phosphorylation and concluded

that p.E64K alters SQ/TQ domain conformation impairing CHK2 activation. Two later independent analyses showed mutually opposite results in yeast assays (Supporting Information Table S2).[35,36] We found p.E64K in one OC and three BC patients, including a carrier who developed three primary tumors (Supporting Information Fig. S4); however, two carriers were also identified in PMC, including a male (aged 68) and female (aged 63). We found no additional functional data for p.T168I, a variant localized to the FHA domain, functionally defective also in our Omnia kinase assay (Supporting Information Table S2). We detected p.T168I in a patient carrying a *BRCA2* mutation diagnosed with BC and OC (Supporting Information Fig. S4). Variant p.R346H, affecting kinase domain, was functionally classified deleterious also by Delimitsou *et al.*[36] and our Omnia kinase assay (Supporting Information Table S2). Moreover, in a BCAC study, Southey found an increased BC risk (OR = 5.06; 95%CI 1.09–23.5; $p = 0.017$) for p.R346C variant at the same position[41] and we observed a segregation of p.R346H with BC in analyzed HBC family (Supporting Information Fig. S4). The p.Y424H kinase domain variant was classified functionally defective by two out of three previous yeast-based analyses and in our Omnia kinase assay (Supporting Information Table S2). We detected p.Y424H in patient with double primary premenopausal BC with multiple cancers in family members. The p.P425L variant, affecting P425 participating in CHK2 kinase domain dimerization,[7] showed also partially reduced Omnia kinase assay activity. We found this variant in BC patients diagnosed at 47 years; however, no other relatives were available for the genetic analysis.

Conceptual differences in functional *CHEK2* assays contribute to discrepant findings for individual VUS, especially in variants sensitive to posttranslational CHK2 modifications. Hence, we think that our assay performed in human nontransformed cells provides an opportunity for realistic functional *CHEK2* VUS analysis. Estimated BC risks associated with functionally deleterious, intermediate and neutral variants (Table 2) revealed a lack of risk association for the latter two groups, supporting our correct functional classification. Altogether, functionally deleterious missense mutations were identified in 15 out of 88 *CHEK2* missense variant carriers (Table 1) constituting 20–25% of pathogenic *CHEK2* mutation in BC patients and 40% in OC patients. However, low number of carriers of functionally deleterious variants limited validity of presented data. The extension of our assay to large-scale *CHEK2* VUS analyses with evaluation of clinical data in their carriers will be required to validate our findings, including lower risk associated with functionally deleterious missense variants in comparison to truncations.

To calculate cancer risk for carriers of deleterious *CHEK2* mutations, we considered *all high-risk patients* and, in parallel, a subgroup of *CPG-wt patients*. The *all high-risk patients* group revealed the real proportion of *CHEK2* mutation carriers and associated cancer risk in a realistic context of all individuals indicated for genetic testing according to current guidelines. The analysis of the *CPG-wt* subgroup (raising the proportion of *CHEK2* mutation carriers by excluding 424 other CPG-mutation carriers of whom 90% carried a *BRCA1/BRCA2* mutation) allows to compare our findings with studies analyzing *BRCA1/BRCA2*-wt patients (Table 3).

We are aware that risk calculations have their specific limitations. Analyzed patients' groups were enriched in high-risk patients from multiple cancer families and, in contrast, PMC group share higher proportion of older noncancer individuals. Both factors can contribute to an overestimated risks found in our study. Other *CHEK2* studies also demonstrated higher OR found in analyses involving patients with familial BC (Table 3) indicating that a precise risk estimation will require a representative number of analyzed individuals and appropriately selected PMC. Higher cancer risks found in our study was affected also by high frequency of LGRs whose identification by panel NGS has been considered problematic[34] or omitted[26] in comparable analyses. Our data urge its careful evaluation in *CHEK2* analyses. Although the OR values calculated in our study must be interpreted with caution (especially in case of missense variants), our data clearly show that germline *CHEK2* mutations carriers are significantly enriched especially in the largest group of female BC patients. Interestingly, deleterious *CHEK2* mutations increased risk of male BC. *CHEK2* was the second most frequently mutated CPG in this small subgroup, preceded by *BRCA2* and followed by *BRCA1*, and *PALB2* (data not shown), indicating that germline *CHEK2* mutations contribute to male BC, as suggested previously.[51,53,54]

Deleterious *CHEK2* mutations were associated with a moderately increased OC risk in our study. However, due to the limited numbers of analyzed OC individuals with *CHEK2* mutations (10 in all patients, 4 in the CPG-negative subgroup), these observations need further validation. A substantial proportion of deleterious missense mutations (4/10) in OC patients indicates that their functional classification will be necessary for proper OC risk assessment.

Our analysis confirmed proposed "*CHEK2* mutation-specific" tumor phenotype, characterized by premenopausal, ductal, grade 2, luminal A or luminal B/HER2-negative tumors, reported in other studies.[25,26,46,55] These tumor characteristics lost in carriers of coincidental *BRCA1/BRCA2* mutations having a stronger effect on tumor phenotype. Nurmi *et al.*[42] identified an additive effect of mutations in moderate-penetrance genes, including *CHEK2*, increasing BC risk in Finnish *BRCA1/BRCA2* mutation carriers. The effect of coincidental alterations in other moderate-penetrance CPG with *CHEK2* mutations are unknown; however, the influence of a polygenic risk score on c.1100delC penetrance has been recently documented.[56]

A strongly increased frequency of second cancers of various origin in *CHEK2* mutation carriers and tumors in their relatives corresponds to documented multiorgan cancer susceptibility in *CHEK2* mutations carriers[5,25] and indicates that family cancer history associated with *CHEK2* mutations must be reconsidered to facilitate the selection of potential *CHEK2* mutation carriers for genetic analyses.

The p.I157T variant did not increase cancer risk in our study; an observation we have previously reported for sporadic BC

Table 3. Analyses of germline variants in the *CHEK2* gene or analyses of selected germline *CHEK2* variants in studies (upper part) and selected meta-analyses (lower part) calculating odds ratio for breast cancer development in mutation carriers

References	Pop.	P: patientsC: controls	Analysis	Odds ratio (95%CI); *p*—evaluated group or *CHEK2* variant
Nurmi *et al.*[42]	FI	P: 3156 BC or OC patients C: 2089 PMC	c.319+2T>A	5.40 (1.58–18.45); 0.007—unselected BC 6.04 (1.65–22.10); 0.007—familial BC
Girard *et al.*[43]	FR	P: 1,207 *BRCA1/2*-negative BC females having sister with BC C: 1,199 noncancer PMC	*CHEK2* (panel NGS)	3.0 (1.9–5.0); 1×10^{-5}—any variant 5.8 (2.0–16.9); 0.001—loss of function variant 2.4 (1.4–4.3); 0.002—likely deleterious missense
Hauke *et al.*[26]	DE	P: 5,589 *BRCA1/2*-negative BC C: 2,189 noncancer PMC	*CHEK2* (panel NGS)	3.72 (1.99–6.94); <0.0001—truncations
Couch *et al.*[24]	US	P: 29,090 BC C: ExAC-NFE non-TCGA	*CHEK2* (panel NGS)	2.31 (1.88–2.85); 3.04×10^{-17}—c.1100delC 2.26 (1.89–2.72); 1.75×10^{-20}—pathogenic variants (p.I157T, p.S428F excluded) 1.48 (1.31–1.67); 1.75×10^{-10}—any variant (p.I157T, p.S428F included) 1.35 (1.12–1.63); 0.0002; bilateral BC
Decker *et al.*[44]	UK	P: 13,087 BC C: 5,488 PMC	*CHEK2* (4 genes)	3.11 (2.15–4.69); 5.6×10^{-11}—truncations 1.36 (0.99–1.87); 0.066—all rare missense 1.51 (1.02–2.24); 0.047—rare missense in any domain 3.27 (1.66–5.83); 0.0014—bilateral BC
Slavin *et al.*[45]	US	P: 2,266 *BRCA1/2*-neg. Fam. BC C: ExAC	*CHEK2*	1.62 (1.03–2.51); 0.004—truncations
Schmidt *et al.*[46]	BCAC	44,777 BC 42,977 PMC	c.1100delC	2.26 (1.90–2.69); 2.3×10^{-20}—invasive BC 2.55 (2.10–3.10); 4.9×10^{-21}—ER-positive BC 1.32 (0.93–1.88); 0.12—ER-negative BC
Southey *et al.*[41]	BCAC	P: 42,671 C: 42,164 PMC	c.349A>G (p.R117G) c.538C>T (p.R180C) c.715G>A (p.E239K) c.1036C>T (p.R346C) c.1312G>T (p.D438Y)	2.26 (1.29–3.95); 0.003—for variant p.R117G 1.33 (1.05–1.67); 0.016—for variant p.R180C 1.70 (0.73–3.93); 0.210—for variant p.E239K 5.06 (1.09–23.5); 0.017—for variant p.R346C 1.03 (0.62–1.71); 0.910—for variant p.D438Y
Cybulski *et al.*[47]	PL	P: 7,494 *BRCA1*-negative BC C: 4,346 PMC	c.1100delC, c.444+1G>A, del5395	3.6 (2.6–5.1)—BC 3.3 (2.3–4.7)—patients with no BC family history 5.0 (3.3–7.6)—patients with BC in first or second degree relatives 7.3 (3.2–16.8)—patients with BC in first and second degree relatives
Desrichard *et al.*[19]	FR	P: 507 *BRCA1/2*-negative BC C: 513 noncancer PMC	*CHEK2*	4.15 (1.38–12.50); 0.007—all *CHEK2* variants 5.18 (1.49–18.00); 0.004—*CHEK2* mutations (p.K244R ex)
Le Calvez-Kelm *et al.*[20]	US, AU	P: 1303 BC ≤45 years C: 1,109 noncancer females	*CHEK2*	6.18 (1.76–21.8)—truncations 2.20 (1.20–4.01)—rare missense
Weischer *et al.*[48]	DK	P: 1,101 BC C: 4,665 PMC	c.1100delC	3.2 (1.0–9.9)—BC (prospective study) 2.6 (1.3–5.4)—BC (case–control study)

(Continues)

Table 3. Analyses of germline variants in the *CHEK2* gene or analyses of selected germline *CHEK2* variants in studies (upper part) and selected meta-analyses (lower part) calculating odds ratio for breast cancer development in mutation carriers (Continued)

References	Pop.	P: patientsC: controls	Analysis	Odds ratio (95%CI); *p*—evaluated group or *CHEK2* variant
Cybulski *et al.*[5]	PL	P: 1,017 BC C: 4,000 PMC	c.1100delC; c.444+1G>A; p.I157T	2.2; $p = 0.02$—for c.1100delC and c.444+1G>A 1.4; $p = 0.02$—for p.I157T
Dufault *et al.*[21]	DE	P: 516 *BRCA1/2*-negative BC C: 1,315 random PMC	*CHEK2*	3.44 (1.19–9.95); 0.016—c.1100delC 3.9 (1.3–10.9)—c.1100delC and c.1214del4
CHEK2 Breast Cancer Case-Control Consortium[49]	UK, NL, FI, DE, AU	P: 10,860 BC C: 9,065 PMC	c.1100delC	2.34 (1.72–3.20); 1×10^{-7} 2.23 (1.60–3.11)—BC with no BC in first degree relative 3.12 (1.90–5.15)—BC with 1 BC in first degree relative 4.17 (1.26–13.75)—BC with ≥2 BC in first degree relative
Vahteristo, 2002[50]	FI	1,035 unselected BC 1885 PMC	c.1100delC	1.48 (0.83–2.65); 0.182 unselected BC 2.27 (1.11–4.63); 0.021 familial BC 6.17 (1.87–20.32); 0.007 bilateral BC
Liang *et al.*[51]	Meta	P: 118,735 BC C: 195,807	c.1100delC	2.88 (2.65–3.16)—female BC 2.87 (1.85–4.47)—early onset BC 3.21 (2.41–4.29)—familial BC 3.13 (1.94–5.07)—male BC
Liu *et al.*[16]	Meta	P: 19,621 BC C: 27,001	p.I157T	1.48 (1.31–1.66); <0.0001—unselected BC 1.48 (1.16–1.89); <0.0001—familiar BC 1.47 (1.29–1.66); <0.0001—early onset BC 4.17 (2.89–6.03); <0.0001—lobular BC
Zhang *et al.*[52]	Meta	P: 9,970/ C:7,526 P: 13,331/C: 10,817 P: 10,543/ C:10,817 P: 4,1,791/C: 50,910	c.444+1G>A p.I157T del5395 c.1100delC	3.07 (2.03–4.63); 9.82×10^{-8}—for variant c.444 +1G>A 1.52 (1.31–1.77); 4.76×10^{-8}—for variant p.I157T 2.53 (1.61–3.97); 6.33×10^{-5}—for variant del5395 3.10 (2.59–3.71); $<10^{-20}$—for variant c.1100delC
Weischer *et al.*[15]	Meta	P: 26,488 C: 27,402	c.1100delC	2.7 (2.1–3.4)—unselected BC 2.6 (1.3–5.5)—early onset BC 4.8 (3.3–7.2)—familial BC

Abbreviations: AU, Australia; BC, breast cancer; BCAC, Breast Cancer Association Consortium; CN, China; DE, Germany; EU, European union; FI, Finland; DK, Denmark; FR, France; meta, meta-analysis; NL, Nederland; PL, Poland; US, USA.

patients.[57] With OR = 1.5 reported in numerous studies (Table 3), is below the threshold considered for moderate-penetrance genes (OR > 2) and together with a high frequency in PMC it negates a clinically considerable effect on BC risk. We noticed a higher proportion of lobular BC in p.I157T carriers (Fig. 3), known from previous studies.[16,58,59] Our functional analysis classified p.I157T as an "intermediate" variant with catalytic activity reaching 48.8% of wild-type CHK2. Hence, an increased cancer risk cannot be ruled out in homozygote p.I157T carriers.

In conclusion, our study demonstrated a substantial clinical relevance of a *CHEK2* analysis in high-risk BC/OC patients, supported by the results of a cell-based functional assay markedly reducing the number of VUS. In addition, the high frequency of non-BC/OC tumors in *CHEK2* mutation carriers and their relatives warrants further investigation by collaborative international efforts.

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Review

CHEK2 Germline Variants in Cancer Predisposition: Stalemate Rather than Checkmate

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Abstract: Germline alterations in many genes coding for proteins regulating DNA repair and DNA damage response (DDR) to DNA double-strand breaks (DDSB) have been recognized as pathogenic factors in hereditary cancer predisposition. The ATM-CHEK2-p53 axis has been documented as a backbone for DDR and hypothesized as a barrier against cancer initiation. However, although CHK2 kinase coded by the *CHEK2* gene expedites the DDR signal, its function in activation of p53-dependent cell cycle arrest is dispensable. *CHEK2* mutations rank among the most frequent germline alterations revealed by germline genetic testing for various hereditary cancer predispositions, but their interpretation is not trivial. From the perspective of interpretation of germline *CHEK2* variants, we review the current knowledge related to the structure of the *CHEK2* gene, the function of CHK2 kinase, and the clinical significance of *CHEK2* germline mutations in patients with hereditary breast, prostate, kidney, thyroid, and colon cancers.

Keywords: checkpoint kinase 2; CHK2; CHEK2; KAP1; WIP1; germline mutation; hereditary cancer; breast cancer; prostate cancer; renal cancer; thyroid cancer; colorectal cancer

1. Introduction

The accumulation of DNA mutations during the continually increasing human life span contributes to rising cancer prevalence worldwide [1]. Cancers are now the first or second leading cause(s) of premature death in individuals between 30 and 69 years in 91 countries of the world [2]. DNA alterations with increased cancer-promoting potentials affect tumor suppressor genes participating in DNA damage repair (DDR) and regulating cell cycle checkpoints [3,4]. Moreover, uncoupling these two processes may cause sustained proliferation of genetically unstable cells resulting in malignant transformation.

Most cancers (over 90%) develop as sporadic tumors during life-long acquisition of DNA mutations. In contrast, less than 10% of cancers forms hereditary tumors that arise as a result of germline mutations in cancer predisposition genes [5]. Typical features—a high overall cancer risk, earlier age at disease onset, and 50% probability of transmitting the mutation to the offspring together with an accumulation of tumors in affected families—increase the medical importance of hereditary cancers and justify genetic counseling in affected families. Moreover, the share of hereditary tumors is higher in several frequent or highly malignant cancer types, including breast, pancreatic, or ovarian cancers. The identification of a causal mutation not only directs tumor-specific surveillance and preventive strategies but also

impacts disease prognosis and targeted treatment [6]. In fact, proper identification and surveillance in mutation carriers has the potential to reduce the bulk of cancer-related mortality associated with several solid tumor types [7].

Fast progress in cancer genetics and the introduction of next-generation sequencing (NGS) have revolutionized the diagnostics of hereditary cancers in the last decade [8,9]. An analysis of individuals at risk using panels of cancer predisposition genes outperforms previous gene-by-gene analyses [10]. The availability of an easy and economically affordable panel NGS analysis together with the widening of testing criteria have brought high volumes of data. Germline variants found in cancer patients not only have confirmed the clinical utility of pathogenic mutations in high-penetrant "first wave" (including *BRCA1*, *BRCA2*, *MLH1*, and *MSH2*) and "second wave" (*PALB2*, *RAD51C*, and *RAD51D*) genes predisposing to common cancers but also have identified dozens of variants with unknown clinical significance (VUS) and variants of the "second wave" moderate penetrance genes (including *ATM* or *CHEK2*) [7,11]. While all cancer predisposition genes are now equal from the perspective of germline testing, the clinical utility of several genes (including *CHEK2*) varies in a broad interval delimited by their penetrance and population-specific prevalence [12]. The determination of penetrance requires careful assessment in the families of mutation carriers and in large populations of cancer patients and corresponding population-specific controls [13]. Moreover, the classification of germline variants and convincing identification of pathogenic mutations are demanding for most cancer predisposition genes.

In this review, we focus on the *CHEK2* gene coding checkpoint kinase 2 protein (CHK2), which was initially recognized as an effector kinase in the ATM-CHK2-p53 pathway in DDR, especially in response to DNA double-strand breaks (DDSB) [14–16]. The competence of the ATM-CHK2-p53 signaling cascade has been hypothesized as a barrier preventing early tumorigenesis [17], inducing cell cycle blockade, apoptosis, or senescence in transformed cells [18]. While initial studies associated *CHEK2* germline mutations with a moderate breast cancer risk, later ones identified a much wider portfolio of cancer types in *CHEK2* mutation carriers [19,20]. Routine genetic testing of *CHEK2* is now included in diagnostic NGS panels targeting various hereditary cancers, and *CHEK2* ranks among genes with the highest frequency of germline mutations. However, the presence of many variants of unknown significance (VUS) with a specific population prevalence prevents precise assessment of the risk associated with particular tumor types in *CHEK2* mutation carriers [21,22]. Thus, finding a *CHEK2* germline variant is sometimes perceived as a hindrance to a conclusive genetic interpretation rather than a gain for the clinical management of carriers. With this in mind, we have also reviewed the clinical importance of germline *CHEK2* mutations in patients with breast, prostate, kidney, papillary thyroid, and colorectal cancers.

2. Structure and Function of CHK2 Kinase

Human CHK2 kinase was identified in 1998 by Matsuoka et al. based on its homology to yeast checkpoint kinases Rad53 (in *Saccharomyces cerevisiae*) and Cds1 (in *Schizosaccharomyces pombe*). This pioneering work and subsequent papers from other laboratories were published with a short delay placed CHK2 downstream of ATM activation in DDR [23–25]. CHK2 kinase is widely expressed in proliferating, renewing cell populations but not in resting or terminally differentiated cells [26].

2.1. The CHEK2 Gene

Tominaga and colleagues [27] localized the *CHEK2* gene to human chromosome 22 (22q12.1), where it spans 54 kb (chr22: 28,687,743–28,742,422; reverse strand; GRCh38). The most expressed transcription variant 1 (NM_007194/ENST00000404276.6) codes for an mRNA consisting of 15 exons with the translation start localized in exon 2. The relevance of alternative splicing variants remains unclear, but their proportion increases in tumor tissues [28]. Putative transcription factor binding sites (including SP1, CCAAT box, C/EBP, AP1, and E2F) were identified in the *CHEK2* promoter spanning the 268-bp region upstream of the transcription start site [29]. CpG islands identified in the

5′ region include a distal (and rarely methylated) CpG island (located −6000 to −8000 from ATG) and a proximal (heavily methylated) CpG island (located −300 to −600 from ATG) [30]. The 3′ portion of the *CHEK2* gene comprising exons 10–14 is duplicated with >90% homology in the human genome (on chromosomes 2, 7, 10, 13, 15, 16, 22, X, and Y) as non-expressed pseudogenes [31]. Munch and colleagues [32] performed a phylogenetic analysis of a *CHEK2* duplicon in anthropoids, indicating a burst of gene duplication in African great apes and humans.

2.2. Structure of CHK2 Kinase Protein

The translation product of the dominant splicing variant consists of 543 amino acids forming the 65 kDa protein. CHK2 comprises three conserved functional domains including a SQ/TQ cluster domain (SCD) at the N-terminus, a forkhead-associated (FHA) domain, and a kinase domain (KD) at the C-terminus [33]. Crystallographic studies have unveiled a nearly complete CHK2 kinase structure in its monomeric and homodimeric forms with the exception of the SCD, extreme C-terminal parts, and several disordered regions, including activation loops (Figure 1) [34–36].

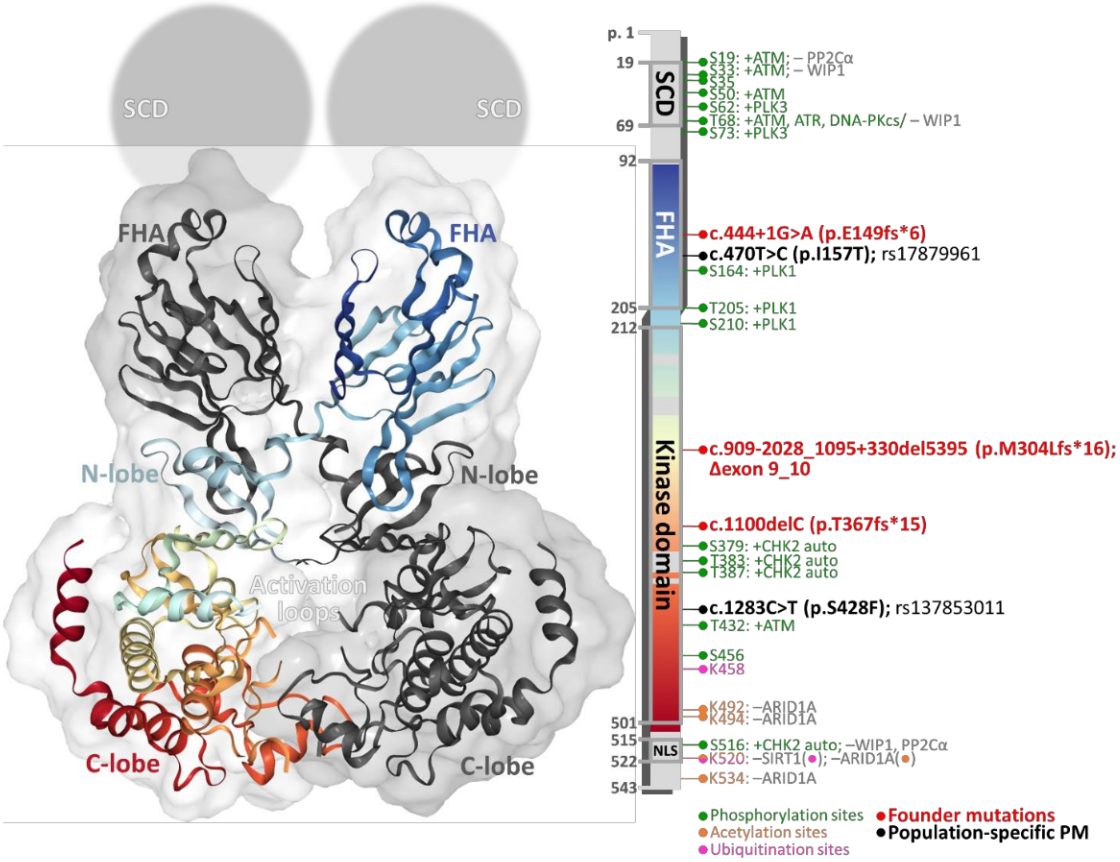

Figure 1. The structure of a CHK2 dimer (left; www.rscb.org/structure/3I6W) consists of intertwined monomers (one subunit is colored in gray, and the second subunit is colored in gradient from the N- to C-terminus; missing parts of 3D structures are colored in gray). The same color-coding of the bar (right) indicates the positions of conserved domains (boundaries reflect the crystallographic analysis by Cai et al. [35]). Lollipops depict known sites of covalent modifications, founder mutations, and variants.

The SCD (residues 19–69) is characterized by seven pairs of serine–glutamine or threonine–glutamine (SQ/TQ) residues phosphorylated by ATM and other kinases [37,38]. It contains the T68 residue important for CHK2 activation; however, the entire SCD (51 amino acids) consists of 22 × S and 5 × T residues representing potential targets for other S/T protein kinases. The FHA domain

(residues 92–205) is arranged in an 11-stranded β sandwich and mediates phosphorylation-dependent protein–protein interactions of CHK2 [34]. A seven-residue linker (residues 206–212) connects FHA and the kinase domain (KD). Almost half of the protein sequence comprises a serine–threonine KD (residues 212–501) consisting of two lobes forming an ATP-binding site at the cleft between them. The N-terminal lobe (residues 213–305) is formed mainly by β-sheet structures and contains a conserved E273 important for catalysis, while the larger C-terminal lobe (306–501) is mostly α-helical. The activation loop (residues 371–391) contains several activating phosphorylation sites (T383 and T387) that participate in substrate binding [35]. The nuclear localization signal (NLS) at the C-terminus is recognized by karyopherin-α2 (KPNA2) importing the CHK2 molecule into the nucleus [39].

2.3. Regulation of CHK2 Kinase Activity

Extensive covalent modifications of amino acid residues (phosphorylation, ubiquitination, and acetylation) and noncovalent interactions (homodimerization and phosphoprotein–protein interactions) influence the catalytic activity, substrate specificity, intracellular trafficking, and the half-life of CHK2 kinase.

2.3.1. Phosphorylation

In the absence of DNA damage stimuli, CHK2 kinase resides in its monomeric inactive form. Upon DNA damage, ATM phosphorylates T68 [37] in the SCD of CHK2, promoting its transient homodimerization. ATM phosphorylation at the T68 priming site is important for full CHK2 activity in cells; however, CHK2 overexpression in bacteria or mammalian cells promotes dimerization and activation independently of ATM or ionizing radiation (IR) [40]. The exploration of CHK2 phosphorylation kinetics demonstrated that T68 phosphorylation occurs 3 min after neocarzinostatin treatment in HCT116 cells, followed by phosphorylation on S19 and S33/35 [38]. In addition, phosphorylation of other residues (S50 and T432) remains unclear [41,42]. Other candidate phosphorylation sites (S120, S260, T225, S379, and S435) were identified in recombinant CHK2 expressed in bacteria and insect cells expression systems using mass spectrophotometry, but their importance is largely unknown [43].

The process of CHK2 kinase activation includes the formation of a transient dimer through reciprocal FHA–KD and FHA–FHA interactions. The I157 residue resides in the center between these interfaces associating intramolecularly with the N-lobe in the KD [35]. The domain-exchanged, intertwined CHK2 homodimer promotes kinase activation by trans-autophosphorylation [35]. Wedged CHK2 molecules *trans*-phosphorylate T383 and T387 residues in activation loops exchanged between protomers (Figure 1), leading in turn to a disruption of homodimer conformation and release of the two catalytically active CHK2 monomers [34–36,44]. A description of another phosphoserine residue, S516, suggested that activating autophosphorylation of CHK2 can occur *in cis* (S516) and *in trans* (T383/387) depending on CHK2 dimerization [40,45]. The phosphorylation of CHK2 T68 has been frequently used as a marker of ATM activation [46], but T68 could be phosphorylated also by ATR in vitro [37] and by the DNA-dependent protein kinase catalytic subunit (PRKDC, alias DNA-PKcs) during mitosis [47]. Other CHK2 phosphorylation sites have been described as the targets of other kinases including polo-like kinase 3 (PLK3) [48,49] or PLK1 [50,51]. PLK3 phosphorylated S62 and S73 in vitro and was proposed to facilitate subsequent phosphorylation on T68 by ATM; however, this possibility has recently been challenged when no impact of PLK3 on checkpoint activation was found [48]. PLK1 in a complex with TP53-binding protein 1 (53BP1) phosphorylates CHK2 on S164, T205, and S210 to prevent its activation in mitosis, with S164 phosphorylation showing the greatest effect. Moreover, a co-localization of CHK2 with PLK1 has been observed during mitosis at centrosomes [52].

2.3.2. Dephosphorylation

The activation of CHK2 in DDR is antagonized by its dephosphorylation by protein phosphatases, including WIP1 phosphatase (protein phosphatase Mg/Mn-dependent 1D; PPM1D) [53–55].

Human WIP1 belongs to a protein phosphatase type 2C family and is a homologue of Ptc2 and Ptc3, which regulate Rad53 in yeast [40,56]. WIP1 efficiently dephosphorylates residues at SQ/TQ sites in the CHK2 SCD, including T68 in a cell culture model; however, WIP1 is unable to dephosphorylate phosphorylated T387 in the activation loop [53]. It has been proposed that, under physiological conditions, this WIP1 activity participates in checkpoint recovery rather than in an inhibition of ATM/ATR-mediated response following DNA damage. It seems that fully active CHK2 kinase phosphorylated on residues in the activation loop is less sensitive to WIP1 dephosphorylation activity. Phosphorylated S516 is more accessible for dephosphorylation by other phosphatases, including PP2Cα [57]. Carlessi and colleagues [58] identified basal CHK2 phosphorylation (including T68) by tonic ATM signaling in undamaged cells and its counteraction by WIP1, PP2A, and PP1. The authors proposed that the activities of these phosphatases maintain the basal state of the ATM/CHK2 regulatory circuit. A recent study of clonal hematopoiesis in cancer patients treated by radiation, platinum, or topoisomerase II inhibitors found that preferentially selected somatic mutations affect all members of this circuit (*ATM*, *CHEK2*, *PPM1D*, and *TP53*) and increase the risk of therapy-related myeloid neoplasm development [59].

2.3.3. Ubiquitination

CHK2 turnover is regulated by ubiquitin-mediated proteasomal degradation. Several E3 ubiquitin-protein ligases targeting CHK2 have been described. Ubiquitination catalyzed by the PIRH2 E3 ubiquitin-protein ligase (p53-induced protein with a RING-H2 domain) requires dephosphorylation of S456 in the CHK2 KD [60] and the presence of MDM2 (mouse double minute 2 homolog), an E3 ubiquitin-protein ligase, and P/CAF (p300/CBP-associated factor, known also as lysine acetyltransferase 2B; KAT2B), which was found to have an intrinsic E3 ligase activity [42,61]. Thus, phosphorylation at S456 increases CHK2 stability after DNA damage. In contrast, ubiquitination of CHK2 by seven in absentia homolog 2 (SIAH2) is independent of S456 phosphorylation and has been proposed as a mechanism regulating CHK2 basal turnover [62]. Ubiquitination of CHK2 catalyzed by the E3 ubiquitin-protein ligase complex containing Cullin 1 (CUL1) in response to DNA damage depends also on the autophosphorylation of S379 [63]. However, CUL1-mediated ubiquitination does not affect CHK2 stability. It has rather been proposed to contribute to CHK2-mediated apoptosis in U2OS cells in response to ionizing radiation. CHK2 has been identified in complexes targeted to DNA damage sites with a CHK2-interaction partner and adaptor protein MDC1 (mediator of DNA damage checkpoint protein 1) and with E3 ubiquitin-protein ligase RNF8; however, it has not been determined when CHK2 is ubiquitinated [63]. Recently, Wand and colleagues [64] described that ARID1A (AT-rich interactive domain-containing protein 1A), a component of SWI/SNF chromatin remodeling complexes, targets CHK2 for polyubiquitination at lysine residues K492, K494, K520, and K534. Thus, a loss of ARID1A by somatic mutations (ranking among the most frequent somatic alterations in various tumors) increases the CHK2 level.

Ubiquitination is opposed by deubiquitinases. Among them, USP28 (ubiquitin-specific peptidase 28) and USP39 have been evidenced to deubiquitinate CHK2, with an apparent impact on CHK2 upregulation upon IR or cisplatin-induced DNA damage [65,66].

2.3.4. Acetylation

Although acetylases modifying CHK2 by acetylation are largely unknown, several reports have described not only CHK2 deacetylation catalyzed by NAD+-dependent histone deacetylase SIRT1 targeting histones but also non-histone proteins implicated in the regulation of many physiological and pathological processes, including DDR and tumorigenesis [67]. A recent study showed that SIRT1 directly deacetylates K520 in CHK2, suppressing its phosphorylation, dimerization, and thus activation. Moreover, this study provides evidence that Chk2 hyperactivity in Sirt1−/− mice is responsible for embryonic lethality that could be rescued by Chek2 co-deletion.

In conclusion, CHK2 covalent modifications affect its catalytic activity, turnover, and targeting. However, it is still not certain how covalent modifications influence CHK2 substrate specificity and direct CHK2 functions depending on the cell cycle phase.

2.4. CHK2 Substrates and Its Effector Pathways

Once activated, CHK2 phosphorylates many intracellular targets carrying a consensus motif containing a hydrophobic amino acid (B^{α}) at position −5 and arginine (R) residue at position −3: B^{α}-X-R-X-X-S/T [68]. Activated CHK2 kinase then participates in the regulation of many intracellular pathways that were comprehensively reviewed by Zannini and colleagues [69]. Our review summarizes CHK2 activities related to the process of tumorigenesis.

2.4.1. CHK2 in the Regulation of the Cell Cycle, Apoptosis, and Senescence

CHK2 is traditionally portrayed as an effector kinase in the ATM-CHK2-p53 pathway mediating response to DDSB (Figure 2) [14–16]. The double-strand DNA breaks, representing highly toxic events in proliferated cells, are recognized by the MRE11-RAD50-NBN1 (MRN) complex recruiting ataxia telangiectasia-mutated (ATM) kinase into the site of DNA damage [70]. This, in turn, activates ATM, a master regulator of the DDR [71]. ATM phosphorylates CHK2 and other proteins orchestrating DDSB repair and DDR. CHK2 overlaps some ATM targets, amplifies an ATM-triggered signal, and increases the DDR regulation fidelity. A parallel DDR pathway, ATR-CHK1-p53 exploiting CHK1 kinase, is activated mainly by the presence of long stretches of single-stranded (ss)DNA or DNA crosslinks, and it targets several overlapping substrates [72,73].

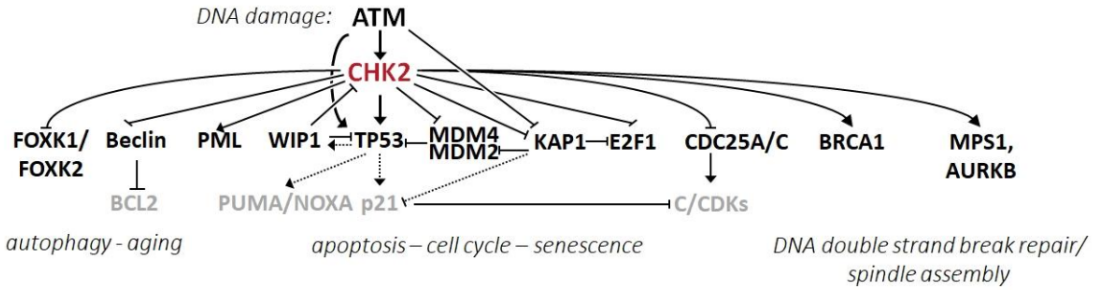

Figure 2. In the presence of DNA damage, especially in the presence of DNA double-strand breaks, sensor protein complexes (not shown) activate the apical kinase ATM phosphorylating CHK2. As an effector kinase, CHK2 phosphorylates numerous substrates (described in the text) participating in critical pathways deregulated in the process of tumorigenesis.

One of the first reported CHK2 targets is the tumor suppressor protein p53. Activated ATM phosphorylates p53 at S15, increasing its stability and activation [74]. Simultaneous phosphorylation of MDM2 disrupts MDM2–p53 interaction and allows p53 stabilization. CHK2 acts on p53 in a similar fashion and phosphorylates p53 at S20, contributing to p53-dependent cell cycle arrest in the G1 phase [75]. Besides, CHK2 phosphorylates MDM4 on S342 and S367 in vivo [76], possibly impacting the transcriptional activity of p53. p53 transactivation targets include the *CDKN1A* gene, coding the inhibitor of cyclin/CDK complexes $p21^{CIP1/WAF1}$ and the *GADD45A* (growth arrest and DNA damage inducible α) gene coding a versatile stress sensor [77–79]. Although CHK2 was initially implicated in the induction of a p53-dependent checkpoint, more recent studies have suggested that the endogenous level of CHK2 does not cause G1 arrest and that the observed checkpoint activation may be an artefact attributable to CHK2 protein overexpression [80]. In agreement with this, no checkpoint defect was observed in HCT116 cells lacking CHK2 and thus the impact of CHK2 on the p53 pathway remains unclear [33,81].

The expressions of p21 and GADD45a are also inhibited by the KRAB (Kruppel-Associated Box Domain)-Associated Protein 1 (KAP1) transcription co-repressor targeting the KRAB-zinc finger

protein superfamily of transcription factors [82]. Upon DNA damage, KAP1 is phosphorylated by ATM and CHK2 (or CHK1) at S824 and S473, respectively [83]. While CHK2 phosphorylates KAP1 dominantly in an etoposide- or ionizing radiation-induced stress response, CHK1 targets the same S473 residue in response to UV radiation. KAP1 S473 phosphorylation relieves its transcriptional repression, which results in increased p21 and GADD45 expressions at the G2/M checkpoint [84]. However, KAP1 S473 also provides a binding site for the E2F1 transcription factor involved in the cell cycle and apoptosis. Increased interaction between KAP1 phosphorylated at S473 and E2F1 decreases the expression of a subset of proapoptotic genes and apoptosis [83]. Besides, CHK2 directly phosphorylates E2F1 at S364, which results in increased E2F1 protein stability and transcriptional activity towards p53-independent apoptosis [85]. Thus, CHK2-activated KAP1 phosphorylation may counteract CHK2-induced E2F1 activity in DDR as a negative regulatory feedback mechanism. Targeting this regulatory network by combination chemotherapy using etoposide and inhibitors of KAP1-S473 phosphorylation may potentiate the cytotoxic effect of chemotherapy [83].

CHK2 has been shown to phosphorylate CDC25 phosphatases, a family of homologous dual-specific enzymes dephosphorylating inhibitory phosphothreonine or phosphotyrosine residues on cyclin-dependent kinases (CDKs) stimulating transition through the cell cycle [86]. The inhibition of CDC25 phosphatases by phosphorylation ensures rapid but transient checkpoint activation, while the activation of p53 is required for longer cell cycle arrest, the induction of senescence, or apoptosis [87]. A CHK2-mediated phosphorylation of CDC25A phosphatase at S123 inhibits dephosphorylation of the cyclin-dependent kinase 2 (CDK2)-cyclin E complex, halting the cell cycle before entry into the S phase [14]. Moreover, CHK2 also phosphorylates CDC25C phosphatase at S216, stimulating an interaction of CDC25C with 14-3-3 proteins. The interaction with 14-3-3 proteins displaces CDC25C from binding and the dephosphorylation of a CDK1-cyclin B complex, required for its activation before mitotic entry [16,88].

CHK2 phosphorylates S117 of the promyelocytic leukemia protein (PML, a tumor suppressor involved in multiple apoptotic pathways) and increases its activity in the induction of γ-radiation-induced apoptosis [89]. In contrast, a fusion protein of PML with retinoic acid receptor α (PML-RARα), resulting from frequent translocation in acute promyelocytic leukemia (t15;17), suppresses CHK2 and inhibits its autophosphorylation [90,91].

2.4.2. CHK2 in the Regulation of DNA Repair and Mitotic Spindle

In response to ionizing radiation, CHK2 phosphorylates breast cancer susceptibility protein 1 (**BRCA1**) at S988, which is believed to modulate the BRCA1 function in DNA repairs towards a homologous recombination (HR) repair instead of non-homologous end joining (NHEJ) [92,93]. Alongside its function in HR, BRCA1 (and other proteins identified as regulators or executors in DDR) has been implicated in mitotic spindle assembly [94]. In case of spindle damage, BRCA1 gets phosphorylated at S988 by CHK2, which leads to protein accumulation and to inhibition of the microtubule-nucleating activity of the centrosome [95–97]. The activity of CHK2 in centrosome regulation includes also the phosphorylation of S/T residues in other regulators, including T288 in the dual specificity protein kinase TTK (alias MPS1), S331 in aurora kinase B (AURKB), or S507 in myosin phosphatase targeting subunit 1 (MYPT1) [94,98]. Cells and organisms lacking CHK2 are viable and fertile, suggesting that its function in mitosis is not essential, and thus, the precise impact of CHK2 on cell division remains to be elucidated.

2.4.3. CHK2 in the Regulation of Autophagy and Aging

CHK2 kinase was also reported to be involved in other processes apart from DDR or cell cycle regulation. In response to oxidative stress, CHK2 has been linked to cell protection via autophagy. High levels of reactive oxygen species (ROS) and hypoxia were reported to trigger the ATM-CHK2 axis and the phosphorylation of Beclin 1 [99]. Beclin 1, coded by the tumor suppressor gene *BECN1*, is an essential regulator of autophagy, and its phosphorylation at S90/S93 by CHK2 has been shown to

disrupt the formation of Beclin 1 (BCL2 autophagy-regulatory complex), reducing ROS production by the autophagy of damaged mitochondria. Thus, the ATM-CHK2-BECN1 autophagy axis may serve as a physiological pathway preventing tissue damage following ischemia [99]. In addition, CHK2 phosphorylates Forkhead transcription factors FOXK1 and FOXK2, which act as transcriptional repressors of autophagy-related genes [100]. CHK2-mediated FOXK phosphorylation induces their binding to 14-3-3 proteins, which, in turn, traps FOXK in the cytoplasm and induces autophagy following DNA damage.

2.4.4. CHK2 in the Regulation of Other Intracellular Pathways

While CHK2 kinase was characterized as a downstream kinase transmitting DDR signal onto effectors over 20 years ago, new functions of CHK2 and the ATM-CHK2 axis have been identified and reviewed by Zannini and colleagues [69]. These "non-canonical" CHK2 activities include stem cell maintenance, regulation of the intracellular response to a viral infection, or the participation of circadian clock regulation.

Despite substantial progress, it should be noted that many canonical as well as novel CHK2 functions have been studied dominantly in model systems involving tumor cell lines. However, little is still known about the real demand for CHK2 functions in particular tissues under physiological and pathological conditions. Animal experiments with *Chek2* knockout (*Chk2*$^{-/-}$) mice demonstrated that *Chk2*$^{-/-}$ mice are viable and fertile, developing a slightly increased tumor incidence with a long latency, and that they are more radioresistant compared with wild type Chk2 mice [101]. This indicates that Chk2 activity is redundant and may be compensated for example by Chk1 kinase sharing overlapping substrates. This hypothesis has supported subsequent experiments demonstrating that double mutant *Chk1*$^{+/-}$/*Chk2*$^{-/-}$ and *Chk1*$^{+/-}$/*Chk2*$^{+/-}$ mice have a progressive cancer-prone phenotype [102].

Interestingly, the prevalence of germline *CHEK2* mutations in cancer patients outnumbers that in *CHEK1* by the order of magnitude. The same is true also for somatic mutations in these two kinases. Individuals carrying bi-allelic *CHEK2* mutations have a normal phenotype; however, they carry an increased cancer risk in comparison with heterozygotes and noncarriers [103,104]. However, the cell-type specific demand for CHK2 activation in human tissues is largely unknown. Recently, van Jaarsveld and colleagues [105] compared CHK2 activation in primary breast and lung cells, describing a significantly higher CHK2 activity in breast than in lung primary cells. These observations can further stimulate investigations revealing tissue-specific cancer development in *CHEK2* mutation carriers.

3. Germline *CHEK2* Variants

Germline mutations in the *CHEK2* gene and their association with cancer development were originally described in 1999 (a year after its discovery) by Bell and colleagues [19], who identified the most studied population-specific *CHEK2* variants c.1100delC and p.I157T in predominantly breast cancer patients from p53-wild type Li-Fraumeni syndrome (LFS) and LFS-like (LFL) families. The observation that *CHEK2* mutations associate with these clinically severe syndromes alongside the functional activity of CHK2 kinase in DDR attracted huge interest, and for a while, *CHEK2* was a candidate for the putative "*BRCA3*" gene. The first functional analysis revealed that c.1100delC completely abrogates CHK2 kinase activity [106]. However, the association between germline *CHEK2* mutations and LFS/LFL was disputed soon afterwards [31,107–111]. Moreover, the CHEK2 consortium (comprising laboratories from the UK, the Netherlands, the USA, and Germany) identified only an incomplete segregation of c.1100delC with cancer phenotypes in breast cancer families [112] and found a high prevalence of heterozygous c.1100delC carriers, exceeding 1% in Netherlands and UK controls. The high prevalence of c.1100delC in northern Europe was also confirmed by a study from Finland [110]. Although these early studies found that the frequency of c.1100delC mutations was enriched among breast cancer patients with early and/or double primary tumors and in multiple cancer families, an incomplete penetrance of c.1100delC in cancer families and a high prevalence of the

variant in controls substantially distorted *CHEK2*'s credit as a clinically considerable predisposing gene. In contrast, Cybulski and colleagues [20] analyzed two founder truncations, c.1100delC and c.444+1G>A, and the p.I157T missense variant in a large group of Polish patients and characterized *CHEK2* as a multi-organ cancer susceptibility gene. Their analysis of 4008 cases with 13 tumor types and 4000 controls found a moderately increased risk of breast, prostate, and thyroid cancer in carriers of truncating *CHEK2* mutations and an increased risk of breast, colon, kidney, prostate, and thyroid cancer for the carriers of p.I157T. Since then, a growing body of evidence has suggested that germline *CHEK2* variants deserve interest from the perspective of clinical oncology as their carriers face an increased risk of various cancer types that display some specific clinicopathological characteristics.

Unfortunately, the identification of c.1100delC (and a few other variants) as a *CHEK2* founder mutation (Figure 1) limited *CHEK2* analyses dominantly to these variants in most pre-NGS studies. Aloraifi and colleagues performed a meta-analysis of protein-truncating variants in moderate-risk breast cancer genes in 2015 and cited only 12 out of 54 published *CHEK2* analyses (22%) that had performed full gene scanning [113]. Recently, a spectrum of *CHEK2* pathogenic/likely pathogenic variants identified in 2508 carriers analyzed by GeneDx in the USA was published by Sutcliffe and colleagues [114]. They showed that nearly 95% of all carriers have some of the 18 variants detected more than 10 times, while the remaining approximately 5% of individuals carried one of the 101 rare germline variants including 17 large intragenic rearrangements. About 73% of individuals carried some of the five most frequent founder variants (including p.I157T and p.S428F). A full gene analysis was largely introduced with NGS panels. However, the identification of copy number variations (CNV), which represent a substantial fraction of *CHEK2* germline mutations (exon 9–10 deletions (denoted also 5395del) in Slavic populations [115] and US patients [114] and exons 2–3 and 6 in Greece [116]), is still not a golden standard. Besides, pseudogene sequences homologous to exons 10–14 limited analyses in early NGS studies [117,118]. Thus, our understanding of *CHEK2*'s contribution to cancer predisposition is incomplete as founder mutations vary among different ethnics and non-founder alterations account for over 25% of *CHEK2* pathogenic variants.

The bottleneck limiting the clinical outcomes of NGS analyses is rare VUS [119]. They currently account for 1228 out of 2195 (55.9%) germline *CHEK2* variants reported in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/?term=chek2; accessed 07-11-2020). As the majority of the *CHEK2* coding sequence comprises established domains, the prioritization of *CHEK2* VUS based on their presence in conserved regions is useless. Some studies have aimed to perform functional analyses challenging the catalytic activity; the activities of putative substrates; protein stability; and dimerization or localization of investigated CHK2 isoforms using in vitro [120,121], bacterial [122], yeast [21,123–125], or human cell models [115]. Besides a handful of exceptions [21,115,123], however, the published studies have only analyzed a single or a few variants, and their results were mutually concordant only in part. Therefore, a systematic analysis of *CHEK2* VUS is highly desirable as rare missense variants or small in-frame deletions are frequent and they may represent 25–50% of all germline *CHEK2* alterations [114,115,122,126,127].

3.1. Ethnic and Geographical Differences in CHEK2 Mutation Frequency

The prevalence of germline *CHEK2* variants substantially varies among different populations and ethnics. These differences can be demonstrated on multiethnic studies utilizing an identical approach. Kurian and colleagues collected data from germline testing in 5900 breast and 937 ovarian cancer patients from California and Georgia [128]. They found that pathogenic *CHEK2* variants were the third most frequent germline alterations in both cancers (following *BRCA1/BRCA2* variants); however, *CHEK2* significantly prevailed in whites over blacks in both breast cancer (2.3% vs. 0.15%) and ovarian cancer (1.3% vs. 0%). An analysis by Caswell-Jin et al. also identified significant differences in the frequency of pathogenic *CHEK2* mutations between whites and non-whites (3.8% vs. 1.0%; $p = 0.002$) tested for hereditary cancer risk [22].

Although *CHEK2* has the highest mutation prevalence among Caucasian individuals of European descent, the spectrum and frequency of founder as well as non-founder mutations vary among particular European populations. The frequency of the European founder mutation c.1100delC declines from the north to the south [129], with carrier frequency in the general population close to 1% in the UK and the Netherlands but very rare in the Mediterranean region [130–132]. The most frequent European *CHEK2* variant, p.I157T, has a population frequency of heterozygous carriers of around 5% in Poles [20], Latvians [133], Hungarians [134], and Russians [135] and around 2–3% in Czechs [136], Slovaks [134], and Germans [126]. Interestingly, the p.I157T allele has developed in some populations independently [137]. This high population frequency rules out the possibility that the p.I157T variant could have a higher than low impact on cancer susceptibility; however, an increased risk with odds ratio (OR) approximately 1.5 in p.I157T carriers has been described systematically in case control studies and meta-analyses for breast cancer (Table 1) and other cancer types. Another central European founder mutation, a deletion of exons 9–10, was described by Walsh et al. [138] in patients of Czech and Slovak origins (Figure 1). A high background frequency of this variant in controls was also found in Poland (0.4%) [139] and Latvia (0.7%) [140].

The lowest frequency of *CHEK2* germline mutations is reported in patients of Asian origin. A panel NGS analysis involving 8085 Chinese breast cancer patients revealed only 18 (0.3%) carriers of pathogenic *CHEK2* mutations [141]; eight of them carried the novel founder nonsense mutation c.C417A (p.Y139*) [142]. Only two carriers (0.24%) of *CHEK2* mutations were identified in a recent analysis of 831 breast cancer patients from Shanghai [143]. Studies of breast and prostate cancer patients from Japan included analyses in control populations that revealed the presence of pathogenic *CHEK2* germline mutations in 0.1% of both female and male noncancer controls [144,145].

Table 1. Analyses of the entire CHEK2 coding sequence (separately or as part of panel next-generation sequencing (NGS)) or analyses of specific variant(s) in breast cancer (BC) patients.

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
		Female breast cancer		
Fostira 2020 [146]	GR	P: 1382 high-risk BC patients C: ExAC/FLOSSIES	*CHEK2* (panel NGS)	*1.7 (0.98–2.7); 0.11—all LoF variants/ExAC* 2.6 (1.44–4.68); 0.003—all LoF variants/FLOSSIES 3.8 (1.86–7.12); 1.2×10^{-3}—missense deleterious/ExAC 5.9 (2.38–14.8); 1.2×10^{-4}—missense deleterious/FLOSSIES
Kurian 2020 [147]	US (66% white)	P: 2,195 postmenopausal BC C: 2322 age-matched PMC	*CHEK2* (panel NGS)	N.D.; *CHEK2* PV found in 0.59% P and 0.26% C
Rogoza-Janiszewska 2020 [148]	PL	P. 2,464 BC diagnosed at <41 C: from Cybulski 2019	c.1100delC; c.444+1G>A; del5395	3.8 (2.53–5.58); <0.0001—BC at < 41 y; all truncations 4.6 (2.44–8.80); <0.0001—BC at < 31 y; all truncations
Kleiblova 2019 [115]	CZ	P: 1526 high-risk female BC C: 3360 PMC	*CHEK2* (panel NGS)	7.94 (3.90–17.47); 4.1×10^{-11}—unilat. BC: truncations 3.90 (1.24–13.35); 0.009—unilat. BC: deleterious missense 8.39 (1.92–28.74); 0.003—bilat. BC: truncations *3.77 (0.08–31.42); 0.26—bilat BC: deleterious missense*
Cybulski 2019 [149]	PL	P: 1,018 hereditary BC C: 4346 PMC	c.1100delC c.444+1G>A del5395	6.9 (3.2–14.7); <0.0001—for c.1100delC 8.4 (3.0–23.3); <0.0001—for c.444+1G>A 6.5 (3.2–13.4); <0.0001—for del53957.2 (4.5–11.6); <0.0001—for all above truncations

Table 1. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Nurmi 2019 [150]	FI	P: 3156 BC C: 2089 PMC	c.319+2T>A; c.444+1G>A; c.1100delC	5.40 (1.58–18.45); 0.007—for c.319+2T>A unselected BC 6.04 (1.65–22.10); 0.007—for c.319+2T>A familial BC
Girard 2019 [151]	FR	P: 1207 *BRCA1/2*$^{-ve}$ BC pts having sister with BC C: 1199 non-cancer PMC	*CHEK2* (WES + panel NGS)	3.0 (1.9–5.0); 1×10^{-5}—any rare variant 5.8 (2.0–16.9); 0.001—LoF variants 2.4 (1.4–4.3); 0.002—likely-deleterious missense
Hauke 2018 [126]	DE	P: 5589 *BRCA1/2*$^{-ve}$ BC C: 2189 non-cancer PMC	*CHEK2* (panel NGS)	3.72 (1.99–6.94); <0.0001—truncations
Momozawa 2018 [145]	JP	P: 7051 BC C: 11,241 PMC	*CHEK2* (panel NGS)	3.2 (1.6–6.8); 3.2×10^{-4}
Decker 2017 [152]	UK	P: 13,087 BC C: 5488 PMC	*CHEK2* (& 3 other genes)	3.11 (2.15–4.69); 5.6×10^{-11}—truncations *1.36 (0.99–1.87); 0.066—all rare missense* 1.51 (1.02–2.24); 0.047—rare missense in any domain 3.27 (1.66–5.83); 0.0014—bilateral BC 3.42 (2.33–5.21); 1.5×10^{-11}—ER^{+ve} BC 3.98 (2.62–6.21)—age at dg < 50 years 3.37 (2.24–5.22)—age at dg = 50–60 years 2.12 (1.35–3.41)—age at dg > 60 years
Slavin 2017 [153]	US (80% white)	P: 2266 *BRCA1/2*$^{-ve}$ fam. BC C: ExAC	*CHEK2* (panel NGS)	1.62 (1.03–2.51); 0.004 – truncations
Couch 2017 [154]	US (white)	P: 29,090 BC C: 25,215 ExAC-NFE	*CHEK2* (panel NGS)	2.31 (1.88–2.85); 3.04×10^{-17}—c.1100delC 2.26 (1.89–2.72); 1.75×10^{-20}—PVs (w/o p.I157T, p.S428F) 1.48 (1.31–1.67); 1.11×10^{-10}—any var (w p.I157T, p.S428F) 1.35 (1.1–1.63); 0.0002; bilateral BC

Table 1. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Schmidt 2016 [155]	BCAC	P: 44,777 population+ hospital-based BC C: 42,977 PMC	c.1100delC	2.26 (1.90–2.69); 2.3×10^{-20}—invasive BC 2.55 (2.10–3.10); 4.9×10^{-21}—ER^{+ve} BC *1.32 (0.93–1.88); 0.12—ER^{-ve} BC*
Naslund-Koch 2016 [156]	DK	2442 BC pts /86,975 individ. (longitudinal study);	c.1100delC	2.08 (1.51–2.85); <0.001
Southey 2016 [157]	BCAC	P: 42,671 C: 42,164 PMC	iCOGS array incl. 6 rare *CHEK2* variants	2.26 (1.29–3.95); 0.003—for p.R117G 1.33 (1.05–1.67); 0.016—for p.R180C 1.70 (0.73–3.93); 0.210—for p.E239K 5.06 (1.09–23.5); 0.017—for p.R346C *1.03 (0.62–1.71); 0.910—for p.D438Y*
Liu Y 2011 [158]	CN (Han)	P: 118 familial BC P: 909 unselected BC C: 1228 healthy PMC	*CHEK2* (dHPLC) for familial BC	5.99 (1.98–18.11); 0.002—for p.H371Y familial BC 2.43 (1.07–5.52); 0.034—for p.H371Y unselected BC
Cybulski 2011 [159]	PL	P: 7494 *BRCA1*$^{-ve}$ BC C: 4346 PMC	c.1100delC; c.444+1G>A; del5395	3.6 (2.6–5.1)—all BC 3.3 (2.3–4.7)—patients with no BC family history 5.0 (3.3–7.6)—patients with BC in 1° or 2° relative 7.3 (3.2–16.8)—patients with BC in 1° and 2° relatives
Desrichard 2011 [122]	FR	P: 507 *BRCA1/2*$^{-ve}$ BC C: 513 non-cancer PMC	*CHEK2* (sequencing)	4.15 (1.38–12.50); 0.007—any variant 5.18 (1.49–18.00); 0.004—deleterious (p.K244R ex)
Le Calvez-Kelm 2011 [160]	US/CA/AU	P: 1242 BC ≤ 45y C: 1109 non-ca PMC female	*CHEK2* (HRM)	6.18 (1.76–21.8)—truncations/splice mutations 2.20 (1.20–4.01)—rare missense
Fletcher 2009 [161]	UK/FI/NL/ RU/DE	P: 1828 bilateral BC C: 7030 PMC	c.1100delC	6.43 (4.33–9.53); <0.0001—second primary for mut. carriers

Table 1. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Weischer 2007 [162]	DK	P + C: 9231 (prospective) P: 1101 BC/4665 PMC (case-control)	c.1100delC	3.2 (1.0–9.9)—BC (prospective study) 2.6 (1.3–5.4)—BC (case-control study)
Cybulski 2006 [163]	PL	P: 3228 BC diagnosed at ≤50 C: 5496 PMC	c.1100delC c.444+1G>A p.I157T	2.3 (1.1–4.8); 0.04—for c.1100delC 2.4 (1.4–4.2); 0.002—for c.444+1G>A 2.4 (1.5–3.7); 0.0001—for any truncation 1.4 (1.1–1.6); 0.002—for p. I157T
Chekmariova 2006 [164]	RU	P: 660 unilat; 155 bilat BC C: 448 middle aged females;	c.1100delC (ASO PCR)	9.8 (1.34–198.26); 0.007 - early onset/bilat BC/C carriers frequencies: 3.4/5.2/0.2%
Cybulski 2004 [20]	PL	P: 1017 BC C: 4000 PMC	c.1100delC; c.444+1G>A; p.I157T	2.2; $p = 0.02$—for c.1100delC and c.444+1G>A 1.4; $p = 0.02$—for p.I157T
Caligo 2004 [130]	IT	P: 939 BC (incl. *BRCA1/2*$^{+ve}$) C: 334 PMC	c.1100delC	*N.S.; frequency of carriers 0.11% (95% CI 0.00–0.59%)*
Dufault 2004 [165]	DE	P: 516 *BRCA1/2*$^{-ve}$ BC C: 500 PMC (1,315 PMC for c.1100delC)	*CHEK2*	3.44 (1.19–9.95); 0.016—c.1100delC 3.9 (1.3–10.9)—c.1100delC and c.1214del4
CHEK2 BC consortium 2004 [166]	UK/NL/FI/ DE/AU	P: 10,860 BC C: 9065 multinatl.	c.1100delC	2.34(1.72–3.20); 1×10^{-7}—all BC 2.23 (1.60–3.11)—BC w/o BC in 1° relative 3.12 (1.90–5.15)—BC with 1 BC in 1° relative 4.17 (1.26–13.75)—BC with ≥2 BC in 1° relatives
CHEK2 BC consortium 2002 [167]	UK/NL/ US/CA	P: 636 unselected BC P: 718 *BRCA1/2*$^{-ve}$ BC C: 1620 multinatl.	c.1100delC	*2.52 (0.78–8.18)—unselected BC* 1.70 (1.32–3.38)—*BRCA1/2*$^{-ve}$ BC
Vahteristo 2002 [110]	FI	P: 1035 unselected BC C: 1885 PMC (blood donors)	c.1100delC	*1.48 (0.83–2.65); 0.182—unselected BC* 2.27 (1.11–4.63); 0.021—familial BC 6.17 (1.87–20.32); 0.007 bilat. vs. unilat. BC

Table 1. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
		Male breast cancer		
Kleiblova 2019 [115]	CZ	P: 48 male BC C: 3360 PMC	*CHEK2* (panel NGS)	20.21 (3.50–80.00); 8.6×10^{-4}—truncations *11.87 (0.25–100.83); 0.1—deleterious missense*
Liang 2018 [168]	meta	P: 1063 male BC C: 31,571	c.1100delC	3.13 (1.94–5.07)
Hallamies 2017 [169]	FI	P: 68 male BC C: 1885 from [110]	c.1100delC	4.47 (1.51–13.18); 0.019
Wasielewski 2009 [170]	NL	P: 71 male BC C: 1692	c.1100delC	4.1 (1.2–14.3); 0.05
CHEK2 consortium 2002 [167]	UK/NL/US/CA	P: 52 male BC families C: 1620 multinatl.	c.1100delC	10.28 (3.54–29.87)
		Meta-analyses		
Yang 2019 [171]	BCAC+ ABCC meta	P: 122,977 + 24,206 BC C: 105,974 + 24,775 PMC	p.I157T	1.28 (1.17–1.39); 9.66×10^{-9}—for Europeans only 1.35 (1.18–1.54); 9.82×10^{-6}—for ER^{+ve} BC *0.95 (0.81–1.12); 0.55—for ER^{-ve} BC*
Liang 2018 [168]	meta	P: 118,735 BC C: 195,807	c.1100delC	2.88 (2.65–3.22)—female BC 2.87 (1.85–4.47)—early-onset BC 3.21 (2.41–4.29)—familial BC 3.13 (1.94–5.07)—male BC
Aloraifi 2015 [113]	meta	P. 7283 C: 13,785	*CHEK2* truncations	3.25 (2.55–4.13)
Han 2013 [172]	meta	P: 15,985 BC C: 18,609	p.I157T	1.58 (1.42–1.75); <0.0001
Liu 2012 [173]	meta	P: 19,621 BC C: 27,001	p.I157T	1.48 (1.31–1.68); <0.0001—unselected BC 1.48 (1.16–1.89); <0.0001—familiar BC 1.47 (1.29–1.66); <0.0001—early onset BC 4.17 (2.89–6.03); <0.0001—lobular BC

Table 1. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Yang 2012 [174]	meta	P: 29,154 BC C: 37,064	c.1100delC	2.33 (1.79–3.05)—unselected BC 3.72 (2.61–5.31)—familiar BC 2.78 (2.28–3.39)—early onset BC
Zhang 2011 [175]	meta	P: 9970/C:7526 P: 13,331/C: 10,817 P: 10,543/C:10,817 P: 41,791/C: 50,910	c.444+1G>A del5395 c.1100delC p.I157T	3.07 (2.03–4.63); 9.82×10^{-8}—for variant c.444+1G>A 2.53 (1.61–3.97); 6.33×10^{-5}—for variant del5395 3.10 (2.59–3.71); $<10^{-20}$—for variant c.1100delC 1.52 (1.31–1.77); 4.76×10^{-8}—for variant p.I157T
Weischer 2008 [176]	meta	P: 26,488 C: 27,402	c.1100delC	2.7 (2.1–3.4)—unselected BC 2.6 (1.3–5.5)—early onset BC 4.8 (3.3–7.2)—familial BC

* *CHEK2* = an analysis of the entire coding sequence (dominantly without copy number variations (CNV)); otherwise specified if certain *CHEK2* variants were genotyped. AU—Australia; ABCC—Asian Breast Cancer Consortium; BC—breast cancer; BCAC—Breast Cancer Association Consortium; CA—Canada; CN—China; CZ—Czech Republic; DE—Germany; DK—Denmark; ES—Spain; EU—European Union; ExAC—Exome Aggregation Consortium; FI—Finland; FLOSSIES—Fabulous Ladies Over Seventy; FR—France; meta—meta-analysis; GR—Greece; IT—Italy; LoF—loss-of-function; JP—Japan; NL—Netherlands; PL—Poland; PMC—population-matched control; RU—Russia; US—the USA. The analyses that failed to demonstrate an association are shown in italics.

3.2. Breast Cancer

Most studies of *CHEK2* germline mutations have dealt with breast cancer patients. The estimated OR for carriers of *CHEK2* mutations varies among the studies considerably depending on analyzed populations, CHEK2 variants, and used controls (Table 1).

The variability of risk estimates is influenced by several important parameters, including the number and (pre)selection criteria of eligible patients, *CHEK2* variants analyzed and considered pathogenic, and control group selection. The estimated lifetime risk of breast cancer for *CHEK2* mutation carriers (mostly c.1100delC) differs according to family cancer history and ranges from 20 to 40% in women without and with a positive family breast cancer history, respectively [150]. More specifically, Cybulski and colleagues [159] estimated the lifetime BC risk for truncating *CHEK2* mutations in Polish patients to be 20% in women without a family cancer history and 28% and 34% in women with a second- and first-degree relatives with BC, respectively. A Danish case-control study determined an absolute 10-year BC risk as 24% in women carrying c.1100delC and older than 60 years undergoing hormone replacement therapy (HRT) with BMI > 25 [162]. Johnson et al. [177] estimated a cumulative risk of 58.8% (95% CI 33.8–85.3) for breast cancer by the age of 80 for first-degree relatives of c.1100delC carriers with bilateral breast cancer from the UK. An international European study predicted the lifetime risk for BC in daughters of c.1100delC carriers and noncarriers with bilateral breast cancer as 37% and 18%, respectively [161]. It can be assumed that breast cancer risk associated with pathogenic *CHEK2* variants in the general population would be at the lower moderate penetrance gene border (OR > 2) but considerably higher (though still in a moderate penetrance range; with OR < 4) for high-risk carriers from families with a positive cancer history. A precise evaluation of the associated risk will require large studies of unselected cancer patients with an appropriately selected population of geographically matched controls. A more precise estimate of individual breast cancer risks associated with germline *CHEK2* mutations could be reached by considering the polygenic risk score (PRS) [178–180].

Breast cancer in the carriers of pathogenic germline *CHEK2* mutations has several recurrently reported clinicopathological characteristics. The most striking is the development of bilateral breast cancer, as shown in some studies (Table 1). A recent meta-analysis by Akdeniz and colleagues [181] computed the relative risk of contralateral breast cancer development as 2.68 (95% CI 1.69–3.65) for c.1100delC mutation carriers versus noncarriers (which was fully comparable with that in *BRCA2* mutation carriers: RR = 2.75; 95% CI 1.77–4.29). A significantly younger cancer onset in *CHEK2* mutation carriers has been reported less consistently [152,154,182]. Published studies have also pointed out a worse breast cancer prognosis for c.1100delC mutation carriers [183–186] but not for p.I157T carriers [187]. Since the first studies, *CHEK2* germline mutations have frequently been associated (in 85–90% of cases) with estrogen receptor positive (ER+) breast cancer subtypes [115,126,139,183,188,189]. Consistent with that, no *CHEK2* mutation carriers were observed in an analysis of 1824 triple negative breast cancer patients [190]. A large analysis conducted by the BCAC consortium estimated the cumulative risk of developing ER+ and ER− breast cancer by the age of 80 for c.1100delC mutation carriers at 20% and 3%, respectively, compared with 9% and 2%, respectively, in the general British population [155].

Although ER+ tumors tend to have a better prognosis in unselected breast cancer patients, ER+ tumors in *CHEK2* mutation carriers were associated with worse breast cancer-specific survival [155,184]. A low or significantly reduced CHK2 expression was found in most breast tumors from mutation carriers [191]. Interestingly, both tumors with low CHK2 expression and tumors from CHEK2 mutation carriers were associated with increased grade, especially with a lower proportion of grade one tumors [115,192]. Bahassi and colleagues [193] offered an interesting hypothesis describing a link between ER positivity and reduced CHK2 expression based on the observation of mouse models. They noticed that the ER stimulated c-MYC transcriptional activity, increasing CDC25A expression that in turn resulted in the S-phase entry and genomic instability in mice homozygous or heterozygous for Chk2 c.1100delC. An association with lobular breast cancer was reported for p.I157T in patients

from Poland [194] and the Czech Republic [115] and from a meta-analysis by Liu and colleagues [173] for pathogenic *CHEK2* mutations in patients from Slovenia [182] and for other germline variants in patients from Bulgaria [195].

All in all, germline *CHEK2* mutations confer increased risk of the development of ER-positive breast cancer with an unfavorable prognosis and an increased risk of bilateral breast cancer. The current NCCN guidelines (National Comprehensive Cancer Network guidelines version 1.2021 for Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic) recommend annual mammogram screenings for women carrying a pathogenic mutation since the age of 40 and recommend an annual MRI check. The risk-reducing mastectomy (RRM) is not generally recommended because of the lack of data confirming its benefits; however, RRM can be considered, especially based on family cancer history [196]. Prophylactic contralateral mastectomy can also be recommended for breast cancer patients with pathogenic *CHEK2* germline mutations. Chemoprevention for unaffected women with pathogenic mutations could be considered as an option [197].

An increased risk of male breast cancer has been documented in few smaller studies (Table 1); however, due to the low overall male breast cancer risk, its increase would not substantiate a specific follow-up, although the association should be considered in case of breast pathology developing in male mutation carriers. The RRM is not recommended in male *CHEK2* mutation carriers [196].

The relatively high frequency of germline *CHEK2* mutations in some populations and the dispensability of CHK2 for normal development results in identification of recessive homozygotes or compound heterozygotes carrying *CHEK2* mutations at both alleles. Sutcliffe and colleagues reported 32 (1.3%) homozygotes among 2508 identified *CHEK2* mutation carriers [114]. The most frequent ones were c.1100delC and p.I157T homozygotes, of whom 66% and 60% were diagnosed with BC, respectively. Rainville summarized data from 31 biallelic mutation carriers identified among 6473 monoallelic *CHEK2* mutation carriers tested by Myriad Genetics, of whom 16/31 were c.1100delC mutation carriers [104]. Compared with monoallelic carriers, biallelic carriers developed breast cancer more frequently (81% vs. 41%; $p < 0.0001$) and more likely before the age of 50 (61% vs. 24%; $p < 0.0001$), they developed secondary breast cancer with a higher frequency (23% vs. 8%; $p = 0.01$), and finally they had a higher risk of developing any primary cancer and multiple primary cancer. The ORs for the development of ductal carcinoma *in situ* (DCIS) and ductal carcinoma were high (OR 8.7, 95% CI 3.7–20.5 and OR 5.0, 95% CI 2.0–12.4, respectively) [104]. Case reports of homozygous carriers, which included other *CHEK2* mutations, have been published episodically [103,198,199], and they indicate an increased risk of the development of variable primary cancers with an early age at onset. These data support an intensified management of homozygous carriers of *CHEK2* pathogenic mutations. In the case of breast cancer, the surveillance follow-up should copy that in *BRCA1/BRCA2* mutation carriers. The prevention of other tumors associated with germline *CHEK2* mutations should be considered. While p.I157T is considered a low penetrance variant, we assume that, based on functional data, homozygotes should be managed in a way similar to c.1100delC heterozygotes [115].

3.3. Prostate Cancer

The association of *CHEK2* with prostate cancer was already proposed in 2003, when Dong and colleagues identified 18 unique *CHEK2* mutations in 15/400 (3.75%) patients with sporadic prostate cancer and in 11/298 (3.69%) patients with familial prostate cancer [200]. This association has been confirmed by subsequent studies (Table 2), and a *CHEK2* gene analysis is currently routinely performed as part of prostate-specific gene panels [201].

Table 2. Analyses of the entire *CHEK2* coding sequence (separately or as part of panel NGS) or analyses of specific variant(s) in prostate cancer (PrC) patients.

Reference	Population	P: Patients C: Controls	Analysis **	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Brandao 2020 [202]	PT PRACTICAL	P: 462 early-onset/familial PrC C: 710 PMC P: 55,162 PrC/C: 36,147	c.349A>G (p.R117G)	*7.7 (0.9–66.6); 0.06—PT PrC p.R117G* 1.9 (1.1–3.2); 0.04—PRACTICAL PrC p.R117G
Momozawa 2018 [144]	JP	P: 7636 C: 12,366	Panel NGS (8 genes)	*2.43 (0.91–6.86); 0.06*
Conti 2017 [203]	AAPC, GH	AAPC–P:4,853 PrC; C: 4678 GH–P: 474; C: 458	GWAS array (rs78554043 = rs17886163; *CHEK2* c.1343T>G; p.I448S)	1.60 (1.27–2.00); 5.02×10^{-5}—for AAPC PrC 2.45 (1.33–4.52); 0.004—for Ghana PrC
Naslund-Koch 2016 [156]	DK	86,975 individuals (longitudinal study); 1340 developed PrC	c.1100delC	*1.60 (1.00–2.56); 0.05*
Southey 2016 [157]	OCAC	P: 22,301 PrC C: 22,320 PMC	iCOGS array incl. 6 rare *CHEK2* variants	*1.46 (0.71–3.02); 0.3—for p.R117G* *1.02 (0.73–1.44); 0.9—for p.R180C* *1.47 (0.41–5.35); 0.6—for p.E239K* *1.07 (0.28–4.07); 0.9—for p.D438Y* 2.21 (1.06–4.63); 0.03—for p.D438Y 3.03 (1.53–6.03); 0.001—for I448S in Africans
Pritchard 2016 [118]	US, UK	P: 692 metastat. PrC C: ExAC/TCGA	Panel NGS	3.1 (1.5–5.6); 0.002—vs. ExAC (excl. p.I157T) 4.7 (2.2–8.5); <0.001—vs. TCGA (excl. p.I157T)
Wang 2015 [204]	meta	P: 6409 PrC C: 11,634	c.1100delC c.444+1G>A p.I157T	3.29 (1.85–5.85); <0.001—c.1100delC *1.59 (0.79–3.20); 0.20—c.1100delC, familial* *1.58 (0.93–2.71); 0.09—c.444+1G>A* 1.80 (1.51–2.14); <0.001—p.I157T
Hale 2014 [205]	meta	P: 5,124 PrC C: 9,258	c.1100delC	1.98 (1.23–3.18); 0.004—unselected 3.39 (1.78–6.47); 0.0001—familial
Cybulski 2006 [206]	PL	P: 1864 PrC (incl. 249 famil.) C: 5496	c.1100delC; c.444+1G>A; 5395del; p.I157T	2.3 (1.1–3.9); <0.001—truncations, sporadic 4.7 (2.5–9.0); <0.001—truncations, familial 1.6 (1.3–2.0); <0.001—p.I157T, sporadic 2.7 (1.8–4.1); <0.001—p.I157T, familial

Table 2. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis **	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Weischer 2007 [162]	DK	P: 116 PrC (prospective) C: 3999 PMC men (prospect.)	c.1100delC	*2.3 (0.6–9.5) PrC prospective study*
Johnson 2005 [177]	UK	P: 469 bilat. BC	c.1100delC	2.41 (1.67–3.36)—risk of PrC for relatives of patients with bilateral BC
Cybulski 2004 [20]	PL	P: 690 PrC C: 4000 PMC	c.1100delC; c.444+1G>A; p.I157T	2.2; 0.04—truncations 1.7; 0.002—p.I157T
Seppala 2003 [207]	FI	P1: 537 unselected PrC; P2: 120 hereditary PrC C: 510 non-PrC men	*CHEK2* (SSCP: heredit. PrC) c.1100delC/p.I157T	*3.14 (0.65–15.16); 0.15—c.1100delC, sporadic* 8.24 (1.49–45.54); 0.02—c.1100delC, hereditary *1.48 (0.89–2.46); 0.13—p.I157T, sporadic* 2.12 (1.06–4.27); 0.04—p.I157T, hereditary
Dong 2003 [200]	US	P1: 400 sporadic PrC; P2: 298 familial PrCC: 510 non-PrC men	*CHEK2* (DHPLC)	2.71 (1.04–7.04); 0.049 *—sporadic PrC *2.66 (0.98–7.28); 0.078 *– familial PrC* *6.84 (0.86–54.1); 0.05 *—sporadic (w/o p.I157T)* *5.74 (0.64–51.5); 0.17 *—familial (w/o p.I157T)*

* Calculated using WINPEPI [208]; ** *CHEK2* = an analysis of the entire coding sequence (dominantly without CNV); otherwise specified if certain *CHEK2* variants were genotyped. AAPC—African Ancestry Prostate Cancer; DK—Denmark; ES—Spain; ExAC—Exome Aggregation Consortium; FI—Finland; GH—Ghana; meta—meta-analysis; JP—Japan; OCAC—Ovarian Cancer Association Consortium; PL—Poland; PT—Portugal; PMC—population-matched control; PRACTICAL - The Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome; TCGA - The Cancer Genome Atlas; US—the USA. The analyses that failed to demonstrate an association are shown in italics.

A study by Pritchard et al. demonstrated that 82/692 (11.8%) men with metastatic prostate cancer carry a mutation in some of the 16 analyzed DNA repair genes. The carriers of *BRCA2* (N = 37; 44%), *ATM* (N = 11; 13%), *CHEK2* (N = 10; 12%), and *BRCA1* (N = 6; 7%) represented over 75% of all mutation carriers. These data confirmed a report by Isaacsson Velho and colleagues [209], who found a similar proportion of mutation carriers (21/150; 14%) among unselected prostate cancer patients with an identical proportion of affected genes: *BRCA2* (N = 9; 43%), *ATM* (N = 3; 14%), *CHEK2* (N = 3; 14%), and *BRCA1* (N = 2; 9%). Moreover, patients with germline mutations had significantly more frequent intraductal histology (47.6% vs. 11.6% in noncarriers; $p = 0.003$) and presence of lymphovascular invasion (52.3% vs. 13.9%; $p < 0.001$). Thus, the authors concluded that genetic testing should be offered to patients with these clinicopathological characteristics. A larger cross-sectional analysis of 1328 men with prostate cancer by Giri and colleagues [210] found 15.6% carriers of germline mutations; 10.9% patients carried a mutation in DNA repair genes (*BRCA2* > *CHEK2* > *ATM* > *BRCA1*), increasing the risk of more advanced tumors (Gleason score ≥ 8). *CHEK2* mutations were less frequent in prostate cancer patients from Japan [144].

Wu and colleagues analyzed survival characteristics in prostate cancer patients carrying germline *CHEK2* mutations [211]. Although they found no association between the *CHEK2* mutation status and early diagnosis or PrC, they noted that c.1100delC mutation carriers are more prevalent among patients with a lethal disease than in patients with low-risk prostate cancer (1.28% vs. 0.16%; $p = 0.004$). Yadav et al. [212] found no significant association between the presence of germline mutations and survival characteristics, but they found that mutations in *ATM*, *BRCA2*, *CHEK2*, *FANCM*, and *TP53* were significantly more frequent in patients with a metastatic disease.

The performed studies present rather compelling evidence that *CHEK2* is a low-to-moderate prostate cancer predisposition gene. Therefore, male carriers of pathogenic *CHEK2* mutations, especially from families with multiple prostate cancers, deserve intensified prostate cancer screening which should include an annual PSA test from the age of 40. A report by Cybulski et al. [213] identified an increased proportion of p.I157T carriers among individuals with elevated PSA or an abnormal digital rectal examination versus individuals with normal assessments (10.2% vs. 4.3%; OR = 2.5; $p = 0.0008$); however, a prostate-specific follow-up needs to be justified in larger studies.

3.4. Kidney Cancer

The p.I157T variant (but not the truncating founder mutations) was recognized to increase kidney cancer risk in a pioneering clinical study by Cybulski and colleagues [20]. Other studies (Table 3) confirmed an association between *CHEK2* germline mutations and renal cell carcinoma later on, with the exception of an analysis by Ge et al. exploiting GWAS datasets.

Table 3. Analyses of the entire *CHEK2* coding sequence (separately or as a part of panel NGS) or analyses of specific variant(s) in renal cell carcinoma (RCC) patients.

Reference	Population	P: Patients C: Controls	Analysis *	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Zlowocka-Perlowska 2019 [214]	PL	P: 835 invasive RCC C: 8304 non-cancer	c.1100delC; c.444+1A>G; 5395del; c.I157T	2.5 (1.5–4.1); 0.0003—for truncations 2.0 (1.6–2.6); <0.001—for p.I157T
Carlo 2018 [215]	US	P: 254 RCC (stage III-IV) C: ExAC	*CHEK2*	3.0 (1.3–5.8); 0.003
Ge 2016 [216]	GWAS	P: 1322 C: 3428	p.I157T	0.63 (0.44–0.89); 0.01
Naslund-Koch 2016 [156]	DK	138/86,975 individuals developed RCC	c.1100delC	3.61 (1.33–9.79); 0.01
Weischer 2007 [162]	DK	P: 33 RCC (prospective) C: 9166 PMC (prospect.)	c.1100delC	9.8 (2.3–41.2) RCC prospective study
Cybulski 2004 [20]	PL	P: 264 RCC C: 4000 PMC	c.1100delC; c.444+1G>A; p.I157T	*1.0*; *p = 0.8—truncations* 2.1; $p = 0.0006$—for p.I157T

* *CHEK2* = an analysis of the entire coding sequence (dominantly without CNV); otherwise specified if certain *CHEK2* variants were genotyped. DK—Denmark; GWAS—genome-wide association study; PL—Poland; US—the USA. The analyses that failed to demonstrate an association are shown in italics.

NGS panel sequencing by Carlo and colleagues in 254 patients with advanced RCC identified 41 carriers of pathogenic germline mutations in renal cancer- or other cancer-associated genes [215]. Among them, germline mutations in *CHEK2* found in nine (3.4%) patients outnumbered the most frequent alterations in RCC-associated mutations (7× FH [2.8%]; 3× BAP1 [1.2%]). Consistently, 7/229 (3.1%) mutation carriers with germline *CHEK2* variants were identified among metastatic clear cell renal cancer patients by Ged and colleagues [217]. *CHEK2* germline mutations were also the most frequent alterations found in 19/844 (2.25%) patients with early onset RCC developed before the age of 60 [218]. Notably, among these, second primary cancers (breast, thyroid, colon, blood, and ovarian) were reported in 13 (68%) individuals. With 3/118 (2.5%) individuals suggestive of inherited RCC, *CHEK2* was the second most frequently altered gene (following *BRIP1*) that is not routinely tested for renal cancer predisposition [219]. Unexpectedly, Gadd and colleagues identified germline *CHEK2* variants in 3/117 (2.6%) and 8/651 (1.2%) patients with Wilms tumors in their discovery and validation sets, respectively [220]. Another report by Ciceri et al. [221] found five carriers of rare missense or splicing *CHEK2* variants among 96 Wilms tumor patients from Italy. While evidence of the association of *CHEK2* germline mutations with an increased RCC risk is currently mounting, larger case control studies in RCC patients are required to confirm and refine the magnitude of the associated risk.

3.5. Papillary Thyroid Cancer

A multiple cancer study by Cybulski et al. [20] identified an increased risk of thyroid cancer, particularly in carriers of *CHEK2* truncations (OR = 4.9) and, to a lesser extent, in carriers of p.I157T (OR = 1.9). Observations from this study were confirmed subsequently (Table 4); however, most of the data originate from Poland only and will require confirmation from other populations.

Table 4. Analyses of the *CHEK2* variants in Polish (PL) patients with papillary thyroid cancer (PTC).

Reference	Population	P: Patients C: Controls	Analysis of Specific *CHEK2* Variants	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Kaczmarek-Rys 2015 [222]	PL	P: 602 differentiated PTC C: 829 PMC	p.I157T	1.81 (1.20–2.72); 0.004—p.I157T heterozygote 12.81 (0.6–248.46); 0.02—p.I157T homozygote
Siolek 2015 [223]	PL	P: 468 unselected PTC C: 468 matched non-cancer	c.1100delC; c.444+1G>A; 5395del; p.I157T	5.7 (1.7–19.3); 0.006—truncations 2.8 (1.7–4.6); 0.0001—p.I157T
Wojcicka 2014 [224]	PL	P: 1781 PTC C: 2081 healthy PMC	p.I157T	2.21 (1.69–2.88); 2.37×10^{-10}—for p.I157T
Cybulski 2004 [20]	PL	P: 173 PTC C: 4000 PMC	c.1100delC; c.444+1G>A; p.I157T	4.9; 0.006—truncations 1.9; 0.04—p.I157T

Recently, Pekova et al. [225] identified pathogenic/likely pathogenic germline *CHEK2* variants in 7/83 (8.4%) Czech pediatric/adolescent patients with papillary thyroid cancer, detecting five (6.0%) p.I157T carriers among them. An interesting report by Zhao and colleagues [226] described a Chinese family with the germline *CHEK2* mutation c.417A>C (p.Y139*; described independently as a recurrent germline mutation in Chinese breast cancer patients [142]) segregating in all four first-degree relatives with papillary thyroid cancer. The authors subsequently analyzed 242 sporadic papillary thyroid cancers and identified two carriers of the p.R180C variant and three carriers of p.H371Y.

Beyond the hereditary cancer genetics, somatic *CHEK2* alterations were characterized as mutations that may contribute to tumor progression in papillary thyroid carcinoma [227].

3.6. Colorectal Cancer

Early studies performed by the *CHEK2* consortium (Meijers-Heijboer and colleagues) identified families of c.1100delC carriers with apparently frequent breast and colorectal cancer and denoted this familial cancer cooccurrence as the "hereditary breast *and* colorectal cancer syndrome (HBCC)" [228]. c.1100delC carriers have a strong association with the HBCC phenotype and a trend increasing colorectal cancer risk in HNPCC and HNPCC-like families (Table 5). However, a follow-up study by Nasem and colleagues [229] failed to confirm this finding as they identified only a single c.1100delC carrier in 113 HBCC individuals. Several studies and meta-analyses have focused on colorectal cancer later on and provided evidence of a low-to-moderate risk for c.1100delC and low risk for p.I157T carriers.

Table 5. Analyses of the entire *CHEK2* coding sequence (separately or as a part of panel NGS) or analyses of specific variant(s) in colorectal cancer (CRC) patients.

Reference	Population	P: Patients C: Controls	Analysis	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Xiang 2017 [230]	meta	Revision of the analysis from [231]		1.8 (1.2–2.7)—unselected CRC *2.4 (0.9–6.6)—familial CRC* *1.7 (0.9–2.9)–sporadic CRC*
Naslund-Koch 2016 [156]	DK	1131/86,975 individuals developed CRC	c.1100delC	*0.86 (0.43–1.72);0.68*
Ma 2014 [232]	meta	P: 3874 CRC/ C:11,630 P: 6042 CRC/ C:17,051	c.1100delC p.I157T	1.88 (1.29–2.73); 0.001 1.56 (1.32–1.84); 1.22×10^{-7}
Han 2013 [172]	meta	P: 3166 CRC C: 9844	p.I157T	1.67 (1.24–2.26); 0.0008
Liu 2012 [233]	meta	P: 4029 CRC C: 13,844	p.I157T	1.61 (1.40–1.87); <0.001—unselected CRC 1.48 (1.23–1.77); <0.001—sporadic CRC 1.97 (1.41–2.74); <0.001—familial CRC
Xiang 2011 [231]	meta	P: 4,194 CRC C: 10,010	c.1100delC	2.11 (1.41–3.16); 0.0003—unselected CRC 2.80 (1.74–4.51); <0.0001—familial CRC *1.45 (0.49–4.30); 0.5—sporadic CRC*
Suchy 2010 [234]	PL	P: 463 HNPCC-related C: 5496 PMC	c.1100delC; c.444+1G>A; del5395; p.I157T	*1.0 (0.4–2.6); 1.0—truncations* 1.7 (1.2–2.4); 0.007—p.I157T
Kleibl 2009 [235]	CZ	P: 631 CRC C: 683 PMC	FHA-coding region c.1100delC;5395del	2.3 (1.3–4.0); 0.003—all variants in FHA domain 2.0 (1.1–3.6); 0.03—p.I157T only *2.3 (0.4–12.8); 0.4—c.1100delC; zero 5395del carriers*
Weischer 2007 [162]	DK	P: 210 (prospective) C: 9007 PMC (prospect.)	c.1100delC	*1.6 (0.4–6.5)—prospective CRC*
Cybulski 2007 [236]	PL	P. 1085 unselected CRC C: 5496 controls	c.1100delC; c.444+1G>A; del5395; p.I157T	*1.0 (0.5–1.8); 0.9—truncations* 1.5 (1.2–2.0); 0.002—p.I157T

Table 5. *Cont.*

Reference	Population	P: Patients C: Controls	Analysis	Odds Ratio (95% Confidence Interval); p—Remark (Statistically Insignificant in *Italics*)
Djureinovic 2006 [237]	SE	P: 174 familial CRC P: 644 unselected CRC C: 760 PMC	c.1100delC	*1.76 (0.34–9.09); 0.6—familial CRC* *1.42 (0.43–4.67); 0.7—sporadic CRC*
Irmejs 2006 [133]	LV	P: 235 C: 978 newborn PMC	p.I157T	1.7 (1.01–2.70); $p < 0.05$
Meijers-Heijboer 2003 [228]	UK/NL/ US/CA	P: 329 CRC C: 1620 [167]	c.1100delC (ASO)	*2.34 (0.95–5.79); 0.07—HNPCC-like families* 5.19 (2.17–12.4); < 0.001—HBCC families

CZ—Czech Republic; DK—Denmark; LV—Latvia; NL—the Netherlands; PL—Poland; SE—Sweden; UK—the United Kingdom, US—the USA. The analyses that failed to demonstrate an association are shown in italics.

Targeted NGS revealed that *CHEK2* was the second most frequently altered gene (following heterozygous *MUTYH*) in the Ambry Genetics ColoNext panel with germline *CHEK2* mutations found in 8/586 colon cancer patients [238]. Subsequent NGS of 450 early-onset colorectal cancer patients from Ohio utilizing a 25-gene panel identified only one *CHEK2* pathogenic mutation; however, the authors reported another 18 *CHEK2* variants as VUS (including six p.I157T) [239]. A recent study of 46 genes in 151 patients with advanced colorectal cancer found 15 carriers of germline mutations. Among them, *CHEK2* with four mutation carriers was the most frequently altered gene [240].

The published data do not provide consistent evidence that germline *CHEK2* alterations substantially contribute to increased colorectal cancer risk. Therefore, the magnitude of colorectal cancer risk needs to be precisely estimated before the formulation of recommendations for a tailored follow-up in mutation carriers. Until then, intensified surveillance may be considered for carriers of *CHEK2* pathogenic mutations from families with multiple appearances of colorectal cancer [241].

3.7. Other Cancers

In 2004, Cybulski and colleagues hypothesized that the portfolio of cancers associating with *CHEK2* germline mutations reaches beyond breast and prostate cancers and identified associations with few other cancer types [20]. However, dozens of isolated studies have reported an association of germline *CHEK2* mutations with an increased, decreased, or no risk in particular types of solid cancers. An increased risk has been documented in patients with melanoma [242], endometrial [243,244], or testicular cancer [245]. An association with pancreatic cancer is less evident [20,246–249], but mutations in genes coding for DDR proteins (including *CHEK2*) in pancreatic cancer patients were associated with improved survival [250–253]. Germline CHEK2 variants were shown to protect against lung cancer, including in patients with a tobacco-related disease [134,254]. Besides solid tumors, an increased *CHEK2* risk has been reported in patients with hematological malignancies [20,255–259].

Some of the analyzed tumor types are a conundrum evergreen for translational research and a nightmare for clinical geneticists. Ovarian cancer ranks among such recurrently queried tumors. With its poor prognosis and inferior treatment outcomes, it is clinically highly desirable to characterize the predisposition factors enabling tailored surveillance, to prevent and/or detect early ovarian cancer, or to start targeted therapy. All ovarian cancer patients are eligible for germline genetic testing. As ovarian cancer associates with breast cancer, the patients are analyzed by overlapping or identical NGS panels and thus ovarian cancer patients probably represent the largest cancer group explored for mutations in the *CHEK2* gene just after breast cancer patients. However, despite many published results, the association of *CHEK2* mutations with ovarian cancer (or its particular non-high-grade subtypes) can be neither confirmed nor rejected, and it illustrates the stalemate situation with the clinical interpretation of germline *CHEK2* variants [115,260–263].

4. Concluding Remarks and Future Directions

The *CHEK2* gene codes for checkpoint kinase CHK2, activated mainly in DDR. Substrates of activated CHK2 include many intracellular targets regulating numerous signaling pathways. However, the quantitative importance of CHK2 in these regulations in particular human tissues remains to be identified.

Pathogenic germline *CHEK2* mutations rank among the most frequent alterations in various tumors. The association of germline *CHEK2* variants has been confirmed for the most frequent gender-specific tumors, including breast and prostate. Despite the high probability of an association with several other cancers, including renal and thyroid cancer, there is no recommendation to prevent these tumors in *CHEK2* carriers. Unfortunately, an association with numerous tumor types and subtypes remains uncertain so far. The major sources of this uncertainty include insufficient numbers of patients with a comprehensive *CHEK2* mutation analysis (including CNV), deficient functional classification of *CHEK2* VUS, and a lack of a precise use of geographically matched population controls for unequivocal

evaluation of the association. The latter point disregarded by many studies is of particular importance if we assume a large population diversity of germline *CHEK2* mutations worldwide, a high frequency of its germline alterations (comparable with that of *ATM* for which the coding sequence is five times larger than that of the *CHEK2* gene), and incomplete penetrance of *CHEK2* mutations.

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