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GENETIC FACTORS IN LYMPHOPROLIFERATIVE MALIGNANCIES

Focus on *CHEK2* gene in lymphomas with comparison to distinct solid tumors

Dizertační práce

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Abstrakt:

Úvod: Gen *CHEK2* (checkpoint kinase 2) se významně podílí na regulaci signální kaskády oprav dvouřetězcových zlomů DNA a kromě jiných interaguje i s proteinem p53. U nosičů mutací genu *CHEK2* bylo prokázáno zvýšené riziko vzniku řady maligních nádorů, ale jeho vztah k riziku vzniku non-Hodgkinských (NHL) a Hodgkinských (HL) lymfomů není znám. Nejčastějším polymorfismem genu *TP53* R72P se u NHL zabývalo několik studií, u HL nebyl zatím zkoumán. **Metody:** Mutační analýza celé kódující sekvence genu *CHEK2* byla provedena u 340 pacientů s NHL a analýza oblasti kódující FHA (forkhead-assodiated) doménu proteinu CHEK2 u 298 pacientů s HL. Výsledky byly porovnány s našimi analýzami *CHEK2* u karcinomů prsu, kolorekta a pankreatu. U pacientů s lymfomy byl také určen genotyp polymorfizmu R72P genu *TP53*. Analýza byla provedena pomocí denaturační vysoce účinné kapalinové chromatografie. **Výsledky:** Četnost mutací v oblasti kódující FHA doménu genu *CHEK2* (exon 2 a 3) byla signifikantně vyšší u pacientů s NHL i HL (19 z 340 – 5,6% a 17 z 298 – 5,7%) než u kontrolní nenádorové populace (19 z 683 – 2,8%; p = 0,03 a 0,04). Alterace v uvedené oblasti zvyšovaly riziko vzniku lymfomů přibližně dvakrát (OR = 2,1; 95% CI 1,2-3,7; p = 0,01) a byly spojeny s horším přežitím bez progrese u pacientů s NHL (p = 0,008). Lepší celkové přežití bylo naopak prokázáno u pacientů s difuzním velkobuněčným B lymfomem a variantou genu *CHEK2* IVS1+43dupA (p = 0,02). Uvedená alterace byla také spojena s lepším přežitím bez progrese ve skupině všech pacientů s NHL (p = 0,01). Alterace v oblasti genu *CHEK2* kódující FHA doménu zvyšovaly také riziko vzniku kolorektálního karcinomu (OR = 2,3; 95% CI 1,3-4,1; p = 0,003), tento vliv nebyl prokázán u karcinomu prsu a pankreatu. Polymorfizmus P72P genu *TP53* neovlivňoval riziko vzniku ani prognózu pacientů s lymfomy. **Závěr:** Alterace genu *CHEK2* v oblasti kódující FHA doménu jsou predispozičním faktorem zvyšujícím riziko vzniku maligních lymfomů a spolu s alterací IVS1+43dupA mohou významným způsobem ovlivňovat prognózu onemocnění.

Klíčová slova: gen *CHEK2* (Checkpoint kinase 2, CHK2), vrozené alterace, gen *TP53* (*p53*), polymorfizmus Arg72Pro (R72P), non-Hodgkinské lymfomy, Hodgkinův lymfom, karcinom prsu, kolorektální karcinom, karcinom pankreatu, predispoziční faktory, prognóza

Summary:

Background: The checkpoint kinase 2 gene (*CHEK2*) codes for an important mediator of DNA damage response pathway that among others interacts with the p53 protein. Mutations in the *CHEK2* gene increase the risk of several cancer types, however, their role in non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma (HL) is not clear. The most frequent *TP53* gene R72P polymorphism was analyzed in several studies in NHL but not in HL. **Methods:** We have performed mutation analysis of the whole *CHEK2* gene coding sequence in 340 NHL patients and the segment coding for *CHEK2* forkhead-associated (FHA) domain in 298 HL patients and compared the results with our analyses of *CHEK2* in breast, colorectal and pancreatic cancers. The *TP53* R75P genotype was assessed in the same lymphoma populations. Both genes were analyzed using denaturing high-performance liquid chromatography. **Results:** The overall frequency of *CHEK2* alterations within FHA-coding region was significantly higher in NHL and HL patients (19/340 - 5.6%; 17/298 - 5.7%, respectively) compared to non-cancer controls (19/683 - 2.8%; p = 0.03 and 0.04, respectively). These alterations were associated with increased risk of lymphoma development (OR = 2.1; 95% CI 1.2-3.7; p = 0.01) and worse progression-free survival (PFS) in NHL patients (p = 0.008). Better overall survival in diffuse large B-cell lymphoma and PFS in all NHL patients was associated with *CHEK2* IVS1+43dupA alteration (p = 0.02 and 0.01, respectively). We have identified the association of *CHEK2* FHA alterations also with colorectal cancer risk (OR = 2.3; 95% CI 1.3-4.1; p = 0.003), but not with breast or pancreatic cancers. The *TP53* R72P polymorphism did not influence lymphoma risk or survival. **Conclusions:** Alterations in the *CHEK2* gene FHA coding region represent moderate genetic predisposition factor increasing the risk of lymphoma and together with IVS1+43dupA alteration may modify lymphoma disease course.

Keywords: Checkpoint kinase 2 gene (*CHEK2*, CHK2), Germ-line mutation, *TP53* gene (*p53*), Arg72Pro polymorphism (R72P), non-Hodgkin lymphoma, Hodgkin lymphoma, Breast cancer, Colorectal cancer, Pancreatic cancer, Genetic predisposition, Prognosis

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1. INTRODUCTION

1.1. Malignant lymphomas

Malignant lymphomas represent a heterogenic group of lymphoid malignancies derived from B cells, T cells or NK cells characterized by different presentation and course of the disease. Lymphomas could be divided into two large subgroups according to the histological characteristics, non-Hodgkin lymphomas (NHL) and Hodgkin lymphomas (HL). Hodgkin lymphoma is characterized by the presence of large Hodgkin and Reed-Sternberg cells (derived from B lymphocytes) that constitute a minority of the cell population in affected lymphatic nodes.

The most common types of non-Hodgkin lymphomas comprise diffuse large B-cell lymphoma (DLBCL, accounts for approx. 30% of NHL cases) and follicular lymphoma (FL, about 20% of NHL cases). Rest NHL cases represent some of over 30 histopathological subtypes each occurring in less than 10% of patients. NHLs are more common in well developed countries with the highest incidence in the USA, Australia, New Zealand and Europe. It is the 7th most common cause of cancer dead worldwide.[1] NHLs accounts for 4% of cancer diagnoses in the United States and stand for the fifth most common cancer diagnosed there.[2] Within the EU, the incidence rate range from 6 to 18 cases per 100 000 inhabitants, mortality rate is 4.1 of 100 000 men and 2.5 of 100 000 women. The annual incidence rate of NHLs in the Czech Republic is 11.6 cases per 100 000 inhabitants.[3] After a dramatic increase in the second half of the 20th century, the incidence remains relatively stable during the last decade.[4]

Based on the histological characteristics, Hodgkin lymphoma could by divided into two distinct subgroups with different clinical features; nodular lymphocyte predominant Hodgkin lymphoma (NLPHL; approx. 5% of HL cases) and classical Hodgkin lymphoma (CHL; approx. 95% of HL cases) with four subtypes [nodular sclerosis (NSCHL), lymphocyte-rich (LRCHL), mixed cellularity (MCCHL) and lymphocyte-depleted (LDCHL) classical Hodgkin lymphoma].[5] The annual incidence of HL is approximately 2.5 cases per 100 000 inhabitants in Europe[6] and 2.7 cases per 100 000 inhabitants in the USA[7] with higher proportion of male patients. The annual incidence rate of HL in the Czech Republic is 2.4 cases per 100 000 inhabitants.[3]

1.2. Risk factors of lymphoma development

Numerous studies were performed to determine the risk factors for NHL and HL development. Environmental and lifestyle factors of NHL reviewed by Alexander et al.[8] are summarized in Table 1. Nearly similar factors have been shown to be associated also with increased risk of HL (e.g. EBV and HIV infection, inherited or acquired immunodeficiency, good socioeconomic status), controversial is the effect of occupation exposure to wood and chemicals (reviewed in[5,9]).

Table 1 Overview of environmental and lifestyle factors evaluated as risk factors of NHL development.

Positive association	Negative association	No association
- red meat intake	- vegetable consumption	- tobacco use
- saturated fat intake	- alcohol consumption	- exposure to the pesticides and other chemicals
- organ or bone marrow transplantation	- UV exposure	- hair dyes
- several autoimmune disorders (rheumatoid arthritis, celiac disease, systemic lupus erythematosus, Sjoegren's syndrome)	- vaccination	- exposure to ionizing radiation
- congenital immunodeficiency syndromes (Wiskott–Aldrich syndrome, severe combined immunodeficiency syndromes)		- occupation
- acquired immunodeficiency syndromes		- body mass index
- HIV, EBV, and Helicobacter pylori infection		- physical activity
		- hormonal and reproductive factors
		- allergy
		- blood transfusion

Alongside above mentioned environmental risk factors, lymphoma development is influenced by so far poorly understood genetic factors. Although the vast majority of lymphoma cases arise in form of sporadic disease, familial clustering is also known (besides the higher risk in lymphoma patients' relatives). Both could be explained by specific genotype of each individual that could at different rate modify the risk of lymphoma development. Family history of hematological malignancy was shown to be a strong risk factor for NHL or HL development in numerous studies. Individuals with first degree relatives affected by hematological malignancy have considerably higher risk of NHL (OR range from 1.5 to 2.9).[10] This association was shown for different types of NHL[11] being even more apparent in

aggressive NHL subtypes.[12] Villeneuve at al.[13] reported stronger association of first degree relatives of patients with hematological malignancy and NHL or HL risk in men and in those relatives of patients diagnosed before the age of 45 years. Chatterjee at al.[14] described even more increased risk of NHL in siblings. Another study of Kadan-Lottick al al.[15] evaluating the risk of NHL in twins found a 40-fold risk increase in monozygotic twins but unchanged risk in dizygotic twins. Further supporting evidence for importance of genetic background in NHL development comes from studies involving analysis of NHL incidence in populations of migrants that reported sustained incidence at the level of mother country unaffected by settlement in a new country.[16,17] Moreover, the difference in frequency of several NHL subtypes in males and females is well documented for a long period of time. The most prominent difference is high incidence of mantle cell lymphoma (MCL) in males (70% of MCL cases), whereas, females tend to predominate in follicular lymphoma (FL).[18]

The hypothesis, that the risk of HL development is also modified by a genetic background[5] is supported by reported increased incidence of HL in monozygotic twins[19] and first degree relatives of lymphoma patients.[20,21]

Numerous studies evaluated the risk of lymphoma in carriers of inherited alterations in particular genes coding for proteins involved in various cellular pathways (e.g. immunomodulation, detoxification, oxidative stress response, or DNA repair) proposed to be impaired in lymphoma pathogenesis.[22-27] The genetic susceptibility to NHL in relation to the germline variation in these genes has been reviewed recently.[28] In respect to the aims of this work, the role of polymorphisms and mutations in critical genes coding for proteins involved in DNA repair pathways in relation to the risk and pathogenesis of lymphomas will be emphasized. The DNA damage response system together with DNA repair mechanisms has been shown to represent a critical anticancer barrier activated upon various cancer-promoting stimuli in precancerous lesions. Impairment of this barrier caused by the selection of clones carrying DNA damage response defects (by acquired somatic genetic and/or epigenetic gene inactivation) or by inherited alterations in genes involved in DNA repair could lead to the progression of tumorigenesis and cancer development.[29-31] Resulting chromosomal/genomic instability belongs to the classical hallmark of cancer cells including lymphomas. It is known that several translocations specifically occur in higher frequencies in certain histopathological NHL subtypes – e.g. t(14;18) in DLBCL, t(8;14) in Burkitt lymphoma, t(14;18) in FL, or

t(11;14) in MCL. Moreover, defects in gene rearrangements during lymphocyte maturation could contribute to the initial lymphoma development.[32]

1.3. Alterations of DNA repair genes and lymphomas

The DNA repair plays an essential role in maintenance of genomic integrity and its failure is a key step toward cancer development due to the accumulation of genetic alterations leading to the initial malignant transformation. Inherited defects of genes coding for proteins involved in DNA repair pathways are responsible for numerous cancer-predisposing syndromes [e.g. Ataxia telangiectasia (OMIM 208900), Bloom syndrome (OMIM 210900), Lynch syndrome 1 (OMIM 120435), or Nijmegan breakage syndrome (OMIM 251260)]. These syndromes are characterized by chromosomal instability and some of them are associated with increased risk of lymphoma development [Ataxia telangiectasia, Bloom syndrome, Nijmegan breakage syndrome, or Mismatch repair cancer syndrome (OMIM 276300)].

1.3.1. Overview of DNA repair mechanisms

The DNA repair processes comprise of several pathways targeting different specific DNA alterations:

A. ***Direct DNA damage reversal*** is a simple mechanism individually repairing lesions without incision of the DNA sugar-phosphate backbone or base excision. This pathway consists of number of different enzymes, for example dioxygenases catalyzing oxidative dealkylation (e.g. ABH2 and ABH3 catalyze the removal of 1-methyladenine and 3-methylcytosine from methylated polynucleotides), or alkyltransferases (e.g. O-6-methylguanine DNA methyl transferase; MGMT).[33]

B. ***Base excision repair*** (BER) responds to the alkylation, deamination or oxidative damage of DNA. DNA glycosylases occuring in form of 11 different mammalian enzymes, e.g. UNG (uracil-DNA glycosylase) or TDG (thymine-DNA glycosylase), recognize the site of DNA damage and catalyze altered base removal creating apurinic or apyrimidinic site (AP site). The DNA backbone is thereafter cleaved by a DNA AP endonuclease or a DNA AP

lyase. Generated single nucleotide gap is in turn filled by DNA-dependent DNA polymerase β. The DNA strand integrity is finally completed by DNA ligase.[34]

C. Contrarily to the BER, ***nucleotide excision repair*** (NER) manages wide variety of lesions by a small set of enzymes. Distortion-sensation complex (consisting of the RPA, XPA, and XPC proteins) recognizes chemically-modified DNA bases, UV induced lesions (e.g. pyrimidines crosslinking), or several types of oxidative damage events (un-repairable by BER) not as a specific DNA alterations but on the level of changes in the DNA structure pattern. In the next step, TFIIH complex induces DNA strand separation (creation of "denaturation bubble") around the site of lesion. Damaged DNA strand (about 25-30 nucleotides) is excised by a group of specific endonucleases (e.g. ERCC5) and the single strand gap filled by DNA-dependent DNA polymerases δ or ε.[35]

D. ***Mismatched repair*** (MMR) serves for removal of mismatched nucleotides misincorporated by the DNA polymerase during replication of genomic DNA or resulting from base damage (e.g. by hydrolytic deamination). Mismatched base pair is recognized by the MutSα heterodimer consisting of MSH2/MSH6 proteins that together with MutLα heterodimer (MLH1 and PMS2 proteins) creates a sliding clamp moving alongside to the DNA molecule. This complex introduces DNA nicks in nascent DNA strand delimiting misincorporated nucleotide and activates EXO1 exonuclease degradating single stranded DNA between formed nicks in reverse direction (from 3' to 5' end). The single strand region is finally resynthesized by DNA-dependent DNA polymerase δ or ε. Mismatches caused by base damage are usually repaired by the activity of BER system.[36]

E. ***Double strand break (DSB) repair pathways*** serves for elimination of DSB, the most serious DNA lesions with strong cancer-promoting potential. DSBs arise from abruption of phosphodeoxyribosyl backbone in DNA molecule and unrepaired could result in chromosomal translocations or loss of chromosomal fragments. The two main DSB repair pathways involve ***homologous recombination*** (HR) and ***non-homologous end-joining*** (NHEJ). Both systems differ in their requirement for a homologous DNA template during repair. HR uses intact DNA duplex as a template, whereas NHEJ does not require a template. Therefore, HR takes place primarily in late S and G2 phases of the cell cycle when sister chromatid is readily available.

During **HR**, undamaged homologous DNA molecule generated by DNA replication in S phase serves as a template for synthesis and rejoining of damaged DNA fragment. HR represents highly accurate error free mechanism of DSB repair. DSBs are recognized by ATM/ATR protein kinases in cooperation with MRN complex (consisting of MRE11, Rad50, and NBS1 proteins) supported by histone H2AX. The MRN complex in cooperation with endonucleases creates single strand regions on both sides of broken DNA molecule. After that, the Rad51 and Rad52 proteins catalyze strand exchange with undamaged homologous strand separated from DNA template. Assembling of the Rad51 nucleoprotein filament is facilitated by five different Rad51 paralogues (Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3). HR process involves establishment of large multiprotein supercomplexes assisted by covalent modification (phosphorylation or ubiquitination) of contributing proteins. The supercomplex assembly is regulated by numerous proteins including BRCA1, BRCA2 or xeroderma pigmentosum (XP) family members. Repair is finished by DNA synthesis, ligation and branch migration. The initial signal is amplified by the CHK1 or CHK2 kinases responsible for signal transduction to DSB repair effector proteins but also for signal transmission to proteins involved in cell cycle arrest, or apoptosis induction in case of repair failure. Primarily, the activation of transcription factor p53 orchestrates mutual regulation of these processes.

NHEJ represent the dominant form of DSB repair in higher eukaryotes. In NHEJ process, protein complex formed by XRCC5 (Ku80) and XRCC6 (Ku70) proteins recognizes and binds to the both ends of damaged DNA and pulls them together. In cooperation with DNA-PKcs XRCC5/6 heterodimer activates XRCC4 and ligase IV complex rejoining the broken DNA. Despite its efficacy, during NHEJ exists considerable risk of rejoining of unrelated DNA fragments resulting in chromosomal translocations.[37-39]

The contribution of DNA repair genes to lymphomas development will be specifically addressed in later sections (1.3.2 and 1.3.3), whereas *CHEK2* and *TP53* genes will be discussed in separate sections (1.4 and 1.5).

1.3.2. Alterations of DNA repair genes and non-Hodgkin lymphoma

The impact of genetic predisposition in NHL patients has been studied in numerous case-control or association studies focused on analysis of single nucleotide polymorphisms (SNPs) in the genes coding for DNA repair proteins or by mutation analyses of entire genes coding for key components of DNA repair pathways.

Three large-scale (SNP) studies of DNA repair genes were performed in population of patients with NHL.

Shen at al. 2006[40] performed a study of 32 SNPs in 18 DNA repair genes in 518 women with NHL and 597 controls from the USA. Genes coding for selected proteins acting in all types of DNA repair processes were selected. The variant D1104H in the *ERCC5* gene (NER pathway) was associated with an increased risk of all NHL subtypes together (OR = 1.46; 95% CI 1.13-1.88). This variant was also significantly associated with several NHL subgroups (B-cell lymphomas, DLBCL, and T-cell lymphomas). Only borderline inverse association was found for *ERCC2* (*XPD*, NER pathway) K751Q variant. Among genes involved in DSB repair, *WRN* (coding for helicase interacting with XRCC5/XRCC6 complex in NHEJ) C1367R mutation reduced the risk of all NHL in general and frequent NHL subtypes (DLBCL, FL). In other genes, positive association was found for *BRCA2* N289H and *XRCC1* R280H variants and the risk of T cell lymphoma, for *XRCC1* R280H variant and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), and for *XRCC3* T241M variant in FL and marginal-zone B-cell lymphoma (MZBL). Lower risk of NHL development was reported for *XRCC1* R194W variant carriers and FL and for *BRCA1* E997G variant carriers and all NHLs.

Second study of Hill at al.[41] examined 1150 NHL cases and 956 controls from the USA for 34 SNPs in 19 DNA repair and related genes. From genes involved in NHEJ, T9I variant in DNA ligase *LIG4* may be associated with the reduced lymphoma risk and K820R variant in *RAG1* with the increased lymphoma risk. Association of K820R mutation in *RAG1* (involved in V(D)J recombination and DNA-repair processes) with the NHL risk was the most significant result. In genes acting in other repair systems, *BRCA2* (HR pathway) N372H mutation was associated with 1.4-fold increased risk, *XRCC1* (BER pathway) R194W mutation with moderately increased risk, and *WRN* V114I mutation with reduced risk of all NHLs. In all these alterations the effect was stronger in subgroups of homozygotes.

Third study involving 561 Australian cases and 506 controls was performed by Shen at al. 2007[42]analyzing 22 SNPs in 14 DNA repair genes. The I143V and K178R variants in *MGMT* showed a significant trend to the elevated risk in individual histological types of NHL, *XRCC1* R194W variant was shown to reduce the risk of DLBCL. The authors also performed metaanalysis of their and two above mentioned studies. They found positive association of *MGMT* I143V variant with FL risk without heterogeneity between studies (OR = 1.3, 95% CI 1.03-1.64; I/V + V/V vs. I/I). This association was not evident for DLBCL. The function of MGMT enzyme (a member of direct DNA damage reversal pathway) is to protects from toxicity of alkylating agents. Other significant results from individual studies were not affirmed.

Similar polymorphisms as those identified by large-scale SNP studies as significantly associated with NHL risk were evaluated in several other studies. Smedby et al.[43] analyzed 19 SNPs in *ERCC2* (NER pathway), *XRCC1* (BER pathway), and *XRCC3* (HR pathway) genes in 430 FL patients and 605 controls. No association of *ERCC2* and *XRCC1* genes variants and FL was found, however, *XRCC3* rs3212024 (reference SNP ID) polymorphism homozygotes showed higher risk of FL. No association of *XRCC1* Q399R polymorphism with malignant lymphomas was found in study of Matsuo at al.[44] and three *XRCC1* polymorphisms in study of Liu at al.[45] Scott at al.[46] analyzed *RAG1* K820R and *BRCA2* N372H polymorphisms (reported in study of Hill at al. as predisposing factors to NHL) but failed to find any association. Comparison of results from individual above mentioned studies are presented in Table 2. Significant results of all studies are overviewed in Table 4. These results show large variability and inconsistency in NHL risk assessment between individual studies.

Table 2 Comparison of the effect of selected alterations in the genes coding for proteins involved in DNA repair on the risk of NHL.

Alteration	Shen at al.[40]	Hill at al.[41]	Shen at al.[42]	Smedby at al.[43]	Liu at al.[45]	Scott at al.[46]
MGMT I143V	NS	NS	Elevated	-	-	-
XRCC1 R194W	Reduced	Elevated	Reduced	NS	NS	-
XRCC1 R280H	Elevated	-	NS	NS	NS	-
XRCC3 T241M	Elevated	NS	NS	NS	-	-
ERCC5 D1104H	Elevated	NS	NS	-	-	-
WRN C1367R	Reduced	NS	NS	-	-	-
BRCA2 N289H	Elevated	NS	NS	-	-	-
BRCA2 N372H	NS	Elevated	NS	-	-	NS
LIG4 T9I	NS	Reduced	NS	-	-	-
RAG1 K820R	NS	Elevated	NS	-	-	NS

NS - not significant result.

Several other studies focused on mutation/SNPs analyses of particular genes coding for proteins of DNA repair:

The ***hMSH2*** [human mutS homolog 2 (E. coli)] gene protein product is involved in MMR pathway; participates in mismatch recognition and coordination of nucleic excision. Polymorphism IVS12-6T>C was shown to be more common in patients with NHL in Ecuador than in control population (22.7% vs. 7.5%; 22 NHL patients and 50 controls; respectively).[47] This finding was not confirmed by a study in Japanese population where higher frequency of this polymorphism was identified (57.3% of 103 NHL cases and 48.9% of 487 controls, non-significant difference).[48] Several case reports revealed pathogenic alterations in the *MSH2* gene. The homozygous truncating mutation Q76X was identified in three siblings who each developed T-cell NHL in early childhood.[49] In two children affected by mediastinal T cell lymphoma and glioblastoma were found intragenic rearrangement involving exons 1-6 and frame-shifting 1 bp deletion at codon 153 acquired from healthy parents.[50] Another inherited *hMSH2* rearrangement was also found in B-cell NHL patient from a HNPCC family [hereditary non-polyposis colorectal cancer (Lynch syndrome 1, OMIM 120435), disorder caused by mutations in MMR genes]. The tumor tissue sample lacked expression of hMSH2 protein and showed loss of the wild-type (wt) *hMSH2* allele.[51] Moreover, the experimental data show that *hMSH2* deficient mice are highly susceptible to lymphoid tumors development.[52] All these results support involvement of *hMSH2* in lymphomagenesis, although the association remains unclear due to the lack of large-scale studies in NHL population.

The ***H2AX*** gene (a H2A histone family member) encodes a key protein participating in the detection of DNA double strand breaks. Many other factors co-localize with phosphorilated H2AX (γH2AX) in a sensoric part of this pathway (incl. ATM, BRCA1, RAD51, MRN complex).[53] The frequency of genetic changes of *H2AX* in NHL patients was estimated in 95 NHL cases and found alterations consequently analyzed in population of 487 NHL cases and 531 controls in Canadian population. Altogether, seven SNPs were identified. Alteration c.-417G>A was negatively associated with NHL risk (OR = 0.54, 95% CI 0.37-0.79). The reduced risk was found in subpopulation of FL and MCL, but not in DLBCL.[54] Limited role of the *H2AX* gene's polymorphisms in lymphomagenesis supports the fact that *H2AX* is neither mutated in lymphomas,[55] nor is affected by common chromosomal aberrations of 11q23 region in MCL.[56]

The ***ATM*** (ataxia-telangiectasia mutated) protein plays crucial role in recognition of DSBs. Germline mutations of *ATM* gene are responsible for the development of recessive disorder ataxia-telangiectasia (A-T) characterized by increased risk of cancer development alongside to neurologic symptomatology (cerebelar ataxia, abnormal eye movements, dysarthria), defects of cell-mediated and humoral immunity, thymic hypoplasia, hypogonadism, growth retardation, and the presence of telangiectasia. Approximately 10-15% of A-T patients develop lymphoid malignancy.[57] The role of *ATM* mutations as a risk factors was recently described in sporadic and familial breast cancer.[58,59] Higher frequency of somatic *ATM* mutations was found in DLBCL and FL tumors than in general population (blood donors).[60] *ATM* mutations occur mainly in MCL (in 40-70% of tumors) and are connected with higher number of chromosomal translocations.[61,62] Briani at al.[63] described patient with heterozygous *ATM* mutation (c.5979_5983delTAAAG, causing frameshift after amino acid 1992 and premature truncation) who developed MCL with somatic mutation of the wt allele (R2263S). Tort at al.[64] investigated MCL tumor tissues from patients with MCL or other distinct lymphoproliferative malignancy in first degree relatives. They failed to find any mutation of *CHK1*, *CHK2*, and *TP53* genes. The only one variation found was *ATM* nucleotide substitution D1853N in one family, but the frequency in healthy population was the same as in sporadic MCL cases (15%).[65] Sipahimalani at al.[66] screened whole coding and promoter sequence of *ATM* in germline DNA of 86 NHL patients and found 79 variants with different frequencies (ranging from 0.5% to 47%). Seventeen selected *ATM* variants were consequently analyzed in 798 cases and 793 controls without any significant association with NHL risk. It could be summarized that common variants of *ATM* do not influence the risk of NHL, but some rare alterations may play significant role in NHL genesis. It has been shown that *ATM* mutation status in tumors does not influence overall survival of MCL patients.[67]

The inherited mutations in the ***NBS1*** (Nijmegan breakage syndrome) gene cause a rare autosomal recessive disease Nijmegan breakage syndrome (NBS). Patients with NBS are characterized by typical facial appearance (bird-like face), microcephaly, radiosensitivity, immunodeficiency, growth retardation, and increased risk of cancer, especially NHL.[68] *NBS1* is a part of MRN complex (MRE11, Rad50, NBS1) which in interaction with ATM recognizes and starts signalization after DNA DSBs. Majority of NBS patients are homozygous carriers of founder mutation c.657del5, heterozygotes are common in patients with different types of cancer (e.g. colorectal, breast, or melanoma).[69] Several studies evaluating the risk of NHL in c.657del5 mutation carriers have been performed (overviewed

in Table 3). The elevated risk of NHL (OR = 8.05, 95% CI 1.71-37.95) in c.657del5 mutations carriers was originally reported in the Polish study of Steffen et al.[69] Analysis of c.657del5 and other four *NBS1* mutations (c.698del4, c.835del4, c.842insT, c.1142delC) in 119 Czech NHL and HL cases performed by Soucek at al.[70] revealed that *NBS1* alterations rarely occur in our population (one carrier of 657del5 mutation was identified in lymphoma cases, no carrier was found in 177 controls). Another germline mutation of *NBS1* - IVS11+2insT - causing deficient function of NBS1 protein was investigated in Japan, however, no carrier was found in 109 lymphoma patients.[71] Frequency of somatic *NBS1* mutations was also investigated in NHL tumors. Low frequency of mutations was reported in a study from the United States,[72] and no mutation was found in a study from Japan.[73] Moreover, deletion of *NBS1* gene was not identified in NHL tumors with structural abnormalities of 8q where the *NBS1* gene is located.[74] The role of *NBS1* mutations seems to be limited to the elevated risk of NHL development in some populations. This finding must by verified by other studies including the analyses of whole coding sequence of *NBS1*.

Table 3 Studies analyzing *NBS1* c.657del5 mutation in NHL patients.

Study	**Country of study**	**No. of cases/ controls**	**Frequency of mutation in cases/controls**	**Population of patients**	**Odds ratio** (95% confidence interval)
Stanulla at al.[75]	Germany	109/-	0/-	Pediatric NHL	-
Rischewski at al.[76]	Germany	55/-	0/-	Pediatric NHL	-
Soucek at al.[70]	Czech rep.	119/177	0.008/0	NHL, HL	-
Resnic at al.[77]	Russia	7/548	0.14/0	Pediatric NHL	-
Steffen at al. 2004[69]	Poland	42/1620	0.05/0.006	NHL	8.05 (1.71-37.95)
Chrzanowska at al.[78]	Poland	212/6984	0.009/0.006	Pediatric NHL	$p<0.05$*
Steffen at al. 2006[79]	Poland	228/1620	0.04/0.006	NHL	5.85 (2.29-15.00)
		37/1620	0.11/0.006	Gastrointestinal lymphoma	19.52 (5.82-65.42)

***** OR not estimated.

The ***MRE11*** [meiotic recombination 11 homolog A (S. cerevisiae)] gene codes for nuclease contributing to formation of MRN complex during HR repair. Individuals with homozygotic mutations of *MRE11* gene develop A-T like disorder (OMIM 604391) and frequently suffer from lymphoid tumors.[80] Fukuda et al.[81] performed mutation analysis of *MRE11* in 21 tumor samples and paired healthy tissues but only one mutation (R572Q affecting the conservative amino acid residue) was identified. Rollinson et al.[82] evaluated the impact of haplotypic variation of different variants in all three MRN members (six *MRE11*, five *NBS1*, and six

RAD50 variants) in 461 non-Hodgkin lymphoma patients and 461 controls. No significant differences in allele or genotype frequencies were found to be associated with elevated risk of NHL, but authors reported protective effect of *MRE11* rs601341 SNP in homozygous form to FL (OR = 0.5; 95% CI 0.26-0.97), and one *MRE11* haplotype to DLBCL (OR = 0.72; 95% CI 0.53-0.97). No association of polymorphisms in genes coding for members of MRN complex with NHL risk was also reported by Schuetz et al.[83]

Table 4 Overview of variants in genes coding for proteins involved in DNA repair that significantly associate with the risk of NHL development.

Study	**No. of cases /controls**	**Gene**	**Alteration**	**Population of patients**	**Odds ratio** (95% confidence interval)
Shen at al.[40]	518/597	*ERCC5*	D1104H	NHL	1.46 (1.13-1.88)
		WRN	C1367R	NHL	0.71 (0.56-0.91)
		BRCA2	N289H	T cell NHL	3.97 (1.60-9.90)
		XRCC3	T241M	FL	1.62 (1.03-2.56)
		XRCC3	T241M	MZBL	2.6 (1.04-6.51)
		XRCC1	R194W	FL	0.45 (0.21-0.95)
		XRCC1	R280H	T cell NHL	3.36 (1.48-7.65)
		XRCC1	R280H	SLL/CLL	2.22 (1.08-4.58)
Hill at al.[41]	1150/956	*LIG4*	T9I	NHL	0.8 (0.7-1.0)
		LIG4	T9I	FL	0.7 (0.5-1.0)
		LIG4	T9I	DLBCL	0.8 (0.6-1.0)
		RAG1	K820R	NHL	1.4 (1.1-1.7)
		RAG1	K820R	FL	1.5 (1.1-2.1)
		BRCA2	N372H	NHL	1.5 (1.0-2.1) [a]
		BRCA2	N372H	FL	1.6 (1.0-2.6) [a]
		BRCA2	N372H	DLBCL	1.5 (1.0-2.4) [a]
		BRCA2	N372H	T cell NHL	2.0 (1.2-3.3)
		XRCC1	R194W	NHL	1.4 (1.1-1.8)
Shen at al.[42]	561/506	*MGMT*	I143V	NHL	1.33 (1.00-1.78)
		XRCC1	R194W	DLBCL	0.50 (0.27-0.93)
Smedby at al.[43]	430/605	*XRCC3*	rs 3212024 [b]	FL	1.8 (1.1-2.8) [a]
Novik at al.[54]	487/531	*H2AX*	c.-417G>A	NHL	0.54 (0.37-0.79) [a]
		H2AX	c.-417G>A	FL	0.40 (0.21-0.74) [a]
		H2AX	c.-417G>A	MCL	0.20 (0.05-0.72) [a]
Steffen at al.[69]	42/1620	*NBS1*	c.657del5	NHL	8.05 (1.71-37.95)
Chrzanowska at al.[78]	212/6984	*NBS1*	c.657del5	Pediatric NHL	$p<0.05$ [c]
Steffen at al.[79]	228/1620	*NBS1*	c.657del5	NHL	5.85 (2.29-15.00)
		NBS1	c.657del5	Gastrointestinal lymphoma	19.52 (5.82-65.42)
Rollinson at al.[82]	461/461	*MRE11*	rs 601341 [b]	FL	0.5 (0.26-0.97)
Cybulski at al.[89]	120/4000	*CHEK2*	I157T	NHL	2.0 (p=0.05)
Worrillow et al.[88]	747/808	*ERCC2*	K751Q	DLBCL	0.56 (0.34-0.92)

If not stated else, the OR is calculated for variant in wt homozygous form vs. heterozygous + recessive homozygous combination; a) The OR calculated for variant in homozygous form vs. wt in homozygous form; b) Reference SNP ID; c) OR not estimated.

No significant association of NHL risk and ***XPD*** gene [Xeroderma pigmentosum, complementation group D (ERCC2), gene involved in NER pathway] polymorphisms was identified.[40-42,84] Analysis of ***BRCA1*** and ***BRCA2*** (breast cancer gene 1 and 2) genes for the three most frequent mutations in 286 DNA of Jewish lymphoma patients discovered only two mutation carriers. This finding suggests that *BRCA1/2* mutations are not associated with increased risk of NHL.[85] From *BRCA1/2* polymorphisms investigated in other studies, positive association was found for *BRCA2* N289H and N372H variants and NHL risk.[40-42] Analysis of ***RAD54*** gene in 24 lymphoma tissues showed only one tumor with a mutation (V444E) which was not observed in 100 healthy controls.[86] A homolog of *RAD54*, the ***RAD54B*** gene, was analyzed in 26 lymphoma samples. One tumor with homozygote *RAD54B* N593S mutation was identified, no mutation in 80 normal individuals was found.[87] Worrillow et al.[88] identified association of ***ERCC2*** (XPD; NER pathway) K751Q polymorphism with decreased risk of DLBCL development (three *ERCC2* polymorphisms evaluated in 747 NHL patients).

1.3.3. Alterations of DNA repair genes and Hodgkin lymphoma

Contrary to the NHL, genes coding for proteins involved in DNA repair were much less studied in HL. Only one study evaluating the association between polymorphisms in DNA repair genes and risk of HL was performed by El-Zein et al.[90] This study involved analyses of polymorphisms in *XPD*, *XPC*, and *XPG* genes (involved in NER pathway), *XRCC1* gene (BER pathway) and *XRCC3* gene (contributing to DSB repair). Positive association was found for polymorphism R399Q of *XRCC1* gene (OR = 1.77; 95% CI 1.16-2.71). Increased risk of HL development was also associated with combined genotypes of *XRCC1*/*XRCC3* genes (OR = 2.38; 95% CI 1.24-4.55) and *XRCC1*/*XPC* genes. Authors concluded that genetic polymorphisms in DNA repair genes could modify the risk of HL development especially when interactions between different pathways are considered. Potential involvement of *ATR* gene (coding for ATM and Rad3-related kinase participating in recognition of DNA double-strand breaks) in HL development was evaluated by a study performed on eight HL cell lines and seven HL tumor specimens.[91] *ATR* alterations (aberrant transcripts) were detected in six HL cell lines and three clinical samples. The cell lines expressing aberrant ATR transcripts showed defective repair of DNA DSBs. This data suggests the possible involvement of the *ATR* gene in lymphomagenesis.

Two polymorphism in DNA repair genes were evaluated as factors modifying the risk of second malignancy after treatment for HL. Mertens et al.[92] did not find any association of *XRCC1* polymorphism at codon 399 with radiotherapy-related cancers in study of 650 HL survivals. However, common polymorphism in the promoter region of *MLH1* gene (mismatch repair) has been found to correlate with higher risk of development of acute myeloid leukemia (OR = 5.31; 95% CI 1.4-20.2) or breast carcinoma (borderline statistical significance, OR = 4.0; 95% CI 0.8-19.4; $p = 0.08$) after methylating chemotherapy for HL.[93]

As a risk factors of HL development were more evaluated polymorphisms in genes whose protein products are implemented in immune functions, e.g. Monroy et al.[94] found association of several functional SNPs in inflammatory genes and HL risk (*COX2*, *IL18*, *IL4* and *IL10*; 38 SNPs were evaluated). Polymorphisms in cytokine genes (*IL10*, *IL6*) were also reported to predict worse treatment outcome by Hohaus et al.[95] No correlation with HL risk was found e.g. in study of Cordano et al.[96] analyzing polymorphism in *IL6* promoter.

1.4. *CHEK2* gene

1.4.1. *CHEK2* gene and protein structure and function

The ***CHEK2*** (check point kinase 2, CHK2, OMIM 604373) gene[97] codes for an important mediator of DNA damage signaling pathway. The CHEK2 protein (CHK2) mediates signal transduction from the apical sensoric part of the pathway, represented by the activation of ATM protein following DSBs, toward cell cycle and apoptosis regulators (p53, Cdc25A, Cdc25C) and protein complexes directly involved in DNA-repair (BRCA1). These proteins represent the key substrates of CHK2 kinase activity (Figure 1).[98]

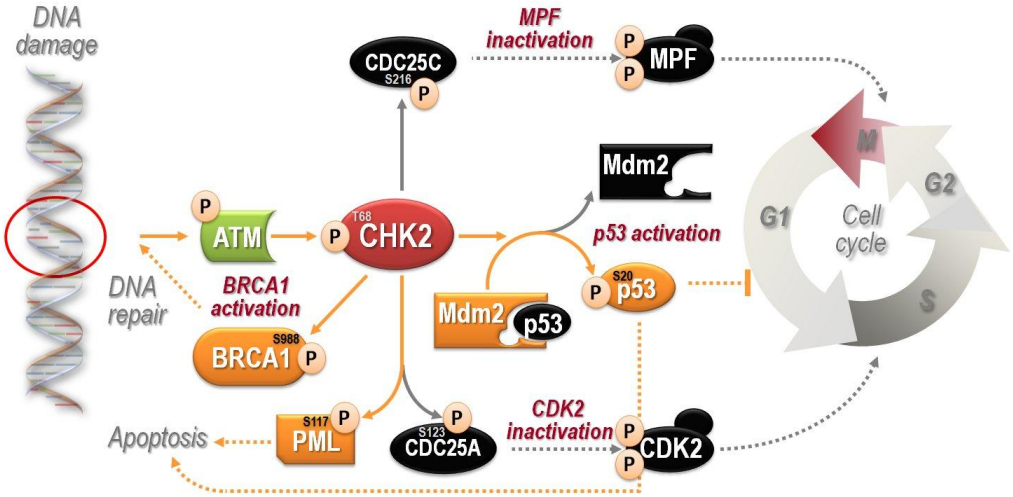

Figure 1 The schematic presentation of the role of CHK2 kinase in coordination of DNA repair processes with cell cycle arrest and apoptosis initiation following DSB.

The *CHEK2* gene localized to chromosome 22q12.1 codes for the 60-kDa protein consisting of 546 amino acid residues.[99] Besides this full-length protein product, numerous alternatively spliced variants were also described.[100] Three functional domains were characterized in CHK2 polypeptide chain (reviewed in Bartek et al.[101]). The N-terminal SQ/TQ cluster domain (residues 20-75) is involved in regulation of CHK2 activity by ATM-mediated phosphorylation in response to genotoxic insults[102] or CHK2 dephosphorylation by oncogenic Wip1 phosphatase abrogating CHK2-mediated proapoptotic signaling.[103] The fork head-associated (FHA) domain (residues 112-175) is critically involved in dimerization of CHK2 molecules in phosphorylation-dependent manner.[104] This process has been recently shown to be necessary for full activation of CHK2 by *trans*-phosphorylation of the activation

segment/T-loop[105] within the kinase domain (residues 225-490) that carries the catalytic serine/threonine kinase activity (Figure 2).

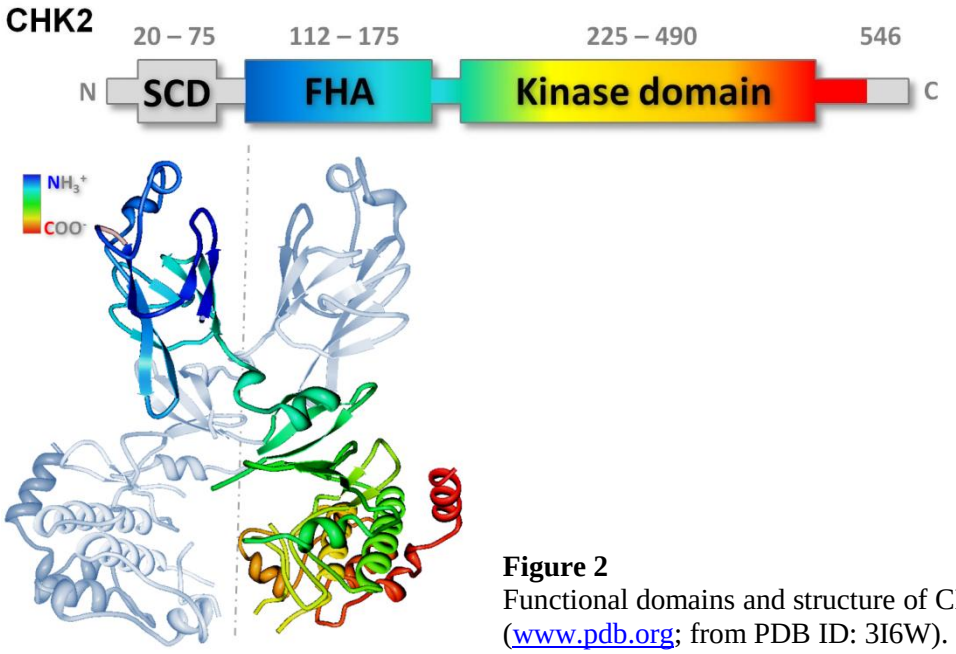

Figure 2
Functional domains and structure of CHK2 dimer (www.pdb.org; from PDB ID: 3I6W).

The initial report of Bell et al.[106] demonstrated that mutation in *CHEK2* are responsible for development of tumors in p53-negative Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) families. This work also described mutation carriers of the two most studied *CHEK2* alterations – c.1100delC (p.380fsX) and c.470T>C (I157T). Later analyses of c.1100delC mutation performed on large cohorts of cancer patients have shown that this alteration acts as a low penetrance allele increasing the risk of different cancer types including breast, colorectal, ovarian, prostate, thyroid, or kidney cancers.[107-112] Later on, numerous alterations of *CHEK2* were detected in diverse populations and distinct types of hereditary and sporadic cancers. The results of breast cancer studies led to the identification of several predominant founder mutations within the *CHEK2* gene and suggested that these mutations were unevenly distributed within the world's populations. The most frequently studied *CHEK2* mutation c.1100delC, that leads to translation of truncated protein lacking kinase domain, is highly incident in Northern and Western Europe[111] and in Russia[113] but its occurrence in Southern Europe,[114,115] South America[116] or China[117] is very low. Similar differences in distribution were found also for other *CHEK2* frequently analyzed mutations located within its FHA domain - c.470T>C (I157T) and IVS2+1G>A (fs154X).[118] Significant impact on CHK2 function is considered in both alterations. The I157T was reported to interfere with

phosphorylated-CHK2 dimerization and its interaction with downstream protein targets[100,119] and IVS2+1G>A results in aberrant splicing of mRNA and production of truncated catalytically non-functional protein.[120] Besides these alterations, many less frequent changes within FHA domain and other *CHEK2* regions were described.[120]

1.4.2. Role of *CHEK2* gene alterations in lymphomas

The risk of NHL was firstly evaluated by genotyping analysis of three *CHEK2* mutations (c.1100delC, I157T and IVS2+1G>A) in the study of Cybulski at al.[89] Reported borderline association of *CHEK2* I157T variant with a higher risk of NHL development (OR = 2.0; p = 0.05) is limited due to the small number (N = 120) of lymphoma cases. Except to this report, all other studies in NHL tumors were focused primarily on the analysis of somatic *CHEK2* mutations in malignant tissue: Hangaishi at al.[121] investigated 109 patients with hematological malignancies including seven NHL samples and found only two *CHEK2* alterations in all patients. Tavor at al.[122] screened a set of 143 hematological malignancies (54 adult T-cell leukemia, 48 NHLs, and 41 childhood acute lymphoblastic leukemia). Just one mutation in exon 11 (S428F) was found in NHL patient by complete mutation analysis of *CHEK2* gene. Tort at al.[123] studied 60 B-NHL tissue samples in which one I157T mutation was detected, however, further analysis from peripheral blood revealed that the affected patient was a carrier of this hereditary mutation. Consequent analysis of control population failed to find any carrier of I157T mutation among 104 healthy Spanish controls. The authors performed also immunohistochemical analysis of CHK2 expression that revealed loss of protein expression in 5 out of 60 NHL tumors without detection of underlying gene mutation, deletion or hypermethylation. Despite that CHK2 protein expression did not differ in diverse histological and proliferation NHL types and was similar in the reactive lymphoid tissue, two tumors with complete absence of CHK2 expression showed the highest number of chromosomal translocations and instability.

Taken together, *CHEK2* somatic mutations are probably rare in NHL tumors but germline variants may be associated with increased NHL risk. This assumption needs to be further investigated by independent studies. Contrary to NHL, the role of germline or somatic alterations of *CHEK2* gene has not been evaluated in HL so far.

1.5. *TP53* gene

1.5.1. *TP53* gene and protein function

The ***TP53*** (tumor protein p53, OMIM 191170) tumor suppressor gene located on the short arm of chromosome 17 codes for the p53 protein initiating the complex signal transduction network that plays critical role in regulation of cell cycle arrest, apoptosis, senescence and DNA repair in response to cell stress of various etiology.[124,125] Germline mutations of *TP53* could be found in majority of Li-Fraumeni families (OMIM 151623) characterized by familial aggregation of early onset tumors including sarcoma, breast carcinoma, brain tumors, adrenocortical carcinoma and leukemia/lymphoma.[126,127] Carriers of *TP53* mutations have 50% risk of cancer development before the age of 40; tumors related to the *TP53* mutations are reviewed in Palmero et al.[128] Mutations in *TP53* belongs to the most frequent genetic alteration in human cancer. The *TP53* has been extensively studied in different tumor types including lymphomas.

1.5.2. The role of *TP53* alterations in lymphomas

Somatic *TP53* mutations are commonly present in wide variety of tumor cells with different frequencies including all types of lymphomas.[129,130] For example, *TP53* mutations are present in approximately 20% of childhood B-cell NHLs,[131] 10 to 20% of diffuse large B-cell lymphomas[132,133] or 6% of follicular lymphoma cases.[134] The presence of somatic *TP53* mutations has been shown to influence clinical course of the disease. Several studies reported worse survival of patients with NHL tumors harboring *TP53* mutations (e.g. Young at al.[132] reported 1.9-fold increased risk of DLBCL specific death in *TP53* mutation carriers).[133] Similar results were published for FL where *TP53* mutations were detected in 6% of FL tumors and their carriers had shorter progression free survival (PFS) and overall survival (OS).[134] Significant difference in median OS of patients with *TP53* point mutations contrary to wild-type *TP53* was also seen in MCL (1.1 vs. 3.1 years respectively; $p = 0.003$).[67] Prevalence of mutation in lymphoid malignancies and their prognostic impact were recently reviewed by Cheung et al.[135]

Interesting results were published by Hosny at al.[136] who isolated a circulating free DNA (cfDNA) from serum of 20 NHL cases and 20 controls and found higher incidence of *TP53*

mutations in NHL patients compared to controls (30% vs. 0%). This suggests that tumor derived cfDNA could serve as a non-invasive strategy for an early detection or follow-up of NHL patients. These promising results are limited by low number of participants in this study.

The most common *TP53* polymorphism R72P (rs1042522; c.215G>C; changes arginin on the position 72 to proline) was studied as a risk and/or prognostic factor for many tumors. This polymorphism resides in the proline-rich domain of p53 protein (residues 64-92) that is important for its pro-apoptotic activity. It has been shown that p53 proteins containing Pro72 or Arg72 differ in their ability to induce apoptosis[137] or cell cycle arrest in G1 phase[138] and in p53-dependent DNA repair efficiency.[139] Association of both p53 polymorphic forms with various types of cancer was tested in large number of studies (reviewed recently by Fancisco et al.).[140] The strongest association was found for the Pro72 variant and hepatocellular carcinoma risk. The role of R72P polymorphism as a risk or prognostic factor of NHL was evaluated in several studies with contradictory results, however, it was not studied in HL patients so far.[41,141-146]

2. AIMS

The causes of malignant lymphomas are largely unknown; however, several environmental or genetic factors were shown to influence the risk of lymphoma development. Contrary to the differences in pathogenesis of lymphoproliferative malignancies, malignant lymphomas are characterized by frequent occurrence of chromosomal aberrations indicating impaired capacity of DNA repair mechanisms. This hypothesis was supported by numerous studies performed in lymphoma patients analyzing the importance of mutations and polymorphisms of genes coding for proteins involved in DNA repair processes. Results of these studies suggest that several aberrations in DNA repair genes contribute to lymphomagenesis and hence they may influence the risk of lymphoma development and disease outcome. Considering this and based on our previous work focused on analysis of germline alterations affecting the *CHEK2* gene coding for CHK2 protein - the critical kinase involved in regulation of cellular response to DNA double strand breaks - we performed mutation analysis of *CHEK2* in Czech non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma (HL) patients. Moreover, we also analyzed the importance of R72P polymorphism in the *TP53* gene coding for master regulator of genetic stability and a core substrate protein of CHK2 kinase activity for lymphoma development and course of disease.

The main tasks of our study were:

- Mutation analysis of entire *CHEK2* gene in NHL patients.
- Identification of a "hot-spot" region of *CHEK2* hereditary mutations in NHL patients and its comparison to that analyzed in solid tumors.
- Mutation analysis of "hot-spot" *CHEK2* hereditary mutation region in HL patients.
- Analysis of the importance of found *CHEK2* alterations for the risk of lymphoma development and prognosis.
- Analysis of *TP53* R72P polymorphism as a risk or prognostic factor in lymphomas.

3. MATERIALS AND METHODS

3.1. Study Populations

Lymphoma patients

The study involves 638 lymphoma cases, 340 NHL and 298 HL patients treated with first line therapy. The only enrollment criterion was histologically confirmed diagnosis of NHL or HL according to the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues.

Unselected NHL samples were collected between May 2000 and June 2008 at the First Department of Medicine – Department of Hematology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague.

HL samples were collected at three Pragues hematological departments between the years 2006 and 2010 (Dept. of Clinical Hematology, University Hospital Kralovske Vinohrady and 3rd Faculty of Medicine, Charles University, 1st Dept. of Medicine – Department of Hematology, General University Hospital and 1st Faculty of Medicine, Charles University and Dept. of Pediatric Hematology and Oncology, Motol University Hospital and 2nd Medical Faculty of Charles University).

Clinical characteristic of NHL and HL patients are displayed in Table 5 and 6. All clinical data were retrieved from the register of lymphoma patients of Czech Lymphoma Study Group (www.lymphoma.cz) and from patients' medical records.

Breast, colorectal and pancreatic cancer patients

Genetic testing was performed in a group of 673 unselected patients with sporadic breast cancer, 631 colorectal cancer patients and 270 incident pancreatic cancer patients (recruited from several oncology and gastroenterology departments throughout the Czech Republic between January 2003 and February 2009). Histologically confirmed diagnosis of breast, colorectal or pancreatic cancer was the only inclusion criterion.

Table 5 Clinical characteristics of NHL patients (N = 340).

Histological subtype	**All NHL cases**	**DLBCL**	**FL**	**MCL**	**MALT**	**B CLL/SLL**	**Other**
Number of patients N (% of all NHL)	340 (100.0)	180 (52.9)	71 (20.9)	19 (5.6)	16 (4.7)	11 (3.2)	43 (13.5)
Age at diagnosis median of years (range)	59.6 (17.4-86.4)	58.7 (20.9.-83.4)	57.3 (28.4-79.4)	63.1 (46.6-81.9)	69.9 (46.1-84.3)	65.8 (47.8-84.5)	58.7 (17.4-86.4)
Gender N (%)							
Male	187 (55.0)	101 (56.1)	36 (50.7)	14 (73.7)	7 (43.8)	8 (72.7)	21 (48.8)
Female	153 (45.0)	79 (43.9)	35 (49.3)	5 (26.3)	9 (56.2)	3 (27.3)	22 (51.2)
Clinical stage N (% of known)							
I	63 (19.4)	45 (25.7)	7 (10.1)	0	5 (31.3)	0	6 (15.8)
II	57 (17.5)	37 (21.1)	12 (17.4)	0	2 (12.5)	0	6 (15.8)
III	46 (14.2)	22 (12.6)	16 (23.2)	2 (11.8)	2 (12.5)	0	4 (10.5)
IV	159 (48.9)	71 (40.6)	34 (49.3)	15 (88.2)	7 (43.8)	11 (100.0)	22 (57.9)
IPI N (% of known)							
Low	122 (38.1)	64 (36.6)	38 (55.9)	2 (11.8)	5 (31.3)	0	13 (38.2)
Low intermediate	93 (29.1)	56 (32.0)	17 (25.0)	6 (35.3)	4 (25.0)	5 (50.0)	5 (14.7)
High intermediate	56 (17.5)	26 (14.9)	9 (13.2)	5 (29.4)	5 (31.3)	4 (40.0)	7 (20.6)
High	49 (15.3)	29 (16.6)	4 (5.9)	4 (23.5)	2 (12.5)	1 (10.0)	9 (26.5)
FLIPI N (% of known)							
Low	-	-	31 (46.3)	-	-	-	-
Intermediate	-	-	21 (31.3)	-	-	-	-
High	-	-	15 (22.4)	-	-	-	-
Bone marrow affection % of known	32.2	17.1	43.5	81.5	37.5	100.0	38.5
Elevated LDH % of known	55.6	48.6	17.6	52.9	31.5	0.3	41.7

DLBCL - diffuse large B-cell lymphoma; *FL* - follicular lymphoma; *MCL* - mantle cell lymphoma; *MALT* - mucosa-associated lymphoid tissue lymphoma; *B CLL/SLL* - chronic lymphocytic leukemia/ small lymphocytic lymphoma; *IPI* - international prognostic index; *FLIPI* - follicular lymphoma prognostic index; *LDH* - lactate dehydrogenase.

Control population

Individual control groups for consequent analyses were selected from two populations – non-cancer controls and blood donors. The subgroup of non-cancer control population consisted of randomly selected adult persons examined at the Department of Clinical Biochemistry and Laboratory Medicine, General University Hospital in Prague between January 2003 and November 2005 excluding those with primary cancer diagnosis.

Control blood donors subgroup comprised of randomly selected fully anonymized healthy individuals enrolled between April 2006 and August 2006 at the Department of Blood Transfusion of the Thomayer Faculty Hospital in Prague.

All patients and controls were of Caucasian origin from the same geographical area. All participating subjects signed informed consent with the genetic testing approved by the local ethical committees.

Table 6 Clinical characteristics of 298 HL patients.

Age at diagnosis median of years (range)	32.2 (14.0-83.7)
Gender N (%)	
Male	150 (50.3)
Female	148 (49.7)
Histological subtype N (%)	
NLPHL	14 (4.7)
NSCHL	199 (66.8)
MCCHL	69 (23.2)
Other	16 (5.4)
Clinical stage N (% of known)	
I	19 (6.5)
II	140 (48.1)
III	62 (21.3)
IV	70 (24.3)
Data not available	7

NLPHL - Nodular lymphocyte predominant Hodgkin lymphoma; *NSCHL* - Nodular sclerosis classical Hodgkin lymphoma; *MCCHL* - Mixed cellularity classical Hodgkin lymphoma.

3.2. Isolation of DNA

Genomic DNA was isolated from the whole peripheral venous blood of patients and controls by Wizard Genomic DNA Purification System (Promega), using QIAamp DNA Blood Mini Kit (Qiagen) or by automated DNA preparation system (MagNA Pure LC 2.0, Roche). Isolation was performed according to the manufacturers' instructions.

3.3. Control of quality and concentration of genetic material

Concentration and purity of isolated DNA was evaluated spectrophotometrically by measurement of absorbances at 260 and 280 nm with correction to 320 nm at spectrophotometer LAMBDA Bio (PerkinElmer) and Nanodrop (Thermo Scientific). Isolated DNA was stored at -20°C.

3.4. Mutation analysis of *CHEK2* gene

3.4.1. PCR amplification

In NHL patients, all 15 individual exons with intron-exonic boundaries were PCR-amplified in 14 fragments (exon 2 and 3 were possible to amplify in one fragment). Because of the presence of large number of pseudogenes with similar sequences to the part of *CHEK2* gene coding for exons 10-14, those exons had to be amplified by nested PCR. Fragment of *CHEK2* gene covering exon 10-14 (9214 bp long) was amplified in first step and consequently used as template for PCR amplification of individual exons 10-14.[120,147]

All primers used for mutation analysis of *CHEK2* gene are listed in Table 7. PCR conditions for all reactions are displayed in Table 8. For the amplification of first non-coding exon (denoted as exon 0) and coding exons 1-9, 50 ng of genomic DNA was used. As a template for amplification of individual exons 10-14, one μl of long-range PCR product was added.

The presence of specific PCR products with simultaneously analyzed negative controls was verified by horizontal electrophoresis in 1.5% agarose gel stained with ethidium bromide. Example of electrophoresis of amplicons covering exon 1-9 are in Figure 3 and of long fragment covering exon 10-14 in Figure 4. PCR-amplified fragments were consequently analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE3500; Transgenomic).

Only the fragment of the *CHEK2* gene covering coding sequence for the FHA domain (exons 2 and 3) and flanking intron-exonic borders that contains majority of different *CHEK2* alterations was analyzed in HL patients.

Table 7 List of primers used for mutation analysis of *CHEK2* gene. Primers were designed according to the publications of Dufault et al.[120] and Offit et al.[147]

Covered exons	Primer (sequence; 5'→3')	Source of primer	Length of amplified fragments (bp)
0*	**CHK32f** (CTAAGTTCCGCTCTCCCTTC) **CHK33r** (CTTAAGATGGGATTCGAACCAC)	Original Original	258
1	**CHK01f** (AACTCACCTTTGTTGTTGGACA) **CHK02r** (CAGAACCTTCCACCTGGTAATAC)	Dufault et al. Original	441
2+3	**CHEK11f** (TCAACAGCCCTCTGATGCATG) **CHEK15r** (ACGCCCAGCAACTTACTCATC)	Original Original	460
4	**CHK05f** (ATCAGTGATCGCCTCTTGTG) **CHK06r** (CAACACCCTGTCTCACAAAGA)	Dufault et al. Dufault et al.	365
5	**CHK07f** (TCACTGTGTCCTCTGCAAAC) **CHK08r** (TTGGGAAGTTATGAAGACGTGTTA)	Dufault et al. Dufault et al.	310
6	**CHK09f** (CTCAGGCAGCCTTGAGTCAAC) **CHK10r** (CCACCACACCTGGCCAATATTATC)	Modified Dufault et al. Modified Dufault et al.	257
7	**CHK11f** (CTTGTGGTTTTCCTCTTGGGA) **CHK12r** (GATGAGAAAGGCAAGCCTACA)	Modified Dufault et al. Dufault et al.	213
8	**CHK13f** (ATTGTCTTCTGTCCAAGTGCG) **CHK14r** (CTCTTCTGAGTTTTAATCCACGGTC)	Modified Dufault et al. Original	268
9	**CHK15f** (AAGTATCTACTGCATGAATCTGAG) **CHK16r** (ATTCGAATCTGGATAAGAGCAG)	Modified Dufault et al. Modified Dufault et al.	301
10-14	**CHK27f** (CGACGGCCAGTCTCAAGAAGAGGACTGTCTT) **CHK29r** (GCTATGACCATGCACAAAGCCCAGGTTCCATC)	Dufault et al. Dufault et al.	9214
10	**CHEK05f** (TGGCAAGTTCAACATTATTCCC) **CHEK04r** (ATTTGTGACTTCATCTAATCACCTCC)	Offit et al. Offit et al.	264
11	**CHK19f** (TGAGAATGCCACTTGATTTCTTT) **CHK20r** (GCACATACACATTTTAGCATACCA)	Dufault et al. Dufault et al.	217
12	**CHK21f** (TTTATCCTTTTCACTGTGATTTGC) **CHK22r** (CATGTCTCTCAGGCAGCAG)	Dufault et al. Dufault et al.	190
13	**CHK23f** (GGAGTTTATTATCCTTCAGACACAGC) **CHK24r** (AGCTCCTTAAGCCCAGACTACAT)	Dufault et al. Dufault et al.	182
14	**CHK30f** (CACTTTACTGGAAGCATATTGAGG) **CHK26r** (CATCAGTGACTGTGAAAAAGCAA)	Original Dufault et al.	309

* First non-coding exon of the *CHEK2* gene was denoted here as exon 0.

Table 8 Conditions for PCR amplification for *CHEK2* fragments covering all individual exons.

Exons	**Reaction volume** (µl)	**Type of enzyme**	**Amount of polymerase per reaction** (U)	**Each primer** (pmol/µl)	**Each dNTP** (mmol/µl)	**Mg** (mmol/µl)	**DMSO** (%)	**Cycle No.**	**Annealing temp.** (°C)
0	20	G	0.5	0.6	0.25	3	5	1	62-55
1	25	G	0.6	0.6	0.25	3	5	1	62-55
2+3	20	G	0.5	0.6	0.25	3	5	1	62-55
4-9*	20	G	0.5	0.6	0.25	3	5	1	62-55
10-14	15	ELT	1.125	0.3	0.5	2.75 (buffer 2)	0	2	61
10	25	G	0.6	0.6	0.25	3	5	3	54
11	20	G	0.5	0.6	0.25	3	5	3	56
12	20	G	0.5	0.6	0.25	3	5	3	55
13	20	G	0.5	0.6	0.25	3	5	3	56
14	25	G	0.6	0.6	0.25	3	5	3	56
Cycle No. 1	95°C 10 min, 15 cycles (95°C 30 sec, 62°C - 0.5°C per cycle 30 sec, 72°C 50 sec), 25 cycles (95°C 30 sec, 55°C 30 sec, 72°C 50 sec), 72°C 10 min, 95°C 10 min, 60°C 5 min, 50°C 5 min, 4°C forever								
Cycle No. 2	94°C 2 min, 10 cycles (94°C 10 sec, 61°C 30 sec, 68°C 8 min), 21 cycles (94°C 10 sec, 61°C 30 sec, 68°C 8 min + 20 sec per cycle), 68°C 7 min, 4°C forever								
Cycle No. 3	95°C 10 min, 40 cycles (95°C 30 sec, 54-56°C 30 sec, 72°C 50 sec), 72°C 10 min, 95°C 10 min, 60°C 5 min, 50°C 5 min, 4°C forever								

* PCR conditions were identical for six fragments covering individual exons 4-9; *G* - AmpliTaq Gold Polymerase (Applied Biosystems); *ELT* - Expand Long Template PCR System (Roche).

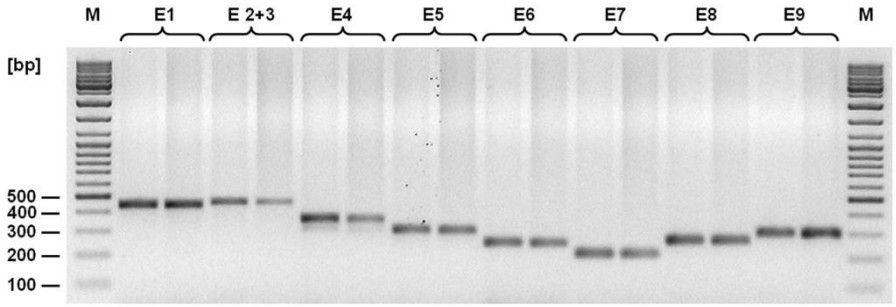

Figure 3 Agarose gel electrophoresis of PCR-amplified fragments of *CHEK2* gene covering exons 1-9 (E1-9; 1.5% agarose gel). DNA control samples. *M* - size marker.

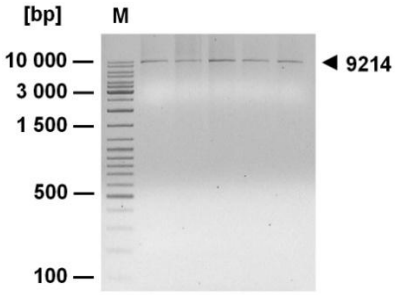

Figure 4
Agarose gel electrophoresis of PCR-amplified fragment of *CHEK2* gene covering exons 10-14 (0.7% agarose gel). Five DNA control samples. *M* - size marker.

3.4.2. Denaturing high-performance liquid chromatography

Denaturing high-performance liquid chromatography (DHPLC) enables identification of alterations by heteroduplex analysis. The presence of alteration in heterozygous form leads to the creation of heteroduplexes with mismatch during PCR amplification of individual fragments with denaturation-renaturation step at the end of amplification. Heteroduplexes thereafter exerts different elution profiles during DHPLC analysis. We have used the WAVE3500 system with computer evaluation by Navigator Software (Transgenomic). As a stationary phase served DNASep Cartridge (Transgenomic) made of alkylated nonporous poly(styren-divinylbenzene) particles that has differential affinity to the single and double-stranded DNA molecules. At the optimal temperature (slightly below the denaturation temperature of individual double-stranded DNA fragments) the mismatch in heteroduplexes causes their easier denaturation and earlier elution in the gradient of acetonitrile (Figure 5).[148] Optimal temperature of individual fragments analysis was software-predicted and verified by optimalization on control samples. Conditions of DHPLC analysis are summarized in Table 9. Samples with aberrant elution profiles on DHPLC were sequenced from independent amplifications (ABI 3130; Applied Biosystems).

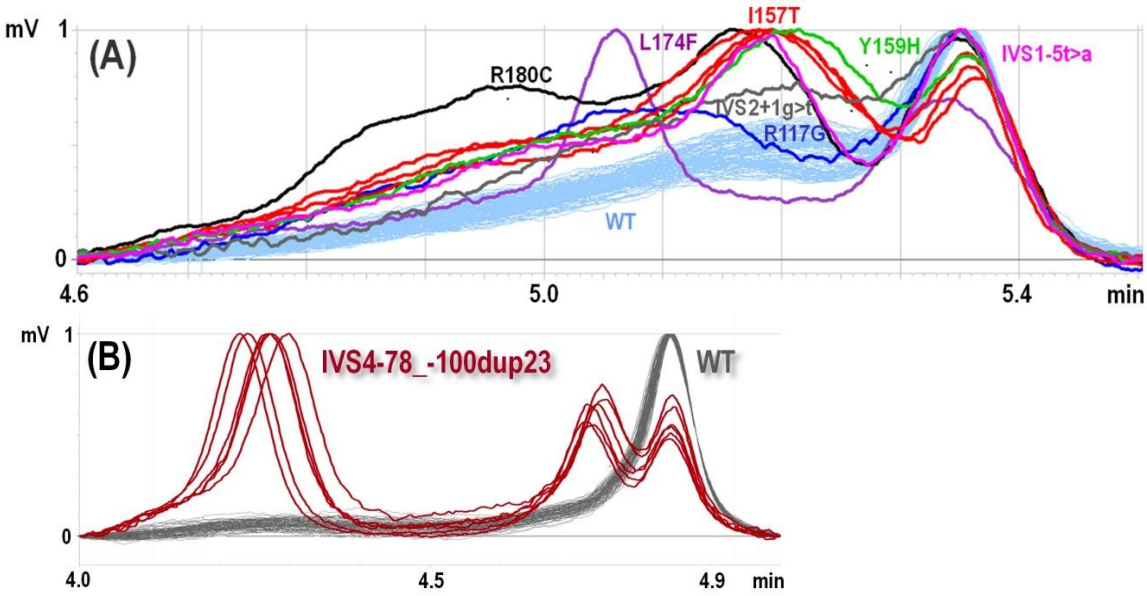

Figure 5 Examples of DHPLC elution profiles of fragments covering exon 2+3 (A) and exon 4 (B) showing good reproducibility and the strong potential of DHPLC analysis in resolution of particular sequence variants.

Table 9 Conditions of DHPLC analysis of *CHEK2* fragments covering individual exons.

Exon	Amplicon length (bp)	Temperature (°C)	Gradient of Buffer B [a] (%)
0	258	64.1	50.3 - 59.3
1	441	57.0	57.7 - 66.7
		61.0	54.7 - 63.7
2+3	460	55.4	58.0 - 67.0
4	365	53.0	56.4 - 65.4
5	310	56.5	55.0 - 64.0
6	257	53.3	53.3 - 62.3
7	213	54.5	51.4 - 60.4
8	268	58.7	53.7 - 62.7
9	301	56.4	54.8 - 63.8
10	264	56.3	53.6 - 62.6
		58.3	50.6 - 59.6
11	217	56.5	51.6 - 60.6
12	190	59.5	50.2 - 59.2
13	182	57.0	49.7 - 58.7
14	309	54.5	55.0 - 64.0
		61.5	48.2 - 57.2

[a] WAVE Optimized Buffer B (Transgenomic) contains 25% of acetonitrile.

3.4.3. Automated sequencing

Prior to the sequencing, PCR products had to be purified to remove the rest of oligonucleotides and dNTPs. Aliquots of 2 µl of PCR products were incubated at 37°C for 15 minutes with 0.8 µl of enzyme mixture ExoSAP-IT (USB Corporation) with consequent inactivation of enzymes at 80°C for 15 minutes. Sequencing reactions were performed in 5µl volumes containing 2.8 µl of mixture of PCR product + ExoSAP-IT, 0.2 µl of 40 µM primers and 2 µl of sequencing mixture BigDye *v*.3.1 (Applied Biosystems) containing all components required for sequencing reaction (including fluorescently dyed ddNTPs). Conditions of sequencing reactions were 95°C 2 min and 20 cycles of: 95°C 5 sec, 60°C 10 sec, and 72°C 4 min. Products of sequencing reaction were precipitated by adding 1.3 µl 3 M Na-acetate, 1.3 µl 0.125 M EDTA (pH 8.0) and 30 µl of 99.6% ethanol with consequent centrifugation (14 000 rpm, 20 min). Formed precipitate was thereafter washed by 60 µl of 70% ethanol with following centrifugation and discharging of the ethanol. Dried DNA precipitate was dissolved in HiDi formamid (Applied Biosystems) and denatured at 95°C for

4 minutes and immediately cooled to 4°C. Samples were analyzed by DNA sequencer ABI Prism 3130 using polymer POP-7 (Applied Biosystems). Results were evaluated in program Sequencing Analysis *v*.2.5.5 (Applied Biosystems).

3.5. Analysis of copy number variants

3.5.1. Multiplex ligation-dependent probe amplification method

The presence of inherited larger genomic rearrangements of *CHEK2* gene was analyzed by multiplex ligation-dependent probe amplification method (MLPA) that enables identification of copy number variations of individual exact sites in DNA sequence (e.g. exons). We have used SALSA MLPA kit P190 CHEK2 (MRC-Holland, www.mrc-holland.com) for the analysis of *CHEK2* gene. Specific pairs of DNA probes were designed to anneal to the specific sequences in individual exons. Initial hybridization of DNA probes with consequent ligation of specific probe pairs is followed by multiplex PCR amplification using the universal primers. Each individual fragment (representing individual exon of the *CHEK2* gene) has a specific length and is amplified proportionally to the amount of template DNA present in the sample. Amplicons of different length could be consequently separated by fragment analysis (Figure 6). The high of signal and area under curve (AUC) in individual position correlates with the amount of specific targeted sequence in the DNA. Comparison of signals in individual peaks between samples and controls enables identification of samples with lower or higher signal of specific fragment that represent loss or gain of genomic material. The MLPA enables amplification of up to 50 different fragments simultaneously in one reaction; therefore (because the *CHEK2* gene consists of only 15 exons), several other probes specific to other genes were introduced to the CHEK2 MLPA kit to utilize the full capacity of the method (probes specific to: *CHEK2* promoter region, *CHEK2* c.1100delC mutation, *PTEN*, *ATM*, *TP53*, *BRCA1/2* promoter regions, *HSC20*, and *XBP1*). In MLPA P190 CHEK2 kit, the first non-coding exon of *CHEK2* gene is numbered as the exon 1 and thus numbers of individual exons are shifted comparing to the consensus numbering (exon 1 corresponds to the exon 2 in MLPA etc.). Individual samples were processed according to the manufacturer instructions and amplified fragments separated on ABI PRISM 3130 analyzer (Applied Biosystems). Raw data were analyzed using Gene Mapper *v*.4.0 software (Applied

Biosystem) and then exported to the Coffalyser *v.*8 software (MRC-Holland) for further analysis involving normalization and statistical evaluation of individual peaks for significantly elevated or decreased signals compared to the median of all samples (Figure 6). Signals with ratio less than 0.6 or more than 1.4 compared to the median signal high in analyzed population were considered as most probably significant losses or gains of corresponding exons. All found deletions and amplification were confirmed by another independent analysis (see below).

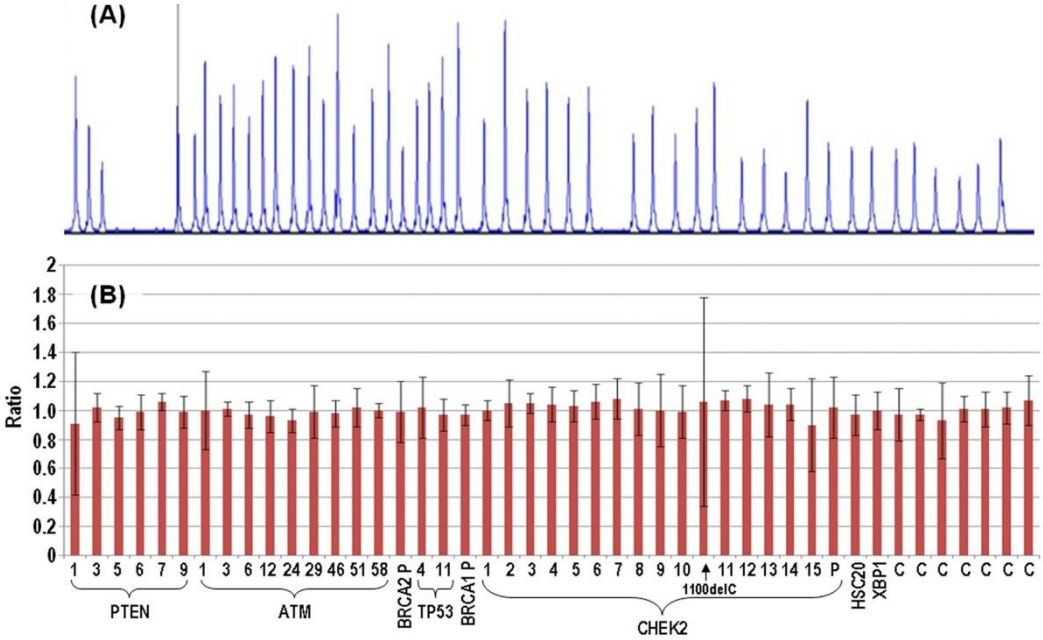

Figure 6 Example of wild type sample MLPA chromatogram (A) and the result of his Coffalyser *v.*8 software evaluation (B). *P* – promoter; *C* – control probes.

3.5.2. PCR-based confirmation of *CHEK2* large deletions

For analysis and confirmation of large deletion involving exon 8 and 9 of *CHEK2* (c.909-2028_1095+330del5395; Met304Leufs15X; Figure 7), method previously published by Walsh et al. was used with minor modifications.[118] Briefly, two primers flanking the deletion (CHEK2delUSF primer located in intron 8 and CHEK2delUSR primer located in intron 10) were used for PCR identification of 1325 bp fragment indicating the large deletion in the *CHEK2* gene. Third primer in the same PCR reaction, CHEK2delUSR2 (annealed to the

sequence in intron 7 lost in the case of deletion), amplified in pair with CHEK2delUSF primer the wild-type *CHEK2* sequence (1195 bp fragment) and served as a positive control of PCR reaction. Horizontal 1% agarose gel electrophoresis stained with ethidium bromide was used for visualization of fragments (Figure 15). Samples with deletions were verified by DNA sequencing of deletion-specific fragment with primer CHEK2delUSR using ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

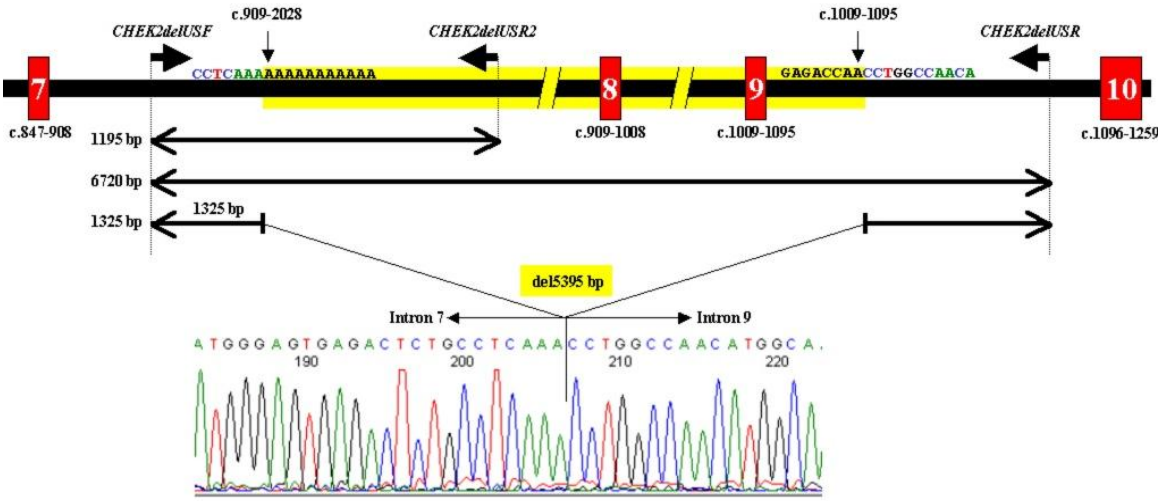

Figure 7 Schematic representation of 5395 bp deletion in *CHEK2* affecting coding exons 8 and 9. Coding exons are depicted in red boxes and their boundaries in bases (starting with c.1 in translation initiation codon) are shown below. The sequencing chromatogram shows sequence of allele with 5395 deletion with depicted deletion site.

3.5.3. Array-based comparative genomic hybridization

Array-based comparative genomic hybridization (aCGH) is a method that enables detection of copy number variations of whole genomic DNA or individual chromosomes with relatively high resolution. DNA from tested sample and from normal control sample are labeled with different fluorophores and hybridized on many thousands of probes printed on glass slide. Sites of gains or losses of genetic material could be identified by comparison of the normal control sample and tested sample signals. Many different types of aCGH chips are commercially available. We have used the NimbleGen chips targeting individual chromosomes residing of the gene of interest. Labeling and hybridization procedure was performed as recommended by manufacturer (Roche - NimbleGen) and performed in

laboratories of Central European Biosystems. The results were evaluated using software analysis in SignalMap (NimbleGen).

3.6. Genotyping of *TP53* R72P polymorphism

The exon 4 of *TP53* gene (where R72P polymorphism is located) was amplified in 363 bp long fragment. PCR reactions were performed in 25 μl reaction mixtures containing 15 pmol of each primer (P42f 5'-ACCTGGTCCTCTGACTGCTCTTTTCAC-3' and P43r 5'-GCCAGGCATTGAAGTCTCAT-3'), 2.0 mM MgSO4, 0.2 mM dNTPs (Invitec), 2% DMSO (Sigma), 0.6 U AmpliTaq Gold DNA polymerase (Life Technologies), and 50 ng of genomic DNA using touch-down PCR protocol (95˚C 10'; 13 cycles of 95˚C 30'', 68˚C - 1˚C/cycle 30'', 72˚C 1' followed by 25 cycles of 95˚C 30'', 55˚C 30'', 72˚C 1' and final extension 72˚C 10'). PCR products were consequently analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE3500; Transgenomic) at 63.9˚C in a gradient of 54.3 - 63.3% WAVE Optimized Buffer B containing 25% of acetonitrile (Transgenomic). DHPLC elution profiles in heterozygotic samples (Figure 8) were confirmed by bidirectional sequencing on ABI3130 (Applied Biosystems) using the above mentioned primers. The homozygotes were distinguished from each other by subsequent DHPLC performed under the same conditions after addition of equimolar amount of PCR amplified from wt sequence and denaturation-renaturation step. Samples of recessive homozygotes had thereafter the type of elution profile as heterozygotes.

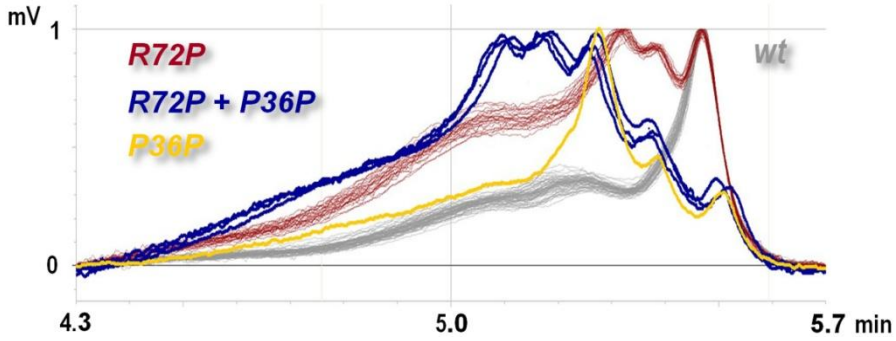

Figure 8 DHPLC elution profiles of samples analyzed for the presence of *TP53* R72P polymorphism.

3.7. Nomenclature of alterations

The nomenclature of alterations reflex guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/). The position of individual alterations is counted according to the reference sequence of *CHEK2* gene (GenBank NG_008150.1) and *TP53* gene (GenBank NG_017013.1). As a first nucleotide was considered the A of the ATG-translation initiation codon. In the *CHEK2*, predominant transcription variant was used as reference sequence (transcription variant a, isoform α, NM_007194.3). The first coding exon of *CHEK2* is designated here as exon 1 regarding to the convention in relevant literature. We have performed also the mutation analysis of the first (non-coding) exon of *CHEK2* transcript, that was designated here as exon 0.

3.8. *In silico* analyses

Biological significance of missense variants was evaluated using freely available web-based program Align-GVGD (http://agvgd.iarc.fr). For each amino acid substitution are counted two scores: Grantham Variation (GV), which express the variability of amino acids in affected position according to the multiple sequence alignment, and Grantham Deviation (GD), that reflects the biochemical difference of the mutant amino acid compared to the wt amino acid. The prediction of significance is thereafter created by combination of these two sores giving GVGD class from C0 (amino acid substitution most probably not influencing protein function) to the C65 (most likely to interfere with protein function, Figure 9).[149,150]

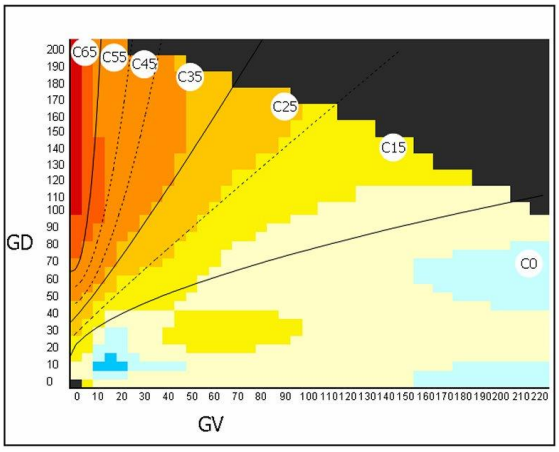

Figure 9
Align-GVGD classifiers (http://agvgd.iarc.fr). *GD* - Grantham Deviation; *GV* - Grantham Variation.

Intronic variants were evaluated using another web-based program, ESE Finder 3.0 (http://rulai.cshl.edu/tools/ESE). Besides identification of exonic splicing enhancer (ESE) motifs, ESE Finder enables to identify splice and branch sites within given DNA sequence. Estimation of possible effect of intronic variant is made by comparison of identified splice or branch sites between wt sequence and sequence with alteration.[151,152]

3.9. Statistical analyses

The two-sided Chi-square tests were used for evaluation of differences in alteration frequencies between analyzed groups. Odds ratios (OR) were calculated from 2 x 2 contingency tables. Differences in patient's clinical characteristics were tested by nonparametric Wilcoxon or Kruskal – Wallis tests or Spearman rank correlation. Survival analysis was performed using Kaplan-Meier method; differences of survival curves were evaluated by Wilcoxon and Log-rank tests, hazard ratio calculated by Cox proportional hazard model. Progression-free survival (PFS) was defined as an interval from the date of diagnosis to the date of progression, relapse or death from any cause or last follow-up date after the first line treatment. Overall survival (OS) was defined as an interval from the date of diagnosis to the date of death from any cause or last follow-up date. Analysis of survival was performed in patients whose survival data were available (numbers of analyzed patients are displayed in each survival figure). All analyses were performed using SW Statistica *v*.9.0 (StatSoft) or NCSS *v*.2007 (NCSS).

4. RESULTS

4.1. Analysis of *CHEK2* gene in lymphomas

4.1.1. Mutation analysis of *CHEK2* gene in NHL patients

Mutation analysis of the whole coding sequence of *CHEK2* gene and of the first non-coding exon was performed in 340 NHL patients in aim to evaluate their potential impact on the risk of NHL development and prognosis. Overall, 26 different alterations of *CHEK2* were identified. Frequencies of individual alterations in NHL patients and non-cancer controls are summarized in Table 10. The most frequent alterations were polymorphisms in front of the first non-coding exon (c.-2161G>A), in exon 1 (c.252A>G), intron 1 (c.319+43dupA) and intron 4 (c.684-78_-100dup23). Frequencies of these polymorphisms were similar in NHL cases as in controls (Table 10).

The *CHEK2* region coding for highly conservative FHA domain (exons 2 and 3) was shown to contain the largest number of different alterations [IVS1-5T>A, IVS2+1G>T, IVS2+24C>T, c.470T>C (I157T), c.538C>T (R180C), c.542G>A (R181H)], moreover, the frequency of alterations in FHA region was significantly higher in NHL patients (19/340; 5.6%) compared to controls (19/683; 2.8%) and associated with elevated risk of NHL development (OR = 2.1; 95% CI 1.1 – 4.0; p = 0.03). When excluding the most frequent alteration within FHA coding region (I157T), the association of the rest five alterations with increased risk of NHL was on the border of statistical significance (p = 0.053). Relatively frequent were also alterations in exon 10 an in its proximity (4/340; 1.2%), but these alterations were not associated with increased NHL risk (p = 0.09).

Alterations in other regions of *CHEK2* gene were rare (with minor allele frequency < 1%). To our knowledge, 14 of *CHEK2* alterations characterized in NHL patients were not described previously (Table 10; Figure 10). No *CHEK2* alteration or group of alterations was associated with a specific histological type of NHL; moreover, no alteration was associated with risk of any NHL subtype. Only borderline association was found for the group of all alterations within the region coding for FHA domain and increased risk of DLBCL (OR = 2.1; 95% CI 0.9 - 4.5; p = 0.07).

Table 10 List of alterations identified in NHL patients and controls with their frequencies and related odds ratios.

Exon/ intron	Alteration	Frequency in NHL N (%)	Frequency in controls N (%)	OR	95% CI	*p* value
5'UTR	c.-2161G>A heterozygotes	13/340 (3.8)	14/376 (3.7)	1.0	0.5-2.2	0.94
	c.-2161G>A homozygotes	3/340 (0.9)	0/376	-	-	-
	c.-2161G>A hetero + homozygotes	16/340 (4.7)	14/376 (3.7)	1.3	0.6-2.7	0.51
5'UTR	c.-2155A>G *	1/340 (0.3)	0/376	-	-	-
E0	c.-2075A>T *	1/340 (0.3)	0/376	-	-	-
E1	c.122C>T (S41F) *	0/340	2/376 (0.5)	-	-	-
E1	c.252A>G (E84E)	22/340 (6.5)	20/376 (5.3)	1.2	0.7-2.3	0.51
i1	IVS1+43dupA heterozygotes	67/340 (19.7)	83/376 (22.1)	0.9	0.9-1.2	0.43
	IVS1+43dupA homozygotes	8/340 (2.4)	4/376 (1.0)	2.2	0.7-7.3	0.21
	IVS1+43dupA hetero + homozygotes	75/340 (22.1)	87/376 (23.1)	0.9	0.7-1.4	0.73
i1	IVS1-5T>A	1/340 (0.3)	0/683	-	-	-
i2	IVS2+1G>T	1/340 (0.3)	0/683	-	-	-
i2	IVS2+24C>T	1/340 (0.3)	1/683 (0.2)	-	-	-
E3	c.470T>C (I157T)	14/340 (0.3)	17/683 (2.5)	1.7	0.9-3.5	0.16
E3	c.538C>T (R180C)	1/340 (0.3)	1/683 (0.2)	-	-	-
E3	c.542G>A (R181H)	1/340 (0.3)	0/683	-	-	-
i4	IVS4-78_-100dup23	23/340 (6.8)	38/376 (10.1)	0.6	0.4-1.1	0.11
i4	IVS4-74G>C *	0/340	1/376 (0.3)	-	-	-
i4	IVS4-4C>G *	0/340	1/376 (0.3)	-	-	-
E5	c.715G>A (E239K)	1/340 (0.3)	-	-	-	-
i8	IVS8+71A>G *	1/340 (0.3)	-	-	-	-
E9	c.1067C>T (S356L) *	1/340 (0.3)	-	-	-	-
E10	c.1100delC	1/340 (0.3)	2/730 (0.3)	-	-	-
E10	c.1201A>G (T401A) *	1/340 (0.3)	0/730	-	-	-
i10	IVS10+1G>C *	1/340 (0.3)	0/730	-	-	-
i10	IVS10+28A>G *	1/340 (0.3)	0/730	-	-	-
E11	c.1336A>G (N446D) *	1/340 (0.3)	-	-	-	-
E12	c.1421G>A (R474H) *	1/340 (0.3)	-	-	-	-
i13	IVS13-31delT *	1/340 (0.3)	-	-	-	-
i14	IVS14+7T>C *	1/340 (0.3)	-	-	-	-
All alterations within FHA coding region (exon 2 and 3)		19/340 (5.6)	19/683 (2.8)	2.1	1.1-4.0	**0.03**
All alterations within FHA coding region excluding I157T		5/340 (1.5)	2/683 (0.3)	5.1	0.98-26.3	0.053
All alterations within exon 10 and in its proximity		4/340 (1.2)	2/730 (0.3)	4.3	0.8-23.8	0.09

* New alterations; *OR* – odds ration; *CI* – confidence interval.

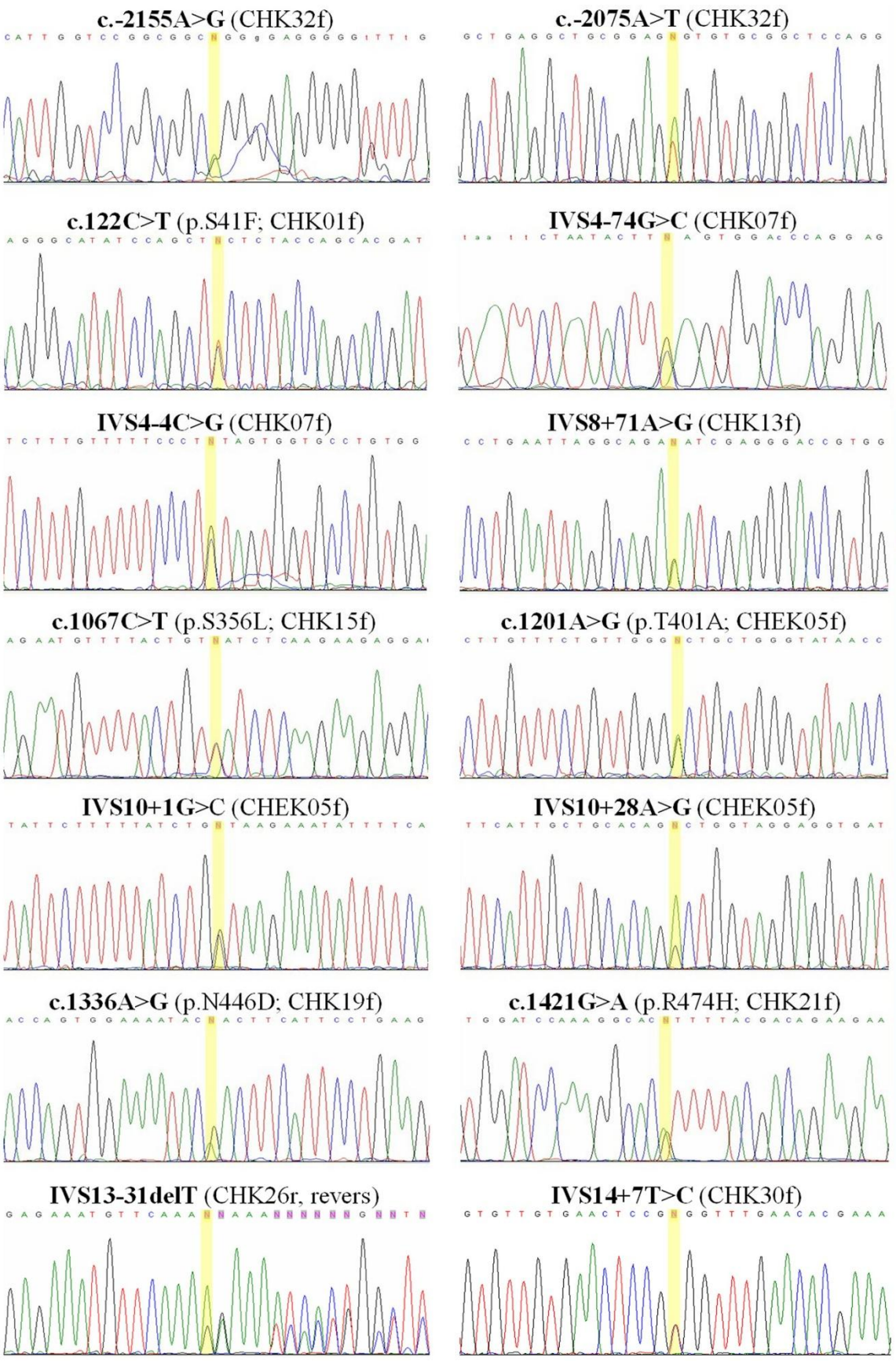

Figure 10 Sequencing chromatograms of novel alterations of *CHEK2* gene characterized in NHL patients and controls (names of primers used for sequencing are in brackets).

We have also evaluated possible correlation of individual *CHEK2* alterations with different clinical characteristics of NHL cases to further analyze the role of *CHEK2* gene and its alterations in NHL. Besides clinical characteristics listed in Table 5, number of lymph nodes areas affected by the tumor, extranodal involvement, maximum tumor diameter, and treatment outcome were evaluated. We have found that carriers of the IVS1+43dupA alteration tend to be of younger age at NHL diagnosis. The median age at diagnosis in IVS1+43dupA carriers (heterozygotes + homozygotes together) vs. all NHL patients without this alteration were 57.3 vs. 60.6 years, respectively ($p = 0.04$). This pattern was even more significant in subgroup of DLBCL patients (median age at diagnosis in alteration carriers 54.5 vs. 59.6 years in non-carriers; $p = 0.02$). In a group of all NHL patients, carriers of IVS1+43dupA in heterozygotic or homozygotic form had higher probability of bone marrow negativity (OR = 2.1; 95% CI 1.2-3.9; $p = 0.02$).

The I157T mutation was associated with worse age-adjusted international prognostic index (AA IPI) in DLBCL. The I157T carriers with DLBCL had a higher probability of high AA IPI (OR = 6.6; 95% CI 1.4-31.2; $p = 0.017$). Moreover, in patients with DLBCL, the I157T mutation was associated with an increased risk to have a higher number of lymph nodes areas affected by the tumor (OR = 9.7; 95% CI 1.8-52.2; $p = 0.008$).

All identified *CHEK2* alterations were analyzed as potential factors influencing the survival of NHL patients. Previously mentioned alteration IVS1+43dupA was associated with better OS in DLBCL patients (either in homozygous or heterozygous form, Figure 11) with $HR_{wt/wt} = 2.2$ (95% CI 1.2 – 4.0; $p = 0.015$). Alteration IVS1+43dupA was also associated with better PFS in all NHL patients ($HR_{wt/wt} = 1.7$; 95% CI 1.1 – 2.6; $p = 0.012$) and DLBCL subgroup ($HR_{wt/wt} = 2.6$; 95% CI 1.4-5.0; $p = 0.002$; Figure 12).

Contrary to the previously mentioned alteration, worse PFS was associated with I157T alteration in all NHL patients ($HR_{I157T} = 3.9$; 95% CI 1.4 – 10.9; $p = 0.008$) and less significantly in DLBCL subgroup ($HR_{I157T} = 7.7$; 95% CI 1.4 – 43.3; $p = 0.02$; Figure 13). Only borderline association was found between I157T mutation and worse OS in DLBCL subgroup ($p = 0.055$; Figure 13).

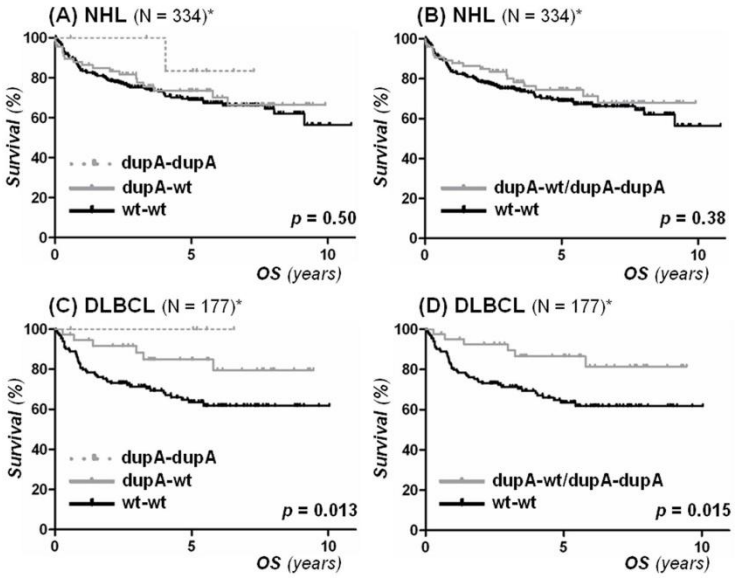

Figure 11
Overall survival (OS) curves of non-Hodgkin lymphoma patients (A, B) and patients with diffuse large B-cell lymphoma (C, D) divided according to the presence of **IVS1+43dupA** alteration (in graph legends abbreviated as dupA). Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

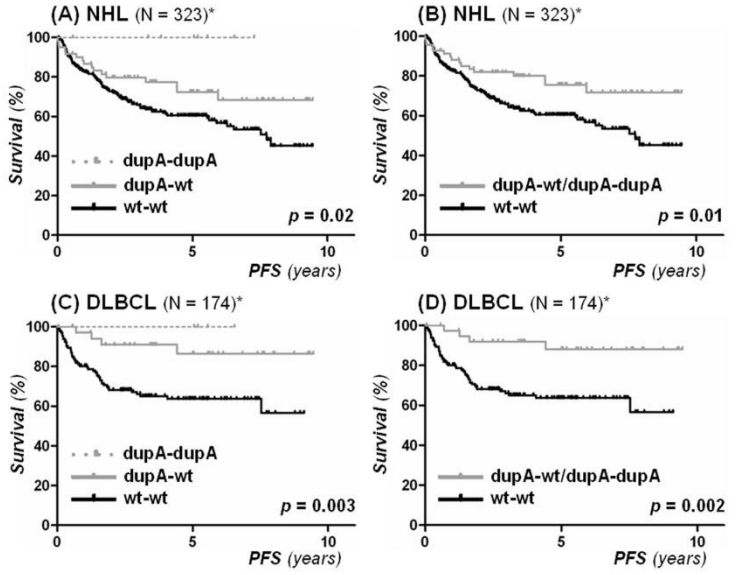

Figure 12
Progression free survival (PFS) curves of non-Hodgkin lymphoma patients (A, B) and patients with diffuse large B-cell lymphoma (C, D) divided according to the presence of **IVS1+43dupA** alteration (in graph legends abbreviated as dupA). Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

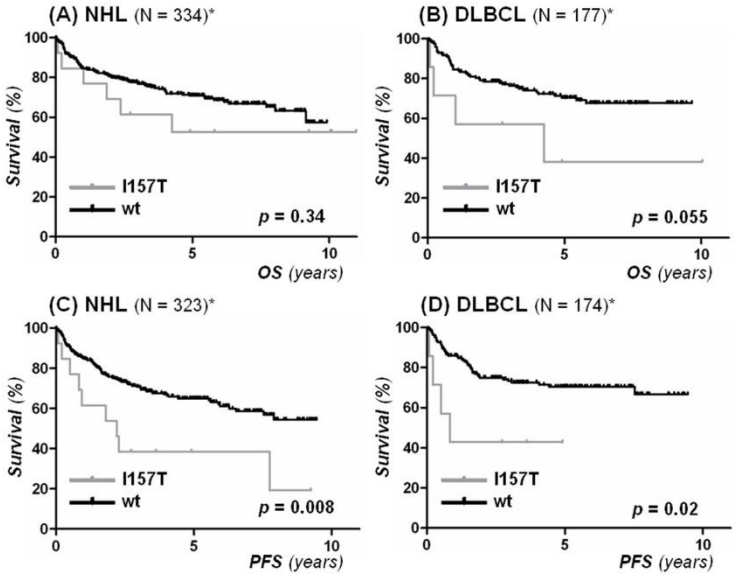

Figure 13
Overall survival (OS) and progression free survival (PFS) curves of non-Hodgkin lymphoma (NHL) patients (A, C) and patients with diffuse large B-cell lymphoma (B, D) divided according to the presence of **I157T** mutation. Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

4.1.2. Analysis of copy number alterations by *CHEK2* MLPA

The MLPA analysis of *CHEK2* gene was successfully performed in 290 NHL patients. Two samples with the large deletion of 5395 bp previously described as a Czech founder mutation in breast cancer patients[118] were identified (Figure 14).

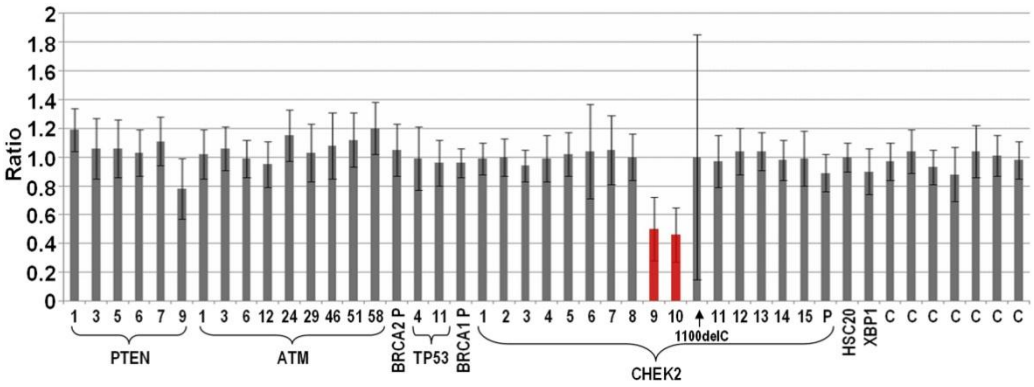

Figure 14 Result of MLPA analysis (kit P190) of L026 sample with del5395 bp of *CHEK2* (coding exons 8+9; in MLPA mix designated as 9+10).

The presence of 5395 bp deletion was confirmed in both samples by PCR based method with consequent sequencing of deletion-specific 1325 bp long fragment (Figure 15).

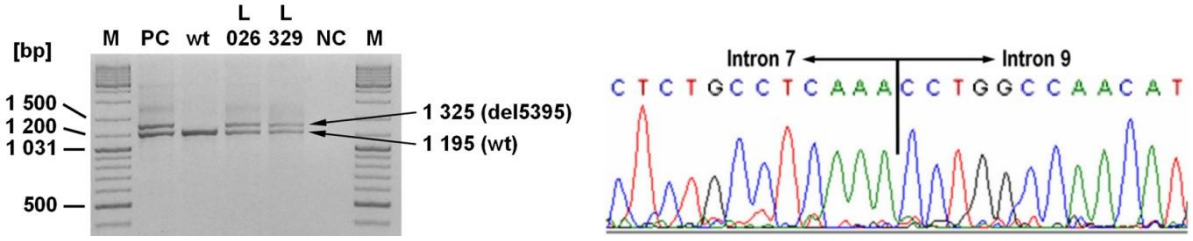

Figure 15 Agarose gel electrophoresis confirming the presence of 5395 bp deletion of *CHEK2* in two NHL samples (L026 and L329) with sequencing result. *M* - size marker; *PC* - positive control; *NC* - negative control.

Further, one sample with deletion of *CHEK2* exon 14 was identified and the presence of c.1100delC mutation of *CHEK2* found by mutation analysis in one NHL sample was confirmed (Figure 16). Surprisingly, beside above mentioned deletions, two gains within the other genes included in the CHEK2 MLPA kit were identified, amplification of genomic regions of *ATM* and *TP53* gene (Figure 16).

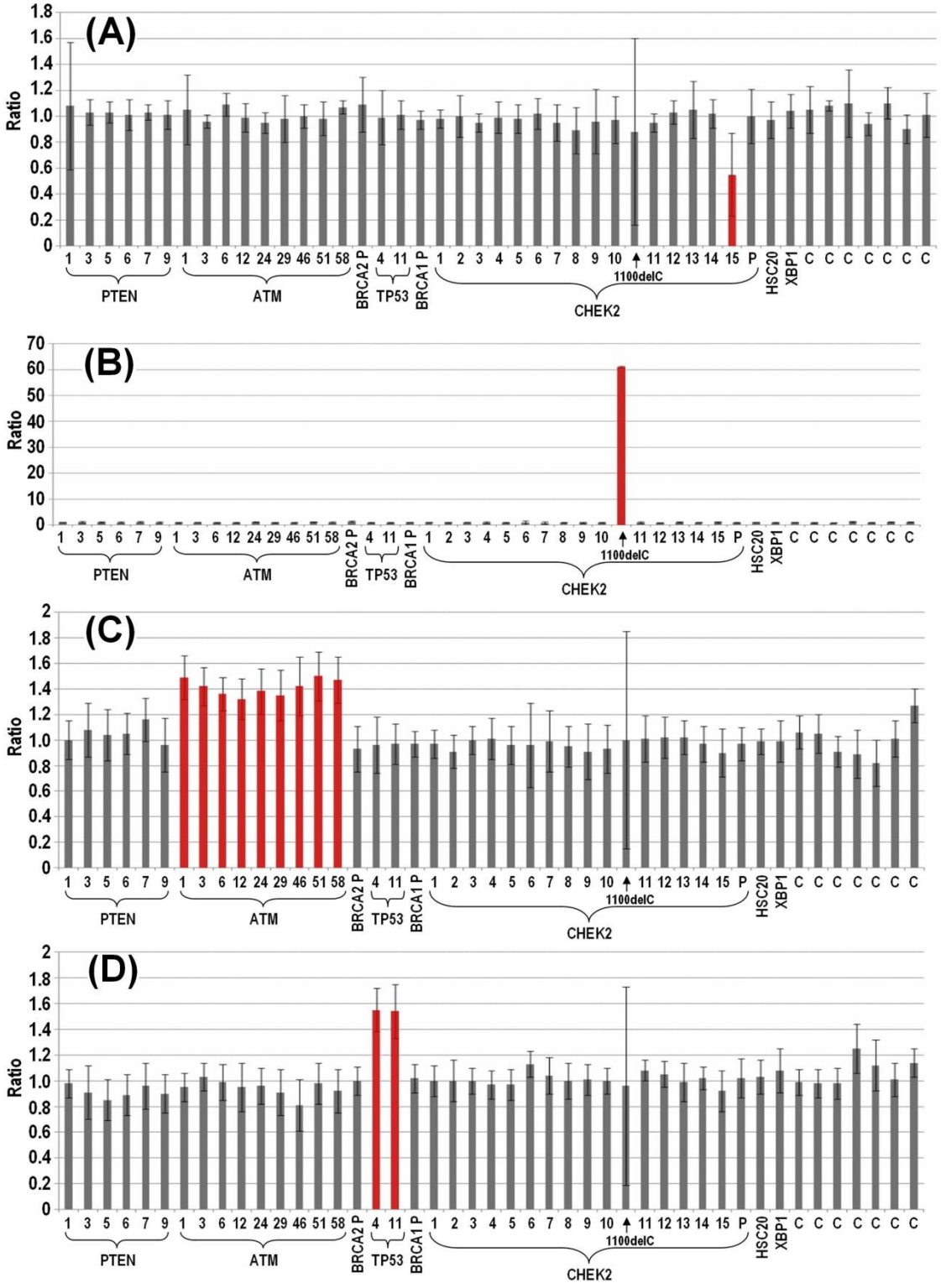

Figure 16 Results of MLPA P190 analysis of samples with *CHEK2* exon 14 deletion (A; in MLPA CHEK2 mix designated as exon 15), *CHEK2* c.1100delC mutation (B), *ATM* amplification (C), and *TP53* amplification (D).

4.1.3. Array-based comparative genomic hybridization (aCGH)

Despite the positive result of MLPA analysis of sample L296 with deletion of exon 14 of *CHEK2* gene, no copy number variant was identified by aCGH of chromosome 22 within the region of *CHEK2* gene in general or exon 14 in particular. On the other hand, aCGH of chromosome 11 and 17 confirmed amplification of large chromosome regions containing also *ATM* and *TP53* genes, respectively (Figure 17). The *ATM* gene was involved in a complex genomic rearrangement of the large parts of chromosome 11. The *TP53* gene was situated in relatively smaller amplified area (approximately 750 kb) of chromosome 17.

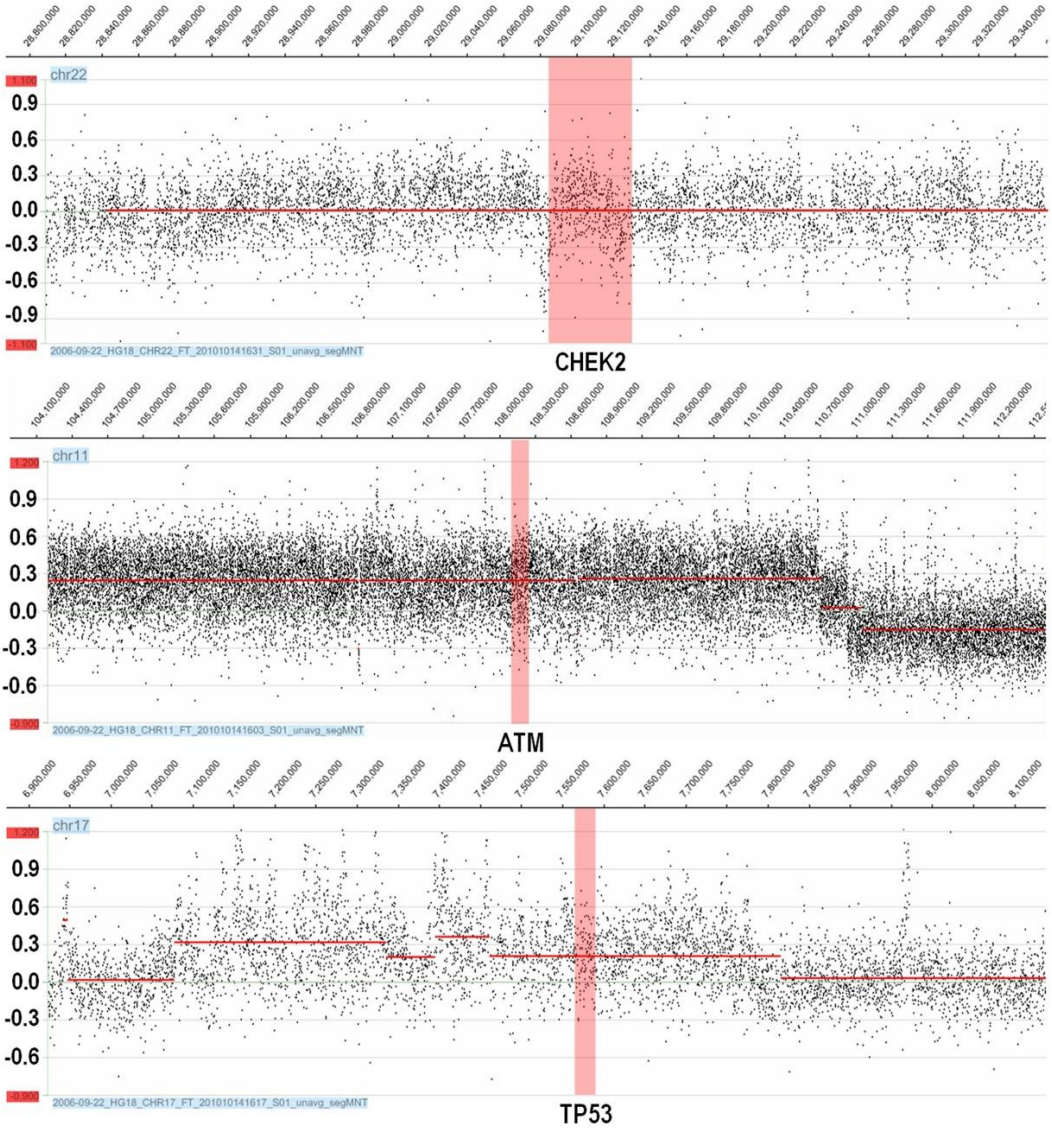

Figure 17 Results of aCGH arrays with pointed sites of *CHEK2*, *ATM* and *TP53* genes. Deviations from the zero level mean gains and losses of genetic material in the specific chromosomal position.

4.1.4. Mutation analysis of *CHEK2* FHA-coding region in HL patients

Analysis of entire *CHEK2* sequence in NHL patients revealed that the majority of alterations reside in a small portion of the gene involving coding sequence of FHA domain. Therefore, mutation analysis only of *CHEK2* FHA-coding region was performed in 298 samples of HL patients in order to evaluate the risk of HL development in *CHEK2* alteration carriers.

We ascertained six different *CHEK2* alterations localized within FHA-coding region (c.470T>C, c.475T>C, c.542G>A) or in its proximity (IVS1-5T>A, IVS2+24C>T, IVS2-54C>T; Table 11). The overall frequency of *CHEK2* alterations in the group of HL patients (5.7%) differed significantly from that characterized in non-cancer controls (2.8%; $p = 0.04$). Presence of any alteration within analyzed region was associated with an increased risk of HL development (OR = 2.11; 95% CI 1.08 - 4.13). The most frequently occurring *CHEK2* alteration – c.470T>C (I157T) – was found in 4.0% of HL patients compared to 2.5% of controls ($p = 0.22$). The frequency of other alterations (excluding the most frequent I157T mutation) was significantly higher in HL cases and associated with an increased risk of HL development (OR = 5.81; 95% CI 1.12 – 30.12; Table 11).

Table 11 Frequencies of alterations identified in *CHEK2* FHA-coding region in HL patients.

Exon/ intron	Alteration	HL patients N (%)	Controls N (%)	OR	95% CI	*p* value
-	None	281 (94.3)	664 (97.2)	Reference (1.00)		
e3	c.470T>C (I157T)	12 (4.0)	17 (2.5)	1.64	0.78-3.49	0.22
e3	c.475T>C (Y159H)	1 (0.3)	0	-	-	-
e3	c.538C>T (R180C)	0	1 (0.1)	-	-	-
e3	c.542G>A (R181H)	1 (0.3)	0	-	-	-
i1	IVS1-5T>A	1 (0.3)	0	-	-	-
i2	IVS2+24C>T	1 (0.3)	1 (0.1)	-	-	-
i2	IVS2-54C>T	1 (0.3)	0	-	-	-
All alterations within coding sequence		14 (4.7)	18 (2.6)	1.82	0.89-3.71	0.12
Alterations excluding I157T		5 (1.7)	2 (0.3)	5.81	1.12-30.12	**0.03**
All alterations		17 (5.7)	19 (2.8)	2.11	1.08-4.13	**0.04**

OR – odds ration; *CI* – confidence interval.

Progression-free survival in HL patients did not differ between *CHEK2* alteration carriers and patients without alteration in analyzed region (Figure 18). Moreover, *CHEK2* FHA alterations did not correlate with any of clinical characteristics of HL patients listed in Table 12.

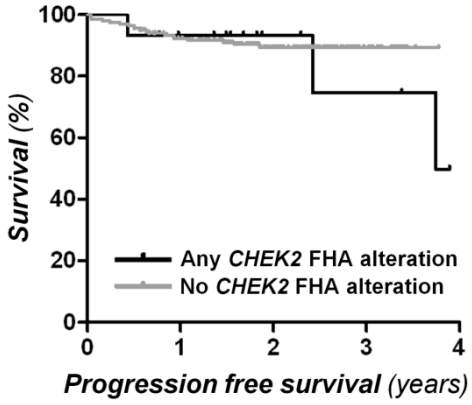

Figure 18
Progression-free survival of 215 HL patients in groups according to the presence of inherited *CHEK2* alterations. No significant difference in PFS was found (*p* values for Log-rank and Wilcoxon tests were 0.53 and 0.97, respectively).

Table 12 Clinical characteristics of HL patients with and without *CHEK2* FHA alterations.

Characteristic	Without *CHEK2* FHA alteration	Any *CHEK2* FHA alteration
Gender N (%)		
Male	141 (50.2)	9 (52.9)
Female	140 (49.8)	8 (47.1)
Age at diagnosis median of years (range)	32.6 (14.0-83.7)	31.9 (17.1-59.0)
Histologic subtype N (%)		
NLPHL	13 (4.6)	1 (5.9)
NS	188 (66.9)	11 (64.7)
MC	64 (22.8)	5 (29.4)
Other	16 (5.7)	0
Clinical stage N (% of known)		
I	18 (6.6)	1 (5.9)
II	132 (48.2)	7 (41.2)
III	57 (20.8)	6 (35.3)
IV	67 (24.5)	3 (17.6)
No data	7	0

NLPHL - Nodular lymphocyte predominant Hodgkin lymphoma; *NS* - Nodular sclerosis classical Hodgkin lymphoma; *MC* - Mixed cellularity classical Hodgkin lymphoma.

4.2. Analysis of *CHEK2* in solid tumors

Alongside to the genetic analyses of *CHEK2* gene in lymphoma patients we analyzed the *CHEK2* gene fragment coding for FHA domain and 1100delC alteration in breast (BC), colorectal (CRC) and pancreatic cancer patients.

4.2.1. Unselected breast cancer cases

We screened the fragment of *CHEK2* gene coding for FHA domain by the DHPLC in 673 unselected breast cancer patients and compared the frequencies of identified alterations with frequencies in non-cancer control group.[153] The results of analysis that identified 10 different alterations in *CHEK2* sequence are summarized in Table 13.

The most frequent alteration was the c.470C>T (I157T) mutation, however, the occurrence of this variant was similar in both groups: 19 cases (2.82%) in sporadic breast cancer patients and 17 cases (2.49%) in non-cancer controls (p = 0.71). Except for this mutation, additional four missense variants in FHA coding sequence (R117G, Y159H, T172A, and L174F) were characterized in four out of 673 breast cancer patients (0.15%) but in none in 682 controls. Transitions c.475T>C (Y159H), c.514A>G (T172A), and c.520C>T (L174F) represented novel alterations not detected previously.

Table 13 Characterized alterations in the *CHEK2* FHA-coding region in sporadic breast cancer (BC) patients.

Exon/ intron	Alteration	BC patients N (%)	Controls N (%)	OR	95% CI	*p* value
-	None	646 (96.0)	664 (97.2)	Reference (1.00)		
e2	c.349A>G (R117G)	1 (0.1)	0	-	-	-
e3	c.470T>C (I157T)	19 (2.8)	17 (2.5)	1.14	0.59-2.21	0.71
e3	c.475T>C (Y159H)*	1 (0.1)	0	-	-	-
e3	c.514A>G (T172A)*	1 (0.1)	0	-	-	-
e3	c.520C>T (L174F)*	1 (0.1)	0	-	-	-
e3	c.538C>T (R180C)	0	1 (0.1)	-	-	-
e3	c.541C>T (R181C)	1 (0.1)	0	-	-	-
i1	IVS1-5T>A*	1 (0.1)	0	-	-	-
i2	IVS2+1G>T*	1 (0.1)	0	-	-	-
i2	IVS2+24C>T*	1 (0.1)	1 (0.1)	-	-	-
All alterations within coding sequence		24 (3.6)	18 (2.6)	1.36	0.73-2.54	0.32
Alterations excluding I157T		8 (1.2)	2 (0.3)	4.1	0.9-19.36	0.053
All alterations		27 (4.0)	19 (2.8)	1.46	0.80-2.65	0.21

*Novel mutations.

We also characterized one known *CHEK2* alteration (R181C) occurring in the coding sequence flanking the C-terminal portion of FHA domain and three intronic variants (IVS1-5T>A, IVS2+1G>T, IVS2+24C>T), each detected in one of breast cancer patients.

The age of breast cancer onset was not different in carriers of *CHEK2* alterations (average 56.1 years) compared to non-carriers (average 55.2 years). Only three of 27 carriers of *CHEK2* alterations with a positive family history of breast cancer were found. No association between *CHEK2* mutations and breast cancer histological type, age of onset, or estrogen receptor expression was found.

4.2.2. Unselected colorectal cancer cases

Eight different variants (Table 14) within the *CHEK2* gene fragment containing coding sequence of FHA domain were found in 39 out of 631 CRC patients (6.2%) contrary to only two *CHEK2* alterations found within the same gene fragment in 19 out of 683 controls (2.8%). Alongside the most frequent c.470C>T (I157T) mutation, and five other alterations (R180C, R181C, IVS1-5T>A, IVS2+1G>A, IVS2+24C>T) two novel gene variants were characterized - the missense mutation c.434G>A (R145Q) and the intronic variant IVS2-54C>T. The frequency of all alterations in patients group elevated the risk of CRC more than two-fold (OR = 2.3; 95% CI 1.3-4.0; $p = 0.003$).[154]

The incidence of I157T mutation was significantly higher in unselected CRC patients - 30/631 (4.8%) than in controls [17/683 (2.5%); $p = 0.03$]. The inheritance of I157T mutation enhanced the risk of CRC two-fold (OR = 2.0; 95% CI 1.1-3.6; Table 14). The incidence of other alterations detected in the *CHEK2* gene region containing FHA domain-coding sequence was also found to differ significantly between CRC patients and controls (10/631 vs. 2/683; $p = 0.02$) and the risk of CRC associated with inheritance of these mutations was enhanced accordingly (OR = 5.6; 95% CI 1.2-25.7; Table 14). The inheritance of any *CHEK2* missense mutation within FHA coding sequence enhanced the risk of CRC more than two-fold (OR = 2.1; 95% CI 1.2-3.7; $p = 0.02$; Table 14). The presence of two *CHEK2* variants (I157T and IVS2+24T>C) was detected in one CRC patient.

Truncating mutation c.1100delC was found in four out of 631 CRC patients (0.6%). Compared to the previously analyzed control cohort[155] (2/730; 0.3%) the difference in frequency of c.1100delC was not statistically significant (OR = 2.3; 95% CI 0.4-12.8; $p = 0.4$). The average age of CRC diagnose in c.1100delC carriers was 60.5 ± 8.5 years (mean ± SD). Positive familial history of cancer was scored in one of four patients carrying c.1100delC (father with gastric cancer diagnosed at the age of 50).[154]

The large deletion of 5395 bp was not identified among 522 CRC patients.[154]

Table 14 Frequency of alterations in the *CHEK2* gene region covering coding sequence of FHA domain in CRC patients.

Exon/ intron	Alteration	CRC patients N (%)	Controls N (%)	OR	95% CI	*p* value
-	None	592 (93.8)	664 (97.2)	Reference (1.00)		
e2	c.434G>A (R145Q) *	1 (0.2)	0	-	-	-
e3	c.470T>C (I157T) *	30 (4.8)	17 (2.5)	2.0	1.1-3.6	**0.03**
e3	c.538C>T (R180C)	0	1 (0.1)	-	-	-
e3	c.541C>T (R181C)	2 (0.3)	0	-	-	-
i1	IVS1-5T>A	1 (0.2)	0	-	-	-
i2	IVS2+1G>A	2 (0.3)	0	-	-	-
i2	IVS2+24C>T	3 (0.5)	1 (0.1)	3.4	0.4-32.4	0.4
i2	IVS2-54C>T *	1 (0.2)	0	-	-	-
All alterations within coding sequence		33 (5.3)	18 (2.6)	2.1	1.2-3.7	**0.02**
Alterations excluding I157T		10 (1.6)	2 (0.3)	5.6	1.2-25.7	**0.02**
All alterations		39 (6.2)[a]	19 (2.8)	2.3	1.3-4.1	**0.003**

a) One carrier of two alterations (I157T and IVS2+24C>T) was found; * Novel alterations.

4.2.3. Pancreatic cancer cases

We analyzed the impact of *CHEK2* alterations within the FHA coding region to the development of sporadic pancreatic cancer in 270 Czech patients.[156] The c.470T>C (I157T) mutation affecting the FHA-coding domain was the most prevalent *CHEK2* alteration in pancreatic cancer cases found in six out of 269 analyzed cases (2.2%). However, this frequency was similar to that noted in controls (2.5%) resulting in non-significant association with the pancreatic cancer risk (p = 0.82; Table 15). The occurrence of other alterations within the *CHEK2* FHA domain-coding sequence or in its proximity was in our study higher among cases (4/269; 1.5%) compared to controls (2/683; 0.3%) but the association with the pancreatic cancer risk was marginally non-significant (p = 0.057; Table 15). All four intronic alterations characterized in this *CHEK2* fragment were found previously in samples of breast and/or colorectal cancer cases.

Analyses of other *CHEK2* hot-spot region in our set of pancreatic cancer patients revealed no carrier of *CHEK2* c.1100delC mutation or the large deletion of 5395 bp.[156]

Table 15 Identified alterations in sequence surrounding the *CHEK2* FHA domain in pancreatic cancer patients.

Exon/ intron	Alteration	Pancreatic cancer patients N (%)	Controls N (%)	OR	95% CI	*p* value
-	None	259 (96.3)	664 (97.2)	Reference (1.00)		
e3	c.470T>C (I157T)	6 (2.2)	17 (2.5)	0.89	0.35-2.29	0.82
e3	c.538C>T (R180C)	0	1 (0.1)	-	-	-
i1	IVS1-5T>A	1 (0.4)	0	-	-	-
i2	IVS2+1G>A	1 (0.4)	0	-	-	-
i2	IVS2+24C>T	1 (0.4)	1 (0.1)	-	-	-
i2	IVS2-54C>T	1 (0.4)	0	-	-	-
Alterations excluding I157T		4 (1.5)	2 (0.3)	5.14	0.94-28.23	0.057
All alterations		10 (3.7)	19 (2.8)	1.35	0.62-2.94	0.451

4.3. Analysis of *CHEK2* alterations in control populations

Population frequency of identified *CHEK2* alterations was established in appropriate number of non-cancer controls. *CHEK2* FHA coding region was evaluated in 683 individuals (17 I157T, one R180C and IVS2+24C>T alterations were identified, Table 10).[153] Mutation analysis of exon 10 of *CHEK2* gene (the site of c.1100delC mutation) was performed in 730 non-cancer individuals in the study of Kleibl el al.[155] (only two c.1100delC mutations were identified). The frequency of c.-2161G>A, E84E, IVS1+43dupA, and IVS4-78_-100dup23 polymorphisms was analyzed in 376 non-cancer individuals (Table 10). One carrier of *CHEK2* 5395 bp deletion was identified among 565 non-cancer cases.[154]

4.4. Analysis of *TP53* R72P polymorphism in lymphomas

Genotyping of *TP53* R72P polymorphism was performed totally in 1387 individuals. The genotype distribution of *TP53* R72P polymorphism among 638 lymphoma cases and 749 non-cancer controls did not differ significantly with similar minor allele frequencies (MAF_{Pro72}) in NHL patients, HL patients and controls (21.8%, 24.3%, and 22.4%, respectively). The genotypes coding for ArgPro, ProPro, or combined ArgPro/ProPro were not associated with the risk of lymphoma in general or NHL and HL separately (Table 16). The genotype coding for ProPro was marginally associated with a lower age at diagnosis of HL compared to ArgArg variant (p = 0.03; median age at diagnosis 29.4 vs. 32.7 years, respectively). The R72P polymorphism was not associated with any other clinical characteristics in NHL and HL patients (listed in Tables 5 and 6) and had no impact on PFS or OS (Figures 19 and 20, respectively) in all lymphoma patients and NHL, HL, DLBCL, FL subgroups. Two other *TP53* variants in exon 4 were identified [silent mutation c.108G>A (P36P) and intronic variant IVS3-9C>T]. The frequency of c.108G>A did not differ between lymphoma patients (13/638; 2.0%) and controls (22/749; 2.9%; p = 0.3). The rare IVS3-9C>T variant of uncertain significance was identified in one HL patient and two control individuals.

Table 16 Distribution of *TP53* R72P genotypes and allele frequencies in lymphoma patients and controls with related odds ratios.

Genotype	**Controls**			**All lymphoma cases**		
	N (%)			**N** (%)	**OR** (95% CI)	***p***
ArgArg	465 (62.1)			392 (61.4)	1.0	
ArgPro	233 (31.1)			199 (31.2)	1.0 (0.8-1.3)	0.95
ProPro	51 (6.8)			47 (7.4)	1.1 (0.7-1.7)	0.75
ArgPro/ProPro	284 (37.9)			246 (38.6)	1.0 (0.8-1.3)	0.82
Arg allele	1163 (77.6)			983 (77.0)	1.0	
Pro allele	335 (22.4)			293 (23.0)	1.0 (0.9-1.2)	0.72
Genotype	**NHL**			**HL**		
	N (%)	**OR** (95% CI)	***p***	**N** (%)	**OR** (95% CI)	***p***
ArgArg	218 (64.1)	1.0		174 (58.4)	1.0	
ArgPro	96 (28.2)	0.9 (0.7-1.7)	0.39	103 (34.6)	1.2 (0.9-1.6)	0.26
ProPro	26 (7.6)	1.1 (0.7-1.8)	0.80	21 (7.0)	1.1 (0.6-1.9)	0.78
ArgPro/ProPro	122 (35.9)	0.9 (0.7-1.2)	0.54	124 (41.6)	1.2 (0.9-1.5)	0.29
Arg allele	532 (78.2)	1.0		451 (75.7)	1.0	
Pro allele	148 (21.8)	1.0 (0.8-1.2)	0.78	145 (24.3)	1.12 (0.9-1.4)	0.36

OR, odds ratio; CI, confidence interval.

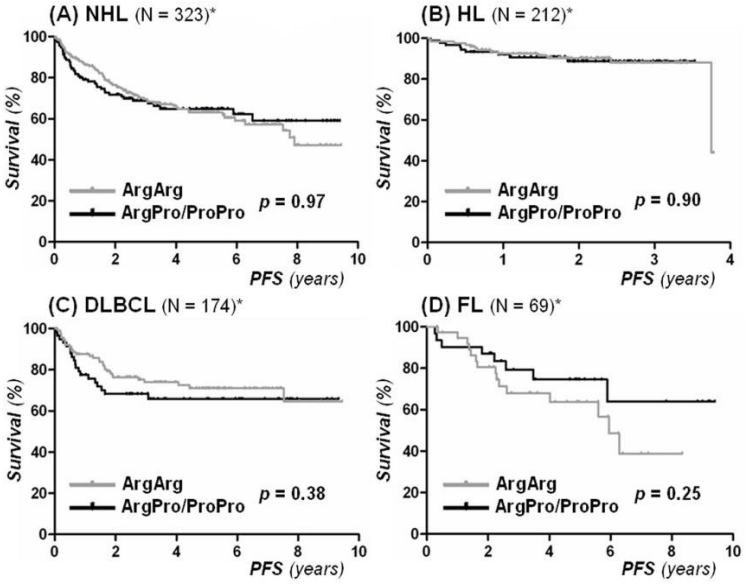

Figure 19
Progression free survival curves (PFS) of (A) non-Hodgkin lymphoma (NHL) patients, (B) Hodgkin lymphoma (HL) patients, (C) patients with diffuse large B-cell lymphoma (DLBCL) and (D) follicular lymphoma (FL) divided according to the genotype coding for R72P polymorphism. Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

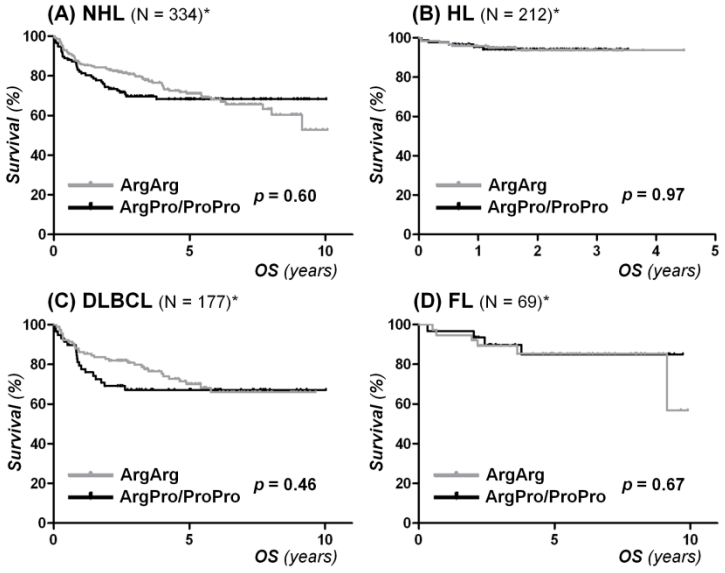

Figure 20
Overall survival curves (OS) of (A) non-Hodgkin lymphoma (NHL) patients, (B) Hodgkin lymphoma (HL) patients, (C) patients with diffuse large B-cell lymphoma (DLBCL) and (D) follicular lymphoma (FL) divided according to the genotype coding for R72P polymorphism. Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

5. DISCUSSION

Only rare cases of lymphoma could be attributable to the apparently familial form of NHL (OMIM 605027) or HL (OMIM 236000). Hence, it could be assumed that no "high"-penetrant NHL or HL-susceptibility gene is involved in lymphomagenesis and therefore the risk of lymphoma development could be influenced by various "low" or "medium" penetrant alleles. Besides many others, alterations in the *CHEK2* gene and polymorphism R72P in the *TP53* gene has been considered as the alleles that associate with genetic risk of several solid tumors, however, their association with hematooncological malignances were shown to be inconsistent, limited, or absent at all.[89]

The genotyping of the *CHEK2* gene was initiated in our laboratory by the analysis of c.1100delC alteration in breast cancer samples (and relevant controls) in 2005.[155] We described that this alteration represents a rare event with little relevance for breast cancer predisposition in clinical settings. Later we introduced the analysis of *CHEK2* genomic fragment coding for FHA domain that harbors two referred pathogenic variants IVS2+1G>A and I157T. We have shown that analyzed *CHEK2* sequence resides numerous rare and probably population-specific sequence variants and represent probable "hot-spot" mutation region of the *CHEK2* gene.[153,154] To verify this assumption, the comprehensive mutation analysis of entire coding sequence of the *CHEK2* gene in a representative population was warranted.

5.1. *CHEK2* gene alterations

5.1.1. The role of *CHEK2* gene alterations in lymphomas

We performed the largest study analyzing the entire *CHEK2* gene in lymphoma patients so far. Based on our results, we confirmed that the widest spectrum of different germline *CHEK2* alterations is clustered within the gene's fragment coding for FHA domain that represent the crucial protein module for proper activation of CHK2 kinase activity in DSB repair pathway.[104] Moreover, we found that carriers of alterations within FHA domain-coding region are at increased risk of lymphoma development. These alterations were associated with increased risk of NHL (OR = 2.1; 95% CI 1.1 - 4.0). This result is in concordance with the

only other study performed in NHL cases by Cybulski at al.,[107] who analyzed the three most studied *CHEK2* alterations (I157T and IVS2+1G>A affecting the FHA domain and c.1100delC affecting kinase domain) and reported positive association of I157T mutation with increased NHL risk (OR 2.0; $p = 0.05$). The I157T mutation was found in 11 out of 120 NHL cases (9.2%) compared to 193 out of 4000 controls (4.8%).

Contrary to the NHL, *CHEK2* gene alterations were not studied in HL lymphoma so far. However, we have identified a positive association of all alterations of the *CHEK2* FHA-coding region with a higher risk of tumor development also in HL cases (OR = 2.1; 95% CI 1.1 - 4.1). Moreover, significant association was identified in the group of all alterations within FHA-coding region excluding the most frequent I157T mutation (OR = 5.8; 95% CI 1.1 - 30.1), however, the association in this group of *CHEK2* alterations was of borderline statistical significance in NHL patients ($p = 0.053$).

Polled analysis of all lymphoma cases revealed even stronger association of alterations within *CHEK2* FHA-coding region with higher risk of lymphoma (Table 17).

Table 17 Analysis of *CHEK2* FHA alterations in pooled lymphoma group.

Alteration	**All lymphomas** N (%)	**Controls** N (%)	**OR**	**95% CI**	***p* value**
None	602 (94.4)	664 (97.2)	Reference (1.00)		
c.470T>C (I157T)	26 (4.1)	17 (2.5)	1.66	0.89-3.10	0.11
All alterations excluding I157T	10 (1.6)	2 (0.3)	5.42	1.18-24.8	**0.03**
All alterations within *CHEK2* FHA-coding region	36 (5.6)	19 (2.8)	2.11	1.19-3.68	**0.01**

The most frequent alteration of FHA-coding region identified among lymphoma patients was c.470T>C (I157T) variant. I157T mutation is localized within the conserved sequence of *CHEK2* FHA domain (localization of identified alterations of *CHEK2* FHA-coding region within the FHA domain is displayed in Figure 21). Although the Align GVGD software prediction classified this mutation to the group of variants with limited impact (Class C25), the *in vitro* functional analyses clearly showed that the mutated I157T CHK2 protein is defective in ability to bind some of its protein targets including the p53 protein[100] or the BRCA1 protein[157] *in vitro* and due to the retained dimerization capacity the I157T heterozygotes exerts impaired substrate binding *in vivo*.[158] Alongside to the previously

mentioned NHL study, the I157T mutation has been also reported to be associated with higher risk of chronic lymphocytic leukemia development (OR = 14.83; $p = 0.0008$).[159]

Alteration c.475T>C (Y159H), described recently as a novel alteration in our BC patient[153] and found thereafter in one HL case, affects also a highly conservative amino acid residue within the FHA-coding region (Align GVGD Class C65) and thus most probably influences the CHK2 protein function.

The c.542G>A (R181H) variant was identified previously in breast and prostate cancer patients from Germany[120] and the USA,[112] however, this variant most likely does not interfere with the function of the CHK2 (Align GVGD Class C0) and together with c.538C>T (R180C; Align GVGD Class C25) may represent neutral *CHEK2* sequence variant affecting non-conservative amino acid residues located in the proximity to the carboxy-terminal end of FHA domain (Figure 21).

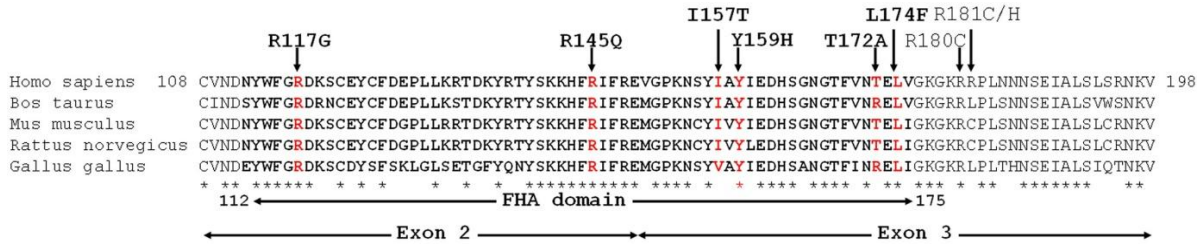

Figure 21 The localization of identified *CHEK2* gene missense mutations in analyzed cancer patients and controls within human CHK2 protein sequence and corresponding sequences of other species.

Identified intronic variants in proximity to the FHA-coding region affecting intron sequences of intron 1 and 2 were previously ascertained in sporadic breast cancer cases.[153] As this variants closely flank to the conservative 5' or 3' splicing sites, it could be assumed that they may contribute to the aberrant splicing of *CHEK2* pre-mRNA. Based on the computer prediction using ESE finder software, we deduced that the intronic variant IVS1-5T>A causes abrogation of splicing site, IVS2+24C>T may interfere with binding sites of splicing factors and that IVS2-54C>T alters the most probable branching site (Figure 22), however, these hypotheses have not been confirmed using functional *in vitro* analyses so far.

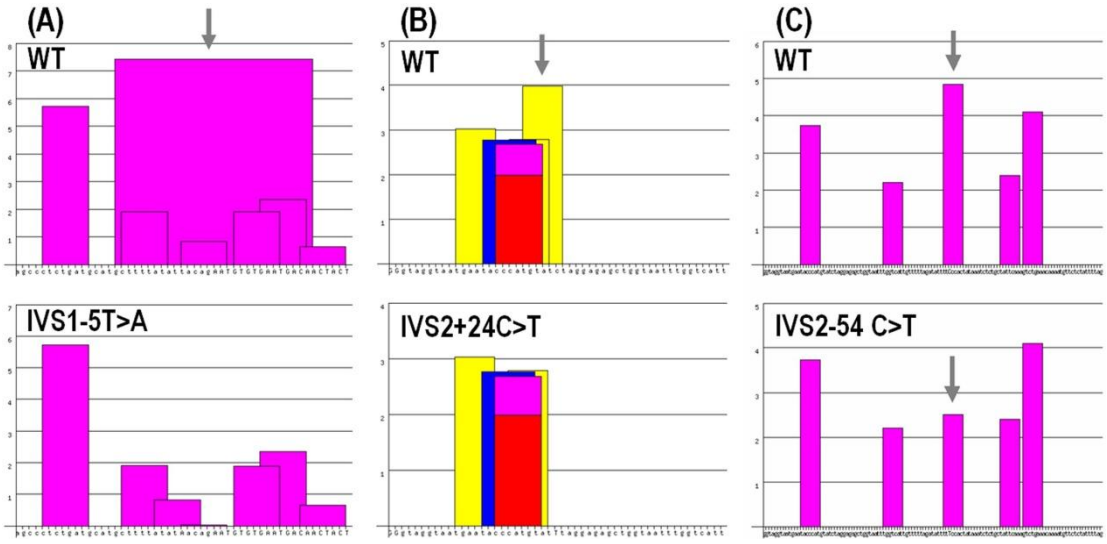

Figure 22 Prediction of the effect of selected intronic variants on CHEK2 pre-mRNA splicing (ESE Finder 3.0; http://rulai.cshl.edu/tools/ESE). (A) IVS1-5T>A and abrogation of splicing site; (B) IVS2+24C>T and splicing factors; (C) IVS2-54C>T and altered branching site.

The IVS2+1G>A transition was identified as the second most frequent frame-shifting *CHEK2* alteration in Polish,[107] German[120,158] and Belarus[158] populations and was shown to contribute to the development of breast, prostate, stomach and thyroid cancers, but this mutation was not identified among lymphoma patients (only in one CRC and one pancreatic cancer patient).[154,156] However, we have detected un-described similar alteration IVS2+1G>T previously,[153] a transversion affecting the same position at the 5' conservative splicing site. It is highly probable that this mutation may also affect the splicing of *CHEK2* pre-mRNA in a similar manner as it has been shown for IVS2+1G>A alteration.[120]

No other *CHEK2* alteration was associated with NHL risk. The most studied *CHEK2* mutation c.1100delC (leading to the translation of truncated protein product lacking kinase domain; p.fs381X) alone or in group with all alterations within the region of exon 10 was not associated with NHL risk. The occurrence of c.1100delC mutation varies substantially among different populations being highly incident in Northern and Western Europe[111] and in Russia[113] but rare in Southern Europe,[115] South America[116] or Asia.[160] Even though this alteration was reported to be associated with higher risk of breast cancer,[111] the frequency of c.1100delC in the Czech population is low (0.3%)[155] and our results indicate that this alteration unlikely contributes to the lymphoma or other cancer development in the Czech population. Within the region of exon 10, one novel interesting alteration was described -

IVS10+1G>C - which, based on the ESE finder prediction and considering the importance and conservativeness of this site, could also interfere with CHEK2 pre-mRNA splicing.

No *CHEK2* alteration was ever evaluated as a factor influencing lymphoma survival. Alterations within the *CHEK2* FHA-coding region and especially the I157T alteration were associated with a worse PFS in all NHL patients ($p = 0.008$) and also in DLBCL subgroup ($p = 0.02$). A borderline association was detected for OS in DLBCL cases. Patients with DLBCL and I157T mutation were at higher risk of high AA IPI and higher number of lymph nodes areas affected by the tumor, however the consequent statistical analysis revealed that the I157T mutation represents an independent prognostic factor in NHL patients.

Even more interesting was identification of IVS1+43dupA alteration as another independent prognostic factor associated with better OS in DLBCL ($p = 0.02$) and PFS in all NHL ($p = 0.01$) and DLBCL subgroup ($p = 0.002$). Better survival corresponded also to the number of altered alleles. This association was caused neither by younger age at diagnosis in IVS2+43dupA carriers (57.3 vs. 60.6 in non-carriers) nor by their higher probability of negative bone marrow. Distribution of the type of chemotherapy according to the intensity and treatment by rituximab was random in all groups analyzed for the differences in survival. The biological rationale how IVS1+43dupA alteration influences the NHL survival is not clear. Computer prediction (ESE finder) did not reveal interaction with splicing of *CHEK2* pre-mRNA, however, another processes influencing gene transcription (at the level of intronic splicing enhancers/silencers) or mRNA metabolism (RNA interference, stability or processing) may be involved.

5.1.2. The role of *CHEK2* gene alterations in distinct solid tumors

We have shown that alterations of *CHEK2* FHA-coding region are associated with higher risk of NHL and HL development. Even more significant association was found for unselected colorectal cancer cases (OR = 2.3; $p = 0.003$).[154] On the other hand, we did not find any association of I157T mutation neither with familiar colorectal cancer nor with patients from APC-negative familial adenomatous polyposis families.[161] Similar results were published for I157T alteration with its considerable higher frequency in CRC patients and controls by Kilpivaara et al. (7.8% and 5.3%, respectively; OR = 1.5) and Cybulski et al. (7.1% and 4.8%, respectively; OR = 1.5).[162,163] Contrary to the sporadic colorectal cancer cases, we did not

identify any association of *CHEK2* alterations with the risk of breast and pancreatic cancers.[153,156] *CHEK2* alterations were not studied in sporadic pancreatic cancer before, while studies from Germany and Belarus,[164] Finland[158] and Poland[107,165] provided the data about contribution of I157T to breast cancer development, the studies from Italy,[114] Germany,[120] UK, the Netherlands and the USA,[166] and the USA on multiethnic cohort[167] failed to find such association. On the other hand, alterations within the FHA-coding region excluding I157T were on the border of statistical significance in both, breast and pancreatic cancers in our study (p = 0.053 and 0.057, respectively). Association of alterations within *CHEK2* FHA-coding region (with or without I157T mutation) with increased cancer risk was apparent also in pooled analysis of all our cancer cases together (N = 2212, Table 18).

Table 18 Analysis of *CHEK2* FHA alterations in all cancer patients in our study (N = 2212).

Alteration	**All lymphomas** N (%)	**Controls** N (%)	**OR**	**95% CI**	***p* value**
None	2099 (94.9)	664 (97.2)	Reference (1.00)		
c.470T>C (I157T)	81 (3.7)	17 (2.5)	1.47	0.88-2.53	0.14
All alterations excluding I157T	32 (1.4)	2 (0.3)	5.0	1.20-20.92	**0.03**
All alterations within *CHEK2* FHA-coding region	112 (5.1) [a)]	19 (2.8)	1.86	1.13-3.06	**0.01**

a) One carrier of two alterations (I157T and IVS2+24C>T) was identified.

5.2. Copy number variants

Deletion of 5395 bp affecting exons 8 and 9 of the *CHEK2* gene was firstly identified by Walsh et al.[118] in the breast cancer families of the Czech origin. Association of this large deletion with higher risk of breast cancer development was confirmed by Cybulski at al. analyzing Polish breast cancer patients.[168] Association of *CHEK2* del5395 with increased cancer risk has been reported also for prostate cancer[169] but was not found in hereditary non-polyposis colorectal cancer[170] or melanoma.[171] We have identified two cases with *CHEK2* del5395 among 290 NHL patients. The low frequency of deletion did not differ between NHL patients and controls (p = 0.27). We have not identified deletion in the Czech colorectal or pancreatic cancer patients[154,156] and thus the role of this alteration could be restricted only to several other cancer types.

The two rearrangements in *TP53* and *ATM* genes incidentally detected during *CHEK2* MLPA were validated using chromosome-specific aCGH. Complex chromosomal rearrangement in sample L327 representing both gain and loss of genetic material within substantial proportion at the long arm of chromosome 11 included also the region of the *ATM* gene. Because this patient had high amount of circulating malignant lymphoma cells in peripheral blood diagnosed by a flow-cytometry at the time of blood sample collection, it could be considered that detected rearrangement is of somatic origin present in genetically unstable malignant cell population.

Amplification in the short arm of chromosome 17 affecting *TP53* gene region was detected in the patient L532 with NHL. The malignant cells were not present in peripheral blood of this patient. The genomic borders of this rearrangement were not resolved yet and future tests including spectral karyotyping or mFISH analysis will be needed.

In patient L296 we repeatedly detected deletion of exon 14 of the *CHEK2* gene using the MLPA analysis; however, further aCGH analysis of chromosome 22 did not revealed any rearrangement in the locus of *CHEK2*. Careful sequencing analysis did not show mutation within the DNA sequences serving as hybridization targets of MLPA probes. This indicates that the decrease in signal of exon 14-specific probes could not be considered as an artefact. One possibility is that the intragenic rearrangement encompasses only small proportion of genomic DNA at the edge of resolution capacity of aCGH analysis (~100 - 120 bp). Further characterization implementing amplification of genomic sequences neighborhooding the exon 14 and MLPA probes-targeted region is in preparation.

5.3. *TP53* R72P polymorphism in lymphomas

The first study evaluating R72P polymorphism in NHL patients was performed by Hishida at al.[141] who reported borderline association of R72P with increased risk of NHL in 103 Japanese patients ($OR_{ArgPro/ProPro}$ = 1.59; 95% CI 0.99-2.57). This observation was confirmed by recent study of Kim et al.[142] involving 945 Korean NHL patients (minor allele frequency - MAF = 34%) that showed increased risk of NHL in carriers of genotypes coding for ProPro (OR = 1.32; 95% CI 1.02-1.72) and ArgPro/ProPro (OR = 1.21; 95% CI 1.02-1.42). The study of Bittenbring et al.[143] involving 311 central European NHL patients found no association between R72P genotypes and the risk of NHL onset. The MAF in this study was comparable

to that in our subgroup of NHL patients (26 and 22%, respectively). In two other large-scale SNP studies involving also analysis of R72P in NHL patients from the USA and Australia[144] and the USA,[41] respectively, no correlation of R72P with NHL risk was found. The lack of prognostic impact of R72P in NHL patients was reported by Wrench et al.[145] in 226 FL patients, and Wang et al.[146] in 215 DLBCL and 192 FL patients. As resulting from forementioned studies and our results, the risk of NHL is not influenced by R72P polymorphism in the *TP53* gene in general; though, the association of R72P with NHL risk could be restricted only to individuals of Asian origin. The R72P polymorphism most probably also does not modify survival in NHL patients.

Contrary to NHL, analysis of R72P in patients with HL has not been performed so far, however, our results based on analysis of 298 HL cases indicate that this *TP53* polymorphism is unlikely to modify the HL risk and disease prognosis.

6. CONCLUSIONS

Results of ours and other aforementioned studies suggest that the clinically meaningful inherited alterations of *CHEK2* gene represent truncating alterations c.1100delC and IVS2+1G>A together with the missense variant I157T. We showed that the occurrence of known variants I157T and IVS2+1G>A affecting the FHA-coding region is accompanied by other rare-occurring (and most probably also population-specific) variants and hence this region represents a mutation hot-spot of the *CHEK2* gene. We have shown that mutations within FHA-coding region and its close neighborhood do represent cancer predisposing loci for the development of lymphoma and colorectal cancer in the Czech population that moderately increase the risk of those cancers.

Our study was the first study evaluating the potential impact of *CHEK2* germline alterations on the lymphoma survival. Especially association of IVS1+43dupA with better prognosis could be of potential clinical interest because of high frequency of this polymorphism in the Czech population (approx. 23%). However, all our results are of limited clinical use and must be verified by other large studies or meta-analyses.

The analysis of *TP53* R72P polymorphism revealed that R72P unlikely modify lymphoma risk and survival in the Czech population.

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LIST OF ABBREVIATIONS

AA IPI	-	age-adjusted international prognostic index
aCGH	-	array-based comparative genomic hybridization
ATM	-	ataxia-telangiectasia mutated
ATR	-	ataxia telangiectasia and Rad3 related
B CLL/SLL	-	chronic lymphocytic leukemia/ small lymphocytic lymphoma
BC	-	breast cancer
BER	-	base excision repair
BRCA1	-	breast cancer gene 1
BRCA2	-	breast cancer gene 2
Cdc25	-	cell division cycle 25
CI	-	confidence interval
CLL/SLL	-	chronic lymphocytic leukemia/small lymphocytic lymphoma
CRC	-	colorectal cancer
DHPLC	-	denaturing high-performance liquid chromatography
DLBCL	-	diffuse large B-cell lymphoma
DNA-PKcs	-	DNA-dependent protein kinase, catalytic subunit (XRCC7)
DSB	-	double strand break
EFS	-	event free survival
ERCC2	-	excision repair cross-complementing rodent repair deficiency, complementation group 2 (XPD)
ERCC5	-	excision repair cross-complementing rodent repair deficiency, complementation group 5 (XPG)
EXO1	-	5'-3' exonuclease and flap-endonuclease
FHA	-	forkhead-associated domain
FL	-	follicular lymphoma
FLIPI	-	follicular lymphoma international prognostic index
H2AX	-	H2A histone family, member X
HL	-	Hodgkin lymphoma
HNPCC	-	hereditary non-polyposis colorectal cancer
HR	-	homologous recombination/hazard ration
CHEK1	-	checkpoint kinase 1 gene
CHEK2	-	checkpoint kinase 2 gene
CHK1	-	checkpoint kinase 1 protein
CHK2	-	checkpoint kinase 2 protein
IPI	-	international prognostic index
LDH	-	lactate dehydrogenase
LIG4	-	ligase IV
MAF	-	minor allele frequency
MALT	-	mucosa-associated lymphoid tissue lymphoma
MCCHL	-	mixed cellularity classical Hodgkin lymphoma

MCL - mantle cell lymphoma
MGMT - O-6-methylguanine DNA methyl transferase
MLH1 - mutL homolog 1 (E. coli) gene
MLPA - multiplex ligation-dependent probe amplification method
MMR - mismatched repair
MRE11 - meiotic recombination 11 homolog A (S. cerevisiae) gene
MRN - MRE11-RAD50-NBS1 complex
MSH2 - mutS homolog 2 (E.coli) gene
MSH6 - mutS homolog 6 (E. coli) gene
MZBL - marginal-zone B-cell lymphoma
NBS1 - Nijmegen breakage syndrome gene 1
NER - nucleotide excision repair
NHEJ - non-homologous end joining
NHL - non-Hodgkin lymphoma
NLPHL - nodular lymphocyte predominant Hodgkin lymphoma
NSCHL - nodular sclerosis classical Hodgkin lymphoma
OMIM - online mendelian inheritance in men
OR - odds ratio
OS - overall survival
p53 - TP53 protein
PFS - progression free survival
PML - promyelocytic leukemia gene
PMS2 - postmeiotic segregation increased gene 2
RAG1 - recombination activating gene 1
RPA - replication protein A1
SNP - single nucleotide polymorphism
TFIIH - transcription factor 2H
TP53 - tumor protein p53 gene
WRN - Werner syndrome gene
WT - wild-type
XPA - Xeroderma pigmentosum, complementation group A
XPC - Xeroderma pigmentosum, complementation group C
XPD - Xeroderma pigmentosum, complementation group D (ERCC2)
XPG - Xeroderma pigmentosum, complementation group G (ERCC5)
XRCC1-7 - X-ray repair complementing defective repair in Chinese hamster cells 1-7
XRCC5 - X-ray repair complementing defective repair in Chinese hamster cells 5 (Ku80)
XRCC6 - X-ray repair complementing defective repair in Chinese hamster cells 6 (Ku70)
XRCC7 - X-ray repair complementing defective repair in Chinese hamster cells 7 (DNA-PKcs)

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I	**Havranek O**, Spacek M, Hubacek P, Mocikova H, Markova J, Trneny M, Kleibl Z. Alterations of *CHEK2* forkhead-associated domain increase the risk of Hodgkin lymphoma. Accepted for publication in the journal Neoplasma (IF_{2009}= 1.192)
II	**Havranek O**, Spacek M, Hubacek P, Mocikova H, Benesova K, Soucek P, Trneny M, Kleibl Z. No association between the *TP53* codon 72 polymorphism and risk or prognosis of Hodgkin and non-Hodgkin lymphoma. Minor revision re-submitted to the journal Leukemia Research (IF_{2009}= 2.358)
III	Kleibl Z, **Havranek O**, Novotny J, Kleiblova P, Soucek P, Pohlreich P. Analysis of *CHEK2* FHA domain in Czech patients with sporadic breast cancer revealed distinct rare genetic alterations. Breast Cancer Res Treat 2008; 112(1):159-64 (IF_{2008}= 5.684; first two authors contributed equally)
IV	Kleibl Z, **Havranek O**, Hlavata I, Novotny J, Sevcik J, Pohlreich P, Soucek P. The *CHEK2* gene I157T mutation and other alterations in its proximity increase the risk of sporadic colorectal cancer in the Czech population. Eur J Cancer 2009; 45:618-24 (IF_{2009}= 4.121; first two authors contributed equally)
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VI	Kleibl Z, **Havránek O**, Novotný J, Kohoutová M, Štekrová J, Matouš M. Analýza nutace c.1100delC genu *CHEK2* v populaci pacientů se sporadickým karcinomem kolorekta a familiární adenomatózní polypózou. Klinická onkologie 2007; 20:224-226

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APPENDIX I

Havranek O, Spacek M, Hubacek P, Mocikova H, Markova J, Trneny M, Kleibl Z.

Alterations of *CHEK2* forkhead-associated domain increase the risk of Hodgkin lymphoma.

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Alterations of *CHEK2* forkhead-associated domain increase the risk of Hodgkin lymphoma

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Running title: Alterations of *CHEK2* FHA domain in Hodgkin lymphoma

Abstract

Checkpoint kinase 2 gene (*CHEK2*) codes for an important mediator of DNA damage response pathway. Mutations in *CHEK2* gene increase the risk of several cancer types, however, their role in Hodgkin lymphoma (HL) has not been studied so far. The most frequent *CHEK2* alterations (including c.470T>C; p.I157T) cluster into the forkhead-associated (FHA) domain-coding region of the *CHEK2* gene. We performed mutation analysis of the *CHEK2* gene segment coding for FHA domain using denaturing high-performance liquid chromatography in 298 HL patients and analyzed the impact of characterized *CHEK2* gene variants on the risk of HL development and progression-free survival (PFS). The overall frequency of *CHEK2* alterations was significantly higher in HL patients (17/298; 5.7%) compared to previously analyzed non-cancer controls (19/683; 2.8%; $p = 0.04$). Presence of any alteration within analyzed region of the *CHEK2* gene was associated with increased risk of HL development (OR = 2.11; 95% CI = 1.08 - 4.13; $p = 0.04$). The most frequent I157T mutation was found in 4.0% of HL patients and 2.5% of controls ($p = 0.22$), however, the frequency of 5 other alterations (excluding I157T) was significantly higher in HL cases and associated with increased risk of HL development (OR = 5.81; 95% CI = 1.12 – 30.12; $p = 0.03$). PFS in HL patients did not differ between *CHEK2* mutation carriers and non-carriers. The predominant I157T mutation together with other alterations in its proximity represent moderate genetic predisposition factor increasing the risk of HL development.

Keywords

Hodgkin lymphoma; checkpoint kinase 2 gene (CHEK2, CHK2); germ-line mutation; genetic predisposition; risk assessment

Introduction

Hodgkin lymphoma (HL) is a malignant disease histologically characterized by the presence of large Hodgkin and Reed-Sternberg cells derived from B lymphocytes that constitute a minority of the cell population in affected lymphatic nodes. The annual incidence of HL in Europe is approximately 2.5 cases per 100 000 inhabitants [1]. Besides known environmental and life style risk factors (such as EBV and HIV infection, immunodeficiency or socioeconomic status), risk of HL development is modified by genetic background [2]. This hypothesis is supported by increased incidence of HL reported in monozygotic twins [3] and first degree relatives of lymphoma patients [4, 5]. The CHK2 protein (coded by *CHEK2* gene, OMIM 604373) is a member of ATM-CHK2-p53 signaling pathway activated upon recognition of DNA double-strand breaks (DSB). CHK2 is responsible for transmission and amplification of the signal from activated ATM kinase to the effector proteins involved in DNA repair, cell cycle arrest and apoptosis [6]. The CHK2 protein contains the N-terminal SQ/TQ cluster domain, the central forkhead-associated (FHA) domain and the C- terminal serine/threonine kinase domain [7]. CHK2 activation is initiated by ATM kinase-mediated phosphorylation of Thr68 that induces homodimerization of CHK2 monomers (via their FHA domains) and consequent autophosphorylation of their kinase domains [8, 9]. It has been shown that mutation of Thr68 or alterations of FHA domain impairs CHK2 dimerization and its activation [10, 11].

The *CHEK2* gene has been considered a multiorgan cancer susceptibility gene predisposing to the development of breast, colon, kidney, prostate, and thyroid cancers [12]. The vast majority of *CHEK2* mutations contributing to cancer predisposition are clustered within the fragment coding for FHA domain. The role of *CHEK2* alterations as a risk factor of HL has never been evaluated; therefore, we performed mutation analysis in the region coding for FHA domain of CHK2 in 298 HL patients and analyzed the impact of characterized *CHEK2* gene variants on the risk of HL development and on progression-free survival (PFS).

Materials and methods

Study population

Two hundred and ninety-eight patients with histologically confirmed diagnosis of HL treated with first-line treatment at three Prague's hematological departments were enrolled to this study between the years 2006 and 2010. Clinical characteristics of patients are summarized in Table 1. Control group of 683 non-cancer individuals was described in detail previously including the results of *CHEK2* mutation analysis [13, 14]. All lymphoma patients and controls were of Caucasian origin from the same geographical area of the Czech Republic. All participating subjects signed an informed consent with genetic testing approved by local ethical committees.

Mutation analysis

Genomic DNA was isolated from whole peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen) or using automated DNA preparation system (MagNA Pure LC 2.0, Roche) according to the manufacturer's instructions. Mutation analysis of FHA-coding region was performed as described previously [13]. Briefly, FHA-coding region of *CHEK2* gene (covering exon 2 and 3) was PCR-amplified in a single fragment and analyzed by denaturing high-performance liquid chromatography (DHPLC, WAVE system, Transgenomic). Samples with aberrant elution profiles on DHPLC were re-amplified and bi-directionally sequenced using ABI 3130 (Applied Biosystemes). Web-based program Align GVGD (http://agvgd.iarc.fr/) was used to predict functional relevance of found *CHEK2* missense variants [15, 16].

Statistical analysis

The two-sided Fisher's exact test was used for the evaluation of differences in alteration frequencies between analyzed groups. Crude odds ratios (OR) were calculated from 2 x 2 contingency tables using unconditional Mantel–Haenszel statistics, differences in clinical characteristics between alteration carriers and non-carriers using Chi-square test and nonparametric ANOVA. Analysis of PFS was performed by Kaplan-Meier method in a subgroup of patients (N = 215) that i) were enrolled to the study at the time of diagnosis and ii) in which the survival data were available. Differences of survival curves were evaluated by Wilcoxon and Log-rank tests. PFS was defined as the interval from the date of diagnosis to the date of progression, relapse or death from any cause or last follow-up date after the first-line treatment. The median follow-up of patients was 22.4 months. All analyses were performed using NCSS 2007 statistical program (NCSS).

Results and discussion

To evaluate the risk of HL development, the mutation analysis of *CHEK2* FHA-coding region was performed in 298 samples of HL patients. We ascertained six different *CHEK2* alterations localized within FHA-coding region (c.470T>C, c.475T>C, c.542G>A) or in its proximity (IVS1-5T>A, IVS2+24C>T, IVS2-54C>T[1]; Table 2). The overall frequency of *CHEK2* alterations in the group of HL patients (5.7%) differed significantly from that characterized previously [13] in controls (2.8%; $p = 0.04$). Presence of any alteration within analyzed region was associated with increased risk of HL development (OR = 2.11; 95% CI = 1.08 - 4.13). The most frequently occurring *CHEK2* alteration – c.470T>C (p.I157T) – was found in 4.0% of HL patients and 2.5% of controls (p=0.22). The

[1] This variant was erroneously referred to as IVS2-55C>T in our previous publications [14, 17].

frequency of other alterations (excluding the most frequent I157T mutation) was significantly higher in HL cases and associated with increased risk of HL development (OR = 5.81; 95% CI = 1.12 – 30.12; Table 2). Progression-free survival in HL patients did not differ between *CHEK2* alteration carriers and patients without alteration in analyzed region (Figure 1). Moreover, *CHEK2* FHA alterations did not correlate with any of clinical characteristic mentioned in Table 1.

Except for the c.542G>A (p.R181H) mutation, all other identified alterations were previously found in Czech breast, colorectal or pancreatic cancer patients [13, 14, 17]. The R181H was identified in breast and prostate cancer patients from Germany [18] and the USA [19], respectively, however, this variant most likely do not interfere with the function of the CHK2 (Align GVGD: Class C0) and together with c.538C>T (p.R180C - identified in one control subject) may represent neutral *CHEK2* sequence variants. Alteration c.475T>C (p.Y159H - previously described in one Czech breast cancer patient) affects highly conservative amino acid residue within the FHA-coding region (Align GVGD: Class C65) potentially influencing protein function [13]. Based on the computer prediction made in our previous studies, we deduced that intronic variants IVS1-5T>A and IVS2+24C>T may interfere with binding sites of splicing factors [13] and that IVS2-54C>T alters the most probable branching site [14], which both could lead to the aberrant splicing of *CHEK2* mRNA, however, these hypotheses have not been confirmed using functional *in vitro* analyses so far. The most frequent c.470T>C (p.I157T) variant is localized within the conserved sequence of *CHEK2* FHA domain. Despite the fact that based on Align GVGD this mutation belongs to the group of variants with limited impact (Class C25), the functional analyses clearly shown that mutated I157T CHK2 protein is defective in ability to bind some of its protein targets including p53 [20] or BRCA1 [21] proteins *in vitro* and due to the retained dimerization capacity the I157T heterozygotes exerts impaired substrate binding *in vivo* [22]. Alongside other previously mentioned solid cancers, the I157T mutation has been also associated with several lymphoproliferative diseases. Rudd et al. [23] described higher risk of chronic lymphocytic leukemia development (OR = 14.83; $p = 0.0008$) in carriers of I157T and Cybulski et al. [12] found increased frequency of I157T in non-Hodgkin lymphoma patients (OR = 2.0; $p = 0.05$).

This is the first study evaluating the potential impact of *CHEK2* I157T and other alterations localized within FHA domain or in its proximity on the risk of HL development. Though we are aware of limited power of our study due to the small number of tested patients, we showed that mutations in this *CHEK2* region represent mild but significant genetic factor increasing the risk of HL in mutation carriers. These results extend our previous observations from studies in breast and colorectal cancer patients that *CHEK2* FHA domain-coding region is affected by numerous distinct rarely-occurring alterations that together with predominant I157T mutation contribute to increased risk of many solid tumors and also at least several lymphoproliferative malignancies, including HL. However, further evaluation of *CHEK2* alterations in HL patients by larger studies is needed.

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Table 1 Clinical characteristics of HL patients (n = 298)

Gender N (%)	
Male	150 (50.3)
Female	148 (49.7)
Age at diagnosis median of years (range)	32.2 (14.0-83.7)
Histological subtype N (%)	
NLPHL	14 (4.7)
NS	199 (66.8)
MC	69 (23.2)
Other	16 (5.4)
Clinical stage N (% of known)	
I	19 (6.5)
II	140 (48.1)
III	62 (21.3)
IV	70 (24.3)
Unknown	7

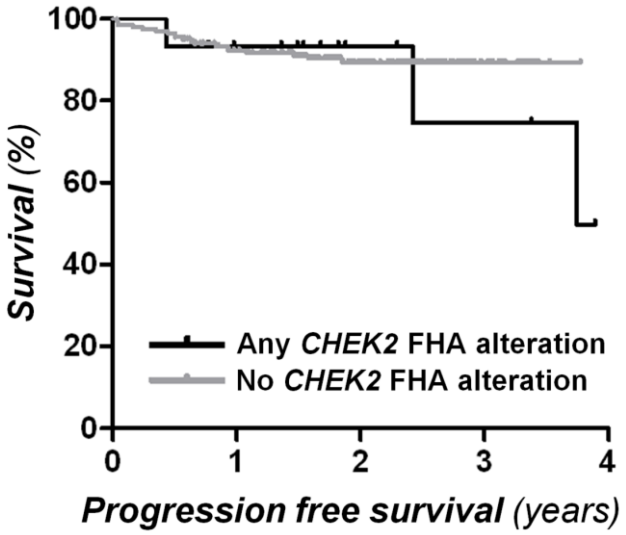
NLPHL - Nodular lymphocyte predominant Hodgkin lymphoma; *NS* - Nodular sclerosis classical Hodgkin lymphoma; *MC* - Mixed cellularity classical Hodgkin lymphoma

Table 2 Frequencies of alterations identified in *CHEK2* FHA-coding region

Exon/ intron	Alteration	HL patients N (%)	Controls N (%)	OR [a]	95% CI [a]	*p* value [a]
-	None	281 (94.3)	664 (97.2)	Reference (1.00)		
e3	c.470T>C (p.I157T)	12 (4.0)	17 (2.5)	1.64	0.78-3.49	0.22
e3	c.475T>C (p.Y159H)	1 (0.3)	0	-	-	-
e3	c.538C>T (p.R180C)	0	1 (0.1)	-	-	-
e3	c.542G>A (p.R181H)	1 (0.3)	0	-	-	-
i1	IVS1-5T>A	1 (0.3)	0	-	-	-
i2	IVS2+24C>T	1 (0.3)	1 (0.1)	-	-	-
i2	IVS2-54C>T [b]	1 (0.3)	0	-	-	-
All alterations within coding sequence		14 (4.7)	18 (2.6)	1.82	0.89-3.71	0.12
Alterations excluding I157T		5 (1.7)	2 (0.3)	5.81	1.12-30.12	0.03
All alterations		17 (5.7)	19 (2.8)	2.11	1.08-4.13	0.04

[a] Common odds ratio (OR) estimate with 95% confidence interval (CI) and significance p by 2-sided Fisher's Exact Test; [b] This variant was erroneously referred to as IVS2-55C>T in our previous publications [14, 17].

Figure 1 Progression-free survival of HL patients in groups according to the presence of inherited *CHEK2* alterations. No significant difference in PFS was found (*p* values for Log-rank and Wilcoxon test were 0.53 and 0.97, respectively).



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Dr.O.Havranek

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Praha

Oznamujeme Vám, že Váš rukopis "Alterations of *CHEK2* forkhead-associated domain increase the risk of Hodgkin lymphoma" bude uverejnený v č. 5/2011 Neoplasmy.

S pozdravom,

Dr.V.Ujházy
hl.redaktor

APPENDIX II

Havranek O, Spacek M, Hubacek P, Mocikova H, Benesova K, Soucek P, Trneny M, Kleibl Z.

No association between the *TP53* codon 72 polymorphism and risk or prognosis of Hodgkin and non-Hodgkin lymphoma.

Minor revision re-submitted to the journal Leukemia Research **(IF_{2009}= 2.358)**

No association between the *TP53* codon 72 polymorphism and risk or prognosis of Hodgkin and non-Hodgkin lymphoma

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Abstract

The role of the *TP53* gene's R72P polymorphism in non-Hodgkin lymphoma (NHL) patients has been analyzed in several studies but it has not been studied in Hodgkin lymphoma (HL) patients. We have evaluated the role of R72P in 340 NHL and 298 HL patients. There was no difference in the R72P frequency between analyzed lymphoma patients and 749 controls. We found no association of R72P with the risk of NHL and HL development [$OR_{ArgPro/ProPro}$ = 0.9 (95%CI 0.7-1.2) and 1.2 (95%CI 0.9-1.5)] or with survival. Our results support the evidence that R72P is not a prognostic factor in Caucasian NHL patients, and they indicate its irrelevance for HL development or prognosis.

Keywords

TP53 gene (*p53*), Arg72Pro polymorphism (R72P), non-Hodgkin lymphoma, Hodgkin lymphoma, Genetic predisposition, Prognosis

1. Introduction

The *TP53* (OMIM 191170) tumor suppressor gene located on the short arm of chromosome 17 codes for the p53 protein that plays a critical role in the complex signal transduction network regulating the cell-cycle arrest, apoptosis, senescence and DNA repair in response to cellular stress of various etiology [1]. Germ-line mutations in *TP53* are responsible for the Li-Fraumeni syndrome 1 (MIM ID #151623), a familial cancer syndrome characterized by an early onset of tumors including sarcoma, leukemia/lymphoma, and breast, brain or adrenocortical tumors [2]. Besides the inherited alterations, somatic mutations in the *TP53* gene are commonly present in a wide variety of cancer types (http://www-p53.iarc.fr/). The frequency of *TP53* mutations is lower in hematological malignancies compared to non-hematological tumors; however, the negative influence of *TP53* mutations on the clinical outcome in lymphoid tumors has been well documented (reviewed in [3]). The prognostic value of the common *TP53* polymorphism - rs1042522 (c.215G>C) - located in exon 4, which changes arginine 72 to proline (R72P) has been analyzed in numerous cancer types, and the prognostic effect of proline-coding genotypes (c.215GC or c.215CC) has been reported only in several of them (reviewed in [4]). Several studies in non-Hodgkin lymphoma (NHL) patients have been published with contradictory results [5-11] but the role of R72P in Hodgkin lymphoma (HL) patients has not been studied so far. Therefore, we performed a

study evaluating the effect of the *TP53* R72P polymorphism on lymphoma risk and survival in Czech NHL and HL patients.

2. Materials and Methods

2.1. Study population

The study involved 638 lymphoma cases including 340 NHL and 298 HL patients treated with first line therapy (Table 1). Histologically confirmed diagnosis of NHL or HL according to the WHO Classification was the only study enrollment criterion. Samples were collected at three hematological departments in Prague between 2000 and 2010. Population frequency of R72P was estimated by analysis of 749 samples of non-cancer individuals. Their characteristics and recruitment were described previously [12]. All cases and controls were of Caucasian origin from the same geographical area in the Czech Republic. All subjects signed informed consent with the participation in the study approved by the local ethical committees.

2.2. Genotyping

Genomic DNA was isolated from whole peripheral blood using standard procedures. The amplicon covering exon 4 of the *TP53* gene was PCR amplified in 25 μl reaction containing 15 pmol of each primer (P42f 5'-ACCTGGTCCTCTGACTGCTCTTTTCAC-3' and P43r 5'-GCCAGGCATTGAAGTCTCAT-3'), 2.0 mM $MgSO_4$, 0.2 mM dNTPs (Invitek), 2% DMSO (Sigma), 0.6 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 50 ng of genomic DNA using touch-down PCR protocol (95˚C 10 min; 13 cycles of 95˚C for 30 sec, 68˚C - 1˚C/cycle for 30 sec, 72˚C for 1 min followed by 25 cycles of 95˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min and final extension 72˚C for 10 min). The PCR products were consequently analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE3500; Transgenomic) at 63.9˚C in a gradient of 54.3 - 63.3% WAVE Optimized Buffer B containing 25% of acetonitrile (Transgenomic). The DHPLC elution profiles in heterozygotic samples were confirmed by sequencing on ABI3130 using BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems). The homozygotes were distinguished from each other by subsequent DHPLC performed under the same conditions after addition of equimolar amount of PCR product amplified from wild-type sequence and denaturation-renaturation step.

2.3. Statistical analysis

The two-sided Chi-square test was used for the evaluation of differences in alterations frequencies between analyzed groups. Odds ratios (OR) were calculated from 2 x 2 contingency tables. Differences in patients' clinical characteristics were tested by the nonparametric Wilcoxon or Kruskal-Wallis tests. Survival analysis (in patients with available data, Fig. 1) was performed using the Kaplan-Meier method; differences of survival curves were evaluated by the Wilcoxon and Log-rank tests. Progression-free survival (PFS) was defined as an interval measured from the date of diagnosis until the date of progression, relapse or death from any cause or until the last follow-up date after the first line treatment. Overall survival (OS) was defined as an interval from the date of diagnosis until the date of death from any cause or until the last follow-up date. The median follow-up of NHL and HL patients was 45.3 and 23.1 months, respectively. All analyses were performed using the SW Statistica 9.0 (StatSoft).

3. Results and discussion

The genotyping of the *TP53* R72P polymorphism was performed on 1387 individuals. The genotype distribution of R72P among 638 lymphoma cases and 749 non-cancer controls did not differ significantly, with similar minor allele frequencies (MAF_{Pro72}) in NHL patients, HL patients and controls (21.8%, 24.3%, and 22.4%, respectively). The genotypes coding for ArgPro, ProPro, or combined ArgPro/ProPro were not associated with the risk of lymphoma in general or NHL and HL separately (Table 2). The genotype coding for ProPro was marginally associated with a lower age at the HL diagnosis compared to the ArgArg variant ($p = 0.03$; median age at diagnosis 29.4 vs. 32.7 years, respectively). The R72P polymorphism was not associated with any other clinical characteristics (listed in Table 1) in NHL and HL patients and had no impact on PFS (data not shown) or OS (Fig. 1) in all lymphoma patients and in NHL, HL, diffuse large B-cell lymphoma (DLBCL), and follicular lymphoma (FL) subgroups in general. Despite the lack of statistical significance, the analysis of OS in DLBCL patients suggests that the Pro allele may be associated with an early survival disadvantage. A worse OS in DLBCL patients has recently been shown for carriers of somatic *TP53* mutations affecting the codons of distinct structurally significant regions in the p53 protein [13]. However, careful meta-analyses and further independent studies will be

necessary to evaluate the prognostic parameters of the Pro 72 allele in DLBCL patients and its potential value in the identification of those patients requiring distinct and more aggressive treatment approaches. Two other *TP53* variants in exon 4 were identified [silent mutation c.108G>A (p.P36P) and intronic variant IVS3-9C>T]. There was no difference in the frequency of c.108G>A between lymphoma patients (13/638; 2.0%) and controls (in 22/749; 2.9%; $p = 0.3$). The rare IVS3-9C>T variant of unknown significance was identified in one HL patient and two control individuals.

The first study evaluating the R72P polymorphism in NHL patients was performed by Hishida et al. [5], who reported a borderline association of R72P with an increased risk of NHL in 103 Japanese patients ($OR_{ArgPro/ProPro} = 1.59$; 95% CI 0.99-2.57). This observation was confirmed by a recent study of Kim et al. [6] involving 945 Korean NHL patients (MAF = 34%) that showed an increased risk of NHL in the carriers of genotypes coding for ProPro (OR = 1.32; 95% CI 1.02-1.72) and ArgPro/ProPro (OR = 1.21; 95% CI 1.02-1.42). A study of Bittenbring et al. [7] involving 311 central European NHL patients found no association between R72P genotypes and the risk of NHL onset. The MAF in this study was comparable to that in our subgroup of NHL patients (26 and 22%, respectively). Two other large-scale SNP studies involving also an analysis of R72P in NHL patients from the USA and Australia [8] and the USA alone [9], respectively, found no correlation of R72P with NHL risk. The lack of the prognostic impact of R72P in NHL patients was reported by Wrench et al. [10] in 226 FL patients, and Wang et al. [11] in 215 DLBCL and 192 FL patients. The above-mentioned studies and our results suggest the risk of NHL is not influenced by the R72P polymorphism in the *TP53* gene in general; however, the association of R72P with NHL risk could be restricted only to individuals of Asian origin. It has been shown that the association between the R72P polymorphism and cancer risk is also modified by ethnicity in various other cancer types, e.g. hepatocellular carcinoma or gastric and lung cancers [4].

Contrary to NHL, an analysis of R72P in patients with HL has not been performed so far, but our results based on an analysis of 298 HL cases indicate that this *TP53* polymorphism is unlikely to modify the HL risk and disease prognosis.

Acknowledgements

We thank to Dr. Drahomira Springer for providing the control blood samples, and hematologists and their patients for their collaboration. The special thanks belong to Ing.

Jaroslava Hajkova for great help with isolation of DNA samples, to Mrs Helena Achylisova for sample handling and to Ing. Stanislav Kormunda for help with statistical analyses.

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Authors' contributions

OH, ZK and MT provided study design; MS, PH, HM, KB and PS provided samples and patient data collection; OH provided the genotyping and statistical analyses; ZK and OH provided the drafting the manuscript and all authors approved final version submitted.

Conflict of interest

The authors declare no conflict of interest.

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Table 1 Clinical characteristics of lymphoma patients (n = 638).

Histological type	NHL	HL
Gender N (%)		
Male	187 (55.0)	150 (50.3)
Female	153 (45.0)	148 (49.7)
Age at diagnosis median of years (range)	59.6 (17.4-86.4)	32.2 (14.0-83.7)
Clinical stage N (% of known)		
I	63 (19.4)	19 (6.5)
II	57 (17.5)	140 (48.1)
III	46 (14.2)	62 (21.3)
IV	159 (48.9)	70 (24.3)
Unknown	15	7
Histological subtype of NHL N (%)		
DLBCL	180 (52.9)	-
FL	71 (21.8)	-
Other	89 (26.2)	-
Histological subtype of HL N (%)		
NS	-	199 (66.8)
MC	-	69 (23.2)
NLPHL	-	14 (4.7)
Other	-	16 (5.4)

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; NS, nodular sclerosis classical Hodgkin lymphoma; MC, mixed cellularity classical Hodgkin lymphoma; NLPHL, nodular lymphocyte predominant Hodgkin lymphoma.

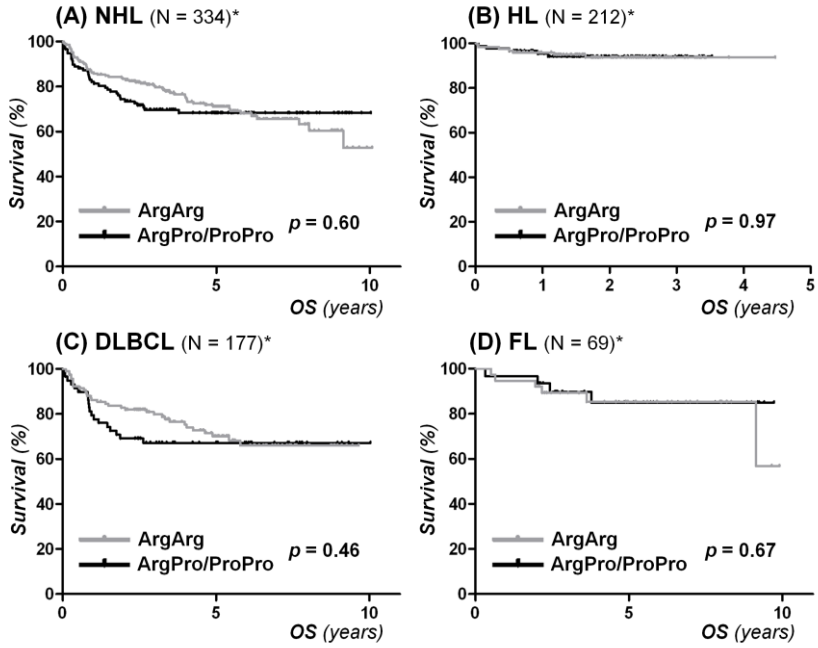
Table 2 Distribution of *TP53* R72P genotypes and allele frequencies in lymphoma patients and controls with related odds ratios.

Genotype	**Controls**	**All lymphoma cases**	
	N (%)	**N** (%)	**OR** (95% CI)	***p***
ArgArg	465 (62.1)	392 (61.4)	1.0	
ArgPro	233 (31.1)	199 (31.2)	1.0 (0.8-1.3)	0.95
ProPro	51 (6.8)	47 (7.4)	1.1 (0.7-1.7)	0.75
ArgPro/ProPro	284 (37.9)	246 (38.6)	1.0 (0.8-1.3)	0.82
Arg allele	1163 (77.6)	983 (77.0)	1.0	
Pro allele	335 (22.4)	293 (23.0)	1.0 (0.9-1.2)	0.72

Genotype	**NHL**			**HL**		
	N (%)	**OR** (95% CI)	***p***	**N** (%)	**OR** (95% CI)	***p***
ArgArg	218 (64.1)	1.0		174 (58.4)	1.0	
ArgPro	96 (28.2)	0.9 (0.7-1.7)	0.39	103 (34.6)	1.2 (0.9-1.6)	0.26
ProPro	26 (7.6)	1.1 (0.7-1.8)	0.80	21 (7.0)	1.1 (0.6-1.9)	0.78
ArgPro/ProPro	122 (35.9)	0.9 (0.7-1.2)	0.54	124 (41.6)	1.2 (0.9-1.5)	0.29
Arg allele	532 (78.2)	1.0		451 (75.7)	1.0	
Pro allele	148 (21.8)	1.0 (0.8-1.2)	0.78	145 (24.3)	1.12 (0.9-1.4)	0.36

OR, odds ratio; CI, confidence interval.

Figure 1 Overall survival curves (OS) of (A) non-Hodgkin lymphoma (NHL) patients, (B) Hodgkin lymphoma (HL) patients, (C) patients with diffuse large B-cell lymphoma (DLBCL) and (D) follicular lymphoma (FL) divided according to the genotype coding for R72P polymorphism.



Log-rank test *p*-values are displayed in each graph. * Number of patients with available survival data.

Minor revision re-submitted to the journal Leukemia Research

Dear Dr. Kleibl,

Your Brief Communication entitled "No association between the TP53 codon 72 polymorphism and risk or prognosis of Hodgkin and non-Hodgkin lymphoma" with manuscript number LR-D-11-00083 has been reviewed by our referees. The editor and referees' comments have been appended below. Please provide me with a letter (revision note) indicating your responses to the referees, and where you have modified the text.

Most sincerely,
Phoebe A. Downing
Editorial Office
Leukemia Research
Reviewers' comments:

Reviewer #1: This Czech study examines the association of the TP53 codon 72 SNP with NHL and Hodgkin's lymphoma (HL) and its association with survival. Previous studies examining the role of the TP53 codon 72 SNP in NHL have given conflicting results and there are no previous studies in HL. No association was found between allelotype and either NHL n=340) or HL (n=298) using a reference population of 749 healthy volunteers from the same racial group. Furthermore, the SNP was not associated with survival in HL (n=212), NHL (n=334), DLBCL (n=177) or FL (n=69).

Major Comments

1. The study involves substantial numbers of patients and is well written.
2. Despite the lack of statistical significance, the Kaplan-Meier curves relating the SNP with OS in DLBCL suggest that the Pro allele may be associated with an early survival disadvantage. This pattern was not observed in FL or HL. This observation should be noted and its possible implications discussed in the context of what is already known about the importance of TP53 mutations as a determinant of outcome in DLBCL.

Reviewer #2: The manuscript has novel value as it represents the first report on the TP53 codon 72 polymorphism in Hodgkin lymphoma. The data on NHL adds to a significant number of cases to those published previously by other authors and is in agreement with the findings of other studies of Caucasian individuals.

In general, the significant grammatical errors throughout, including the abstract, do not prevent the reader from understanding the data but do make it harder to read. It would be nice to see these addressed.

In terms of specific issues that must be addressed,

In the statistical methods, the definition of PFS is confused. Is PFS measured from diagnosis or from time of first treatment? 'Progression-free survival (PFS) was defined as an interval from the date of diagnosis to the date of progression, relapse or death from any cause or last follow-up date after the first line treatment.'

In the final sentence of penultimate paragraph, relating to risk association differing between ethic groups, the authors should mention and reference that this has recently been reported for the TP53 codon72 polymorphism in colorectal cancer. 'As resulting from above mentioned studies and our results, the risk of NHL is not influenced by R72P

polymorphism in the TP53 gene in general; though, the association of R72P with NHL risk could be restricted only to individuals of Asian origin.'

From the Editor:

To make your revisions please use the files that our Editorial Office sent you. Also explain in your letter where you have made your changes. No tracking changes or highlights in this version. Thank you.

Sincerely,
Terry Hamblin, DM, FRCP
Editor-in-Chief

APPENDIX III

Kleibl Z, **Havranek O**, Novotny J, Kleiblova P, Soucek P, Pohlreich P.

Analysis of *CHEK2* FHA domain in Czech patients with sporadic breast cancer revealed distinct rare genetic alterations.

Breast Cancer Res Treat 2008; 112(1):159-64 **(IF_{2008}= 5.684; first two authors contributed equally)**

Breast Cancer Res Treat (2008) 112:159–164
DOI 10.1007/s10549-007-9838-7

EPIDEMIOLOGY

Analysis of *CHEK2* FHA domain in Czech patients with sporadic breast cancer revealed distinct rare genetic alterations

Zdenek Kleibl · Ondrej Havranek · Jan Novotny · Petra Kleiblova · Pavel Soucek · Petr Pohlreich

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Abstract The *CHEK2* gene mutations I157T (c.470T > C) and IVS2 + 1G > A affecting the forkhead-associated domain (FHA) have been shown to increase the risk of breast cancer development in several populations. We analyzed the *CHEK2* gene segment coding for FHA domain in 673 unselected breast cancer patients and 683 controls from the Czech Republic using the denaturant high-performance liquid chromatography. The found frequency of predominant FHA alteration I157T did not differ between breast cancer patients (19/673; 2.82%) and controls (17/683; 2.49%; $P = 0.71$). Besides this mutation we characterized another nine alterations—six located within FHA coding sequence and three occurring in introns 1 or 2). Eight variants occurred once each in patients with breast cancer and two were present in controls. Three alterations found in breast cancer patients were novel missense variants (Y159H, T172A, and L174F) affecting highly conservative residues in FHA domain. Despite the lack of association of I157T mutation with breast cancer development in our population we deduced that the FHA domain is the subject of rare population-specific alterations that might modify risk of various cancers.

Keywords Breast cancer · *CHEK2* (*Chk2*) gene · FHA domain · Mutation analysis

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Introduction

Checkpoint kinase 2 [CHEK2, Chk2, (OMIM 604373)] is an important mediator of DNA damage signaling pathway whose defects have been found to contribute to the development of breast and other cancers [1, 2]. The CHEK2 protein mediates signal transduction from the apical sensoric part of the pathway, represented by the activation of ataxia-telagiectasia mutated (ATM) protein following DNA damage, toward cell cycle and apoptosis regulators (p53, Cdc25A, Cdc25C) and protein complexes directly involved in DNA-repair (BRCA1) that are phosphorylated by CHEK2 kinase activity [3]. The *CHEK2* gene localized to chromosome 22q12.1 codes for the 60-kDa protein consisting of 546 amino acid residues [4]. Besides this full-length protein product, numerous alternatively spliced variants were also described [5]. Three functional domains were characterized in CHEK2 polypeptide chain (reviewed in [6]). The N-terminal SQ/TQ cluster domain (residues 20–75) is involved in regulation of CHEK2 activity by ATM-mediated phosphorylation in response to genotoxic insults [7] or CHEK2 dephosphorylation by oncogenic Wip1 phosphatase abrogating CHEK2-mediated proapoptotic signaling [8]. The

forkhead-associated (FHA) domain (residues 112–175) is critically involved in dimerization of CHEK2 molecules in phosphorylation-dependent manner [9]. This process has been recently shown to be necessary for full activation of CHEK2 by *trans*-phosphorylation of the activation segment/T-loop [10] within the kinase domain (residues 225–490) that carries the catalytic serine/threonine kinase activity.

Despite the initial studies assuming that *CHEK2* may be the gene responsible for development of tumors in p53-negative Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) families [11], later analyses performed on larger cohorts including LFS/LFL families have shown that *CHEK2* acts as a low penetrance gene and alterations of this gene contribute to the mild cancer risk increase in different (breast, colorectal, ovarian, prostate, thyroid, kidney) cancers [1, 2, 12]. Numerous alterations of *CHEK2* were detected in diverse populations and distinct types of hereditary and sporadic cancers. The results of breast cancer studies led to the identification of several predominant founder mutations within the *CHEK2* gene and suggested that these mutations were unevenly distributed within the world populations. The c.1100delC mutation most frequently studied in *CHEK2* that leads to translation of truncated protein lacking kinase domain, is highly incident in Northern and Western Europe [13] and in Russia [14] but its occurrence in Southern Europe [15, 16], South America [17] or China [18] is very low. Similar differences in distribution were also found in other *CHEK2* frequently analyzed mutations located within its FHA domain—c.470T > C (I157T) and IVS2 + 1G > A (fs154X) [19]. Significant influence on CHEK2 function is considered in both alterations. The I157T was reported to interfere with phosphorylated-CHEK2 dimerization and its interaction with downstream protein targets [5, 20] and IVS2 + 1G > A resulted in aberrant splicing of mRNA and production of truncated catalytically nonfunctional protein [21]. Besides these alterations, many less frequent changes within FHA domain were described [21].

To evaluate the frequency and spectra of gene alterations in *CHEK2* FHA domain we performed the mutation analysis of the *CHEK2* gene segment encoding this domain in the Czech patients with sporadic breast cancer and relevant non-cancer controls.

Materials and methods

Genetic testing was performed in a group of 673 unselected patients with sporadic breast cancer and in a group of 524 controls previously described in the study of *CHEK2* c.1100delC mutation [22]. The remaining subgroup of 159 controls was formed by blood donors (69 females and 90 males). All individuals gave the informed consent prior genetic testing. Clinical and histopathological data were obtained from patients' log files. All patients were of the Czech origin and were living in Prague area.

Genomic DNA from peripheral blood was isolated by standard procedure (Wizard DNA extraction blood kit; Promega, Madison, WI) according to the supplier's instruction. The *CHEK2* gene fragment coding the FHA domain was amplified in a single 460 bp PCR fragment covering exons 2 and 3, together with intron 2. PCR amplifications were performed in 20 µl reaction mixtures containing 10 pmol of each primer [CHEK11F: 5′-TCAACAGCCCTCTGATGCATG-3′; CHEK15R: 5′-(GC-clamp GCCGC)TTCCAGTAACCATAAGATAATA-3′; Generi Biotech, Hradec Kralove, CR], 2.5 mM $MgCl_2$, 5% DMSO, 0.2 mM dNTPs (Applied Biosystems, Foster City, CA), 0.6 U Gold Taq DNA polymerase (Roche, Basel, Switzerland) with 100 ng of genomic DNA in 35 cycles involving the touch-down PCR protocol (64–56°C). Following denaturation/renaturation step, 5 µl aliquot of the PCR reactions were resolved using denaturant high-performance liquid chromatography (DHPLC; WAVE 3500 System; Transgenomic, Omaha, NE) on the DNASep cartridge (Transgenomic) at 55°C in a gradient of 58–67% acetonitrile-containing Buffer B (Transgenomic). The samples that gave abnormal chromatograms comparing to simultaneously run wild-type controls were sequenced from independently amplified PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a ABI3100 sequencer (Applied Biosystems).

Results

We screened the fragment of *CHEK2* gene coding for FHA domain by the DHPLC (Fig. 1a) in 673 breast cancer patients and 683 non-cancer controls. The results of analysis that identified 10 different alterations in *CHEK2* sequence are summarized in Table 1. The most frequent alteration was the 470C > T (I157T) mutation, however, the occurrence of this variant was similar in both groups: 19 cases (2.82%) in sporadic breast cancer patients and 17 cases (2.49%) in non-cancer controls ($P = 0.71$). Except for this mutation, additional four missense variants in FHA coding sequence (R117G, Y159H, T172A, and L174F; Fig. 1b) were characterized in four out of 673 breast cancer patients (0.15%) but in none of 683 controls. The R117G mutation has been previously described in 2/737 [23] and 1/516 [21] patients with hereditary breast/ovarian cancer and in 1/68 sporadic breast cancer patients [24]. In all these studies R117G variant was not present in simultaneously analyzed control cohorts. In our study, this substitution was detected in

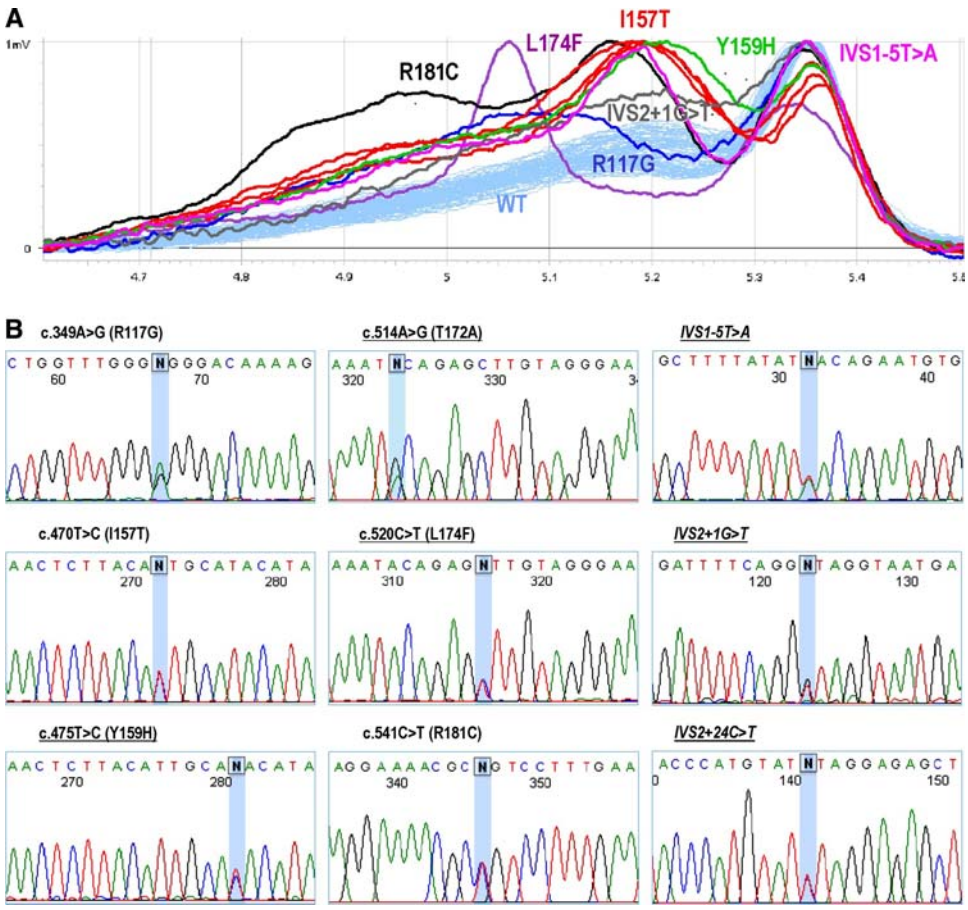

Fig. 1 (**a**) The representative view on DHPLC chromatograms in Navigator software (Transgenomic) shows multiple different chromatographic profiles corresponding to various identified alterations in analyzed 460 bp PCR products covering coding sequence of *CHEK2* FHA domain. (**b**) Sequencing chromatograms of characterized *CHEK2* variants found in our population of BC patients. The newly described alterations are underlined

Table 1 Characterized alterations in the *CHEK2* gene in sporadic breast cancer (BC) patients and controls

Exon/intron	Genetic change	Amino acid change	BC patients ($N = 673$)	Controls ($N = 683$)	P^b
e2	c.349A > G	R117G	1 (0.15%)	0	0.31
e3	c.470T > C	I157T	19 (2.82%)	17 (2.49%)	0.71
e3	c.475T > C[a]	Y159H	1 (0.15%)	0	0.31
e3	c.514A > G[a]	T172A	1 (0.15%)	0	0.31
e3	c.520C > T[a]	L174F	1 (0.15%)	0	0.31
e3	c.538C > T	R180C	0	1 (0.15%)	0.32
e3	c.541C > T	R181C	1 (0.15%)	0	0.31
i1	IVS1-5T > A[a]	?	1 (0.15%)	0	0.31
i2	IVS2 + 1G > T[a]	fs154X?	1 (0.15%)	0	0.31
i2	IVS2 + 24C > T[a]	?	1 (0.15%)	1 (0.15%)	1.00
All alterations within coding sequence			24 (3.57%)	18 (2.64%)	0.32
Alterations excluding I157T			8 (1.19%)	2 (0.29%)	0.053
All alterations			27 (4.01%)	19 (2.78%)	0.21

All mutations were heterozygous

[a] Novel mutations

[b] Test of difference between two proportions [software STATISTICA 5.1 ('98 Edition); StatSoft Inc]

one patient with bilateral breast cancer (Table 2). Transitions c.475T > C (Y159H), c.514A > G (T172A), and c.520C > T (L174F) are referred for the first time. All these alterations represent the missense variants of highly conservative amino acid residues within FHA domain (Fig. 2). We also characterized the two other known

Table 2 Clinical and histopathological characteristics of breast cancer patients carrying alterations in the *CHEK2* gene

Patient	CHEK2 alteration	Age of diagnosis	Histology of breast cancer	Stage	ER	Other cancer	Cancer family history
4	I157T	45	Lobular & ductal	I	+	None	Negative
29	I157T	62	Ductal	IIA	−	None	S_1-breast, S_2-breast
31	I157T	50	Ductal	IIA	+	None	M-leukemia
64	Y159H	83	Unknown	IIA	−	None	Negative
68	I157T	41	Lobular	IIA	−	None	Negative
128	I157T	78	Ductal	I	+	None	Negative
175	I157T	51	Unknown	I	+	None	Negative
193	IVS2 + 1G > T	50	Unknown	I	+	Breast	Negative
225	IVS2 + 24T > C	76	Ductal	I	+	None	Negative
249	T172A	47	Ductal	I	−	None	Negative
296	I157T	61	Ductal	IIA	+	None	Negative
310	I157T	49	Ductal	IIA	+	None	MS-breast
328	I157T	70	Ductal	IIA	+	None	D-unspecified gynecological ca
362	I157T	62	Lobular & ductal	I	+	None	MS-breast
415	I157T	45	Ductal & comedo	IIB	+	None	M-pancreatic, FS-unspecified ca
418	I157T	76	Ductal	IIB	+	None	Negative
442	IVS1-5T > A	54	Ductal	IIB	+	None	Negative
451	I157T	61	Ductal	I	+	None	Negative
461	I157T	50	Ductal	I	+	None	Negative
480	I157T	53	Ductal	I	+	None	S-breast, M-unknown ca, DMS-ovarian
500	I157T	41	Ductal	I	−	Cervical	Negative
549	I157T	51	Tubular	I	+	None	M-rectal ca, D-lymphoproliferative disease
553	R117G	37	Ductal	IIA	+	Breast	F-unknown primary ca
560	I157T	43	Ductal	IIA	−	None	F-bones
570	R181C	59	Tubular	I	+	None	F-lung
576	L174F	55	Lobular & ductal	IIB	+	None	Negative
683	I157T	64	DCIS	I	n.d	Colorectal	M-uterine ca, MS-unspecified gynecological ca

The average age of all 673 breast cancer patients in the dataset was 55.1 years (mean 54 years) and 56.1 years (mean 53.5 years) in patients carrying *CHEK2* mutation. In the set of 673 analyzed breast cancer patients the estrogen receptor (ER) expression was positive in 74.9%, in patients carrying any *CHEK2* alterations the positivity was found in 76.9%

Abbreviations: ca—cancer; D—daughter; DCIS—ductal carcinoma in situ; DMS—daughter of mother's sister; F—father; FS—father's sister; M—mother; MS—mother's sister; n.d.—not determined; S—sister

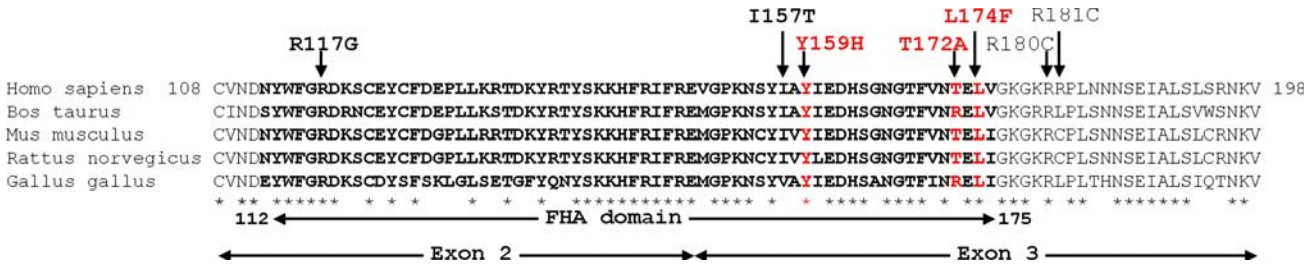

Fig. 2 The localization of identified *CHEK2* gene missense mutations in analyzed patients and controls within human CHEK2 protein sequence and corresponding sequences of other species showing that all identified genetic changes are affecting highly conservative amino acids. The newly described alterations and affected amino acids are in red. Fragment of CHEK2 coded by exons 2–3 (aa 108–198) is shown. The position of FHA domain (aa. 112–175) is marked by bold capitals in protein sequences

CHEK2 alterations (R181C and R180C) occurring in the coding sequence flanking the C-terminal portion of FHA domain. Each alteration was found once in the group of patients and controls, respectively. These uncommon alterations have been previously reported in patients with hereditary breast cancer/ovarian cancers [21, 23] and

prostate cancer [25]. Missense mutation R180C was also found in unaffected control individuals [21, 25].

In our analysis, we also characterized three intronic variants (IVS1-5T > A, IVS2 + 1G > T, IVS2 + 24C > T), each detected in one of breast cancer patients. The IVS2 + 24C > T transition was also identified in one sample from control cohort. The IVS2 + 1G > T transversion is located in the position where known splice site frame-shifting mutation IVS2 + 1G > A (fs154X) (not detected in our set of 1,355 samples) occurs [21].

The age of breast cancer onset was not different in carriers of *CHEK2* alterations (average 56.1 years) compared to non-carriers (average 55.2 years; Table 2). Only three of 27 carriers of *CHEK2* alterations with a positive family history of breast cancer were found. In our analyzed cohort no association between *CHEK2* mutations and breast cancer histological type, age of onset, or estrogen receptor expression was found.

Discussion

The most frequently studied alteration in *CHEK2* gene is 1100delC mutation leading to approximately twofold increase in risk of breast cancer [13, 26]. Other two mutations I157T and IVS2 + 1G > A were analyzed less frequently, and in studies involving hereditary, sporadic and male breast cancer patients contradictory results were reported. While studies from Germany and Belarus [27], Finland [28] and Poland [2, 29] provided the data about contribution of I157T to breast cancer development, the studies from Italy [16], Germany [21], UK, The Netherlands and USA [23], and USA on multiethnic cohort [30] failed to find such association. Mutation IVS2 + 1G > A was shown to be less frequent than I157T in all breast cancer studies.

We performed the case–control study aimed at analysis of the *CHEK2* locus, that harbors I157T and IVS2 + 1 G > A mutations. The frequency of I157T in our sporadic breast cancer group was slightly higher than that reported by Dufault et al. [21] in *BRCA1/2* negative breast/ovarian cancer patients (1.9%) and controls (1%) in Germany. On the other hand, the frequency of I157T in our population was substantially lower compared to Polish population. Occurrence of I157T variant (17/683; 2.49%) in our control group was comparable to the frequency found by Brenan et al. [31] during independent analysis implementing Czech control cohort (16/683; 2.51%). The risk of breast cancer development in carriers of I157T mutation was considered to be lower (OR = 1.4) compared to the risk of 1100delC mutation carriers [28]. The complete screening of *CHEK2* gene in cancer patients is limited to several studies that, with some exceptions [21, 25] were performed on small populations sizes. Interestingly, many of them repeatedly reported diverse missense variants (alongside the I157T) within sequence coding *CHEK2* FHA domain [21, 23–25, 30, 32, 33]. These variants accounted for substantial proportion of identified types of *CHEK2* alterations in patients cohorts, however had occurred rarely in analyzed control populations. We found four such alterations (R117G, Y159H, T172A, and L174F); three of them were novel gene alterations. These rare missense variants with different population-specific spectra may indicate that FHA domain is a subject of numerous genetic changes that are evolutionally young. We have not detected the IVS2 + 1G > A transition that was identified as the second most frequent frame-shifting *CHEK2* alteration in Polish [2], German [21, 27] and Belarus [27] populations and was shown to contribute to the development of breast, prostate stomach and thyroid cancers. In the same position, we have detected previously undescribed IVS2 + 1G > T transversion in patient with bilateral breast cancer (diagnosed at the age of 50 and 63). It is probable, that this mutation may also affect the splicing of *CHEK2* pre-mRNA, however, due to the lack of patients' RNA samples we were not able to prove this hypothesis. The other alteration identified in intron 2 (IVS2 + 24T > C) was observed once in both breast cancer and control cohort, whereas alteration identified at the end of intron 1 IVS1-5T > A was detected once in breast cancer patients group only. We performed the analysis of putative *cis*-regulating RNA elements within the site of these intronic alterations using the ESE finder software [34]. In both cases, the in silico prediction of analyzed intron alterations showed loss of one splicing factor binding sites—SRp55 (SFRS6) for IVS2 + 24T > C and SRp40 (SFRS5) for IVS1-5T > A. However, at least the functional mRNA-based analysis of these alterations is essential for definition of their impact on CHEK2 mRNA splicing.

Mutation analysis demonstrated that the I157T mutation is the most prevalent alteration of the *CHEK2* gene in Czech Republic, however, the frequency of this variant is similar in a group of breast cancer patients and analyzed controls. Also, the clinical and histopathological characteristics of *CHEK2* mutation carriers with breast cancer did not differ significantly from non-carriers. We failed to confirm strong co-segregation of I157T mutation with development of lobular type of breast cancer recently reported by Huzarski et al. [35]. Recently, Cybulski et al. [2] suggested that different *CHEK2* mutations might contribute to the development of cancer in different organs. Despite we failed to find association between breast cancer development and mutations in FHA domain, we characterized a set of probable population-specific *CHEK2* alterations that may be relevant for population specific cancer development.

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APPENDIX IV

Kleibl Z, **Havranek O**, Hlavata I, Novotny J, Sevcik J, Pohlreich P, Soucek P.

The *CHEK2* gene I157T mutation and other alterations in its proximity increase the risk of sporadic colorectal cancer in the Czech population.

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The *CHEK2* gene I157T mutation and other alterations in its proximity increase the risk of sporadic colorectal cancer in the Czech population

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ABSTRACT

Checkpoint kinase 2 (*CHEK2*) gene codes for an important mediator of DNA damage response pathway. Its mutations increase risk of several types of cancer. We analysed selected *CHEK2* mutations in 631 Czech colorectal cancer (CRC) patients.

The increased risk of CRC was associated with mutations in *CHEK2* gene region involving fork head-associated domain [39/631 (6.2%) cases versus 19/683 (2.8%) controls; odds ratio (OR) = 2.3; 95% confidence interval (CI) = 1.3–4.0; $p = 0.003$], and with the most frequent I157T mutation [30/631 (4.8%) cases versus 17/683 (2.5%) controls; OR = 2.0; 95% CI = 1.1–3.6; $p = 0.03$]. Prevalence of 1100delC mutation in CRC patients (4/631) did not differ from that in the control population (2/730; $p = 0.4$). The deletion of 5395 bp was not found in any of the successfully analysed CRC cases. We observed no association of analysed mutations with CRC family history. We conclude that the I157T and other alterations in its proximity predispose to sporadic but not to familial CRC in the Czech population.

1. Introduction

Colorectal cancer (CRC) is the most frequent cancer diagnosed in adult population in the Czech Republic ranking our country at the second place in the world incidence of CRC (incidence in 2005 = 77.9 per 100,000 persons).[1] The vast majority of CRC diagnoses arise in the form of sporadic disease; however, the hereditary predisposition to CRC could be found in about 5% of cases.[2] The causal role of mutations in APC gene (OMIM 175100) or mismatch repair genes (OMIM 120435) in CRC is now well established. In contrast, the role of mutations in low penetrance genes is not clear and is currently intensively studied. In comparison to the major predisposing genes, the low penetrance alleles display several distinct characteristics. Alongside the mild elevation of cancer risk (increase in RR ~ 2), the substantial regional differences in distribution

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and frequency (and hence clinical importance), and predisposition to wider spectrum of cancer diagnoses are frequently noted. Recently, mutations in checkpoint kinase 2 [*CHEK2*, Chk2, (OMIM 604373)] were shown to increase the susceptibility for CRC development.[3,4]

CHK2 is a nuclear phosphoprotein involved in genome integrity maintenance, and regulation of cell cycle, apoptosis and senescence (reviewed in [5]). Activation of CHK2 is initiated by its phosphorylation by ataxia-telangiectasia-mutated (ATM) kinase following DNA damage.[6] Three distinct structural/functional domains within CHK2 polypeptide were characterised. The N-terminal SQ/TQ domain (residues 20–75) contains the Thr^{68} targeted by ATM kinase.[7] The conservative fork head-associated domain (FHA; residues 112–175) promotes homodimerisation of CHK2 following Thr^{68} phosphorylation.[8] Autocatalytically activated kinase domains (residues 225–490) of CHK2 homodimer catalyse phosphorylation of CHK2-targeted downstream effectors.[9] The substrates of CHK2 kinase activity include several critical cell cycle and apoptosis regulators and DNA repair proteins (p53, PML, E2F1 and BRCA1).

Mutation analyses indicate that *CHEK2* acts as the multiorgan cancer susceptibility gene contributing to the development of numerous cancers, including breast, colorectal, prostate, ovarian, thyroid and kidney cancer.[10–13] The frameshifting 1100delC mutation leading to the translation of truncated protein product lacking kinase activity (fs381X) has been the most studied gene alteration in *CHEK2*, especially in patients with breast cancer. Its occurrence varies substantially among different populations being highly incident in Northern and Western Europe[14] and in Russia,[15] but rare in Southern Europe,[16] South America[17] or Asia.[18] Three other founder mutations in *CHEK2* were primarily shown to influence the development of breast cancer.[19] The c.470T>C (I157T) affects CHK2 FHA domain and reduces CHK2 activation in response to DNA damage.[7] The IVS2+1G>A (fs154X) leads to splicing aberration resulting in frame shift and synthesis of truncated protein [20] The large deletion of 5395 bp causes synthesis of protein with truncated kinase domain.[11]

Previously, we have shown that 1100delC, IVS2+1G>A and I157T mutations are not significantly associated with breast cancer development in the Czech population; however, we characterised several rare alterations within or flanking to FHA-coding sequence of *CHEK2*.[21,22] Here, we summarise the results of analyses of *CHEK2* gene loci harbouring selected mutations including I157T, and other alterations in its neighbourhood, 1100delC, IVS2+1G>A and the 5395 bp deletion in CRC patients from the Czech Republic.

2. Patients and methods

2.1. Patients

The study involved 631 CRC patients and 683 unrelated non-cancer individuals. All CRC cases and controls were of Czech origin. CRC patients (367 males and 264 females) were recruited from six oncology departments throughout the Czech Republic since September 2004 to February 2006. This study was coordinated by the Department of Oncology, General Teaching Hospital and 1st Faculty of Medicine Charles University in Prague. Histologically, confirmed CRC diagnosis was the only inclusion criterion for group of cases. Data on personal and family history, clinical and histological characteristics of disease and its therapy were retrieved from medical records. A family history of cancer was available in 576 of 631 analysed cases. Positive family history (at least one cancer case in the first or second degree relative) was recorded in 279 patients (48.4%); history of CRC in at least one in the first or second degree relative was present in 100 patients (17.4%).

Control group consisting of two populations – 524 non-cancer controls and 159 blood donors – was described previously, including the results of mutation analysis of *CHEK2* fragment containing FHA domain-coding exons 2–3.[22] Briefly, the subgroup of non-cancer control population (250 males and 274 females), aged 59.0 ± 16.6 years (mean ± SD), consisted of randomly selected adult persons examined at the Department of Clinical Biochemistry and Laboratory Medicine, General Teaching Hospital in Prague between January 2003 and November 2005 excluding those with primary cancer diagnosis. Control blood donors subgroup comprised randomly chosen fully anonymised healthy individuals (69 females and 90 males) enrolled between April 2006 and August 2006 in the Department of Blood Transfusion of the Thomayer Faculty Hospital in Prague. The frequency of 1100delC mutation in control group was also assessed in our previous report.[21] This group (consisting of 730 non-cancer individuals) represented enlarged set of samples of the above-described control group of 524 non-cancer controls. All examined individuals were asked to read and sign the Informed Consent in agreement with the requirements of the Ethical Committee of the General Teaching Hospital.

2.2. DNA extraction

Genomic DNA was isolated from peripheral blood lymphocytes by the phenol/chloroform extraction method or using Wizard DNA extraction blood kit (Promega) according to the supplier's instruction. DNA samples were stored at –20 °C.

2.3. Genotyping

2.3.1. Analysis of CHEK2 gene fragment containing coding sequence for FHA domain

The PCR-amplified *CHEK2* gene fragment (covering FHA-coding exons 2 and 3 with adjacent intronic sequences of introns 1 and 3, and whole sequence of intron 2) was analysed using denaturant high-performance liquid chromatography (DHPLC; WAVE 3500; Transgenomic) as described in details previously.[22] Both I157T and IVS2+1G>A alleles were screened in this analysis. Samples showing aberrant DHPLC chromatograms were bidirectionally sequenced from independently amplified samples using ABI 3130 sequencer (Applied Biosystems).

2.4. Analysis of 1100delC mutation

Mutation 1100delC was detected by DHPLC as we reported previously.[21] Analysis involved DNA amplification using nested PCR (to avoid random coincidence of numerous pseudogenes with high homology to *CHEK2* sequence) followed by DHPLC analysis. Presence of mutation was confirmed by DNA sequencing.

Table 1 – Frequency of alterations in the CHEK2 gene region covering coding sequence of FHA domain.

Exon/intron	Genetic change	Protein change	CRC patients (*N* = 631)	Controls[a] (*N* = 683)	OR[b]	95% CI[c]	*p* Value[d]
e2	c.434G>A[f]	R145Q	1 (0.2%)	0	–[e]		
e3	c.470T>C[g]	I157T	30 (4.8%)	17 (2.5%)	2.0	1.1–3.6	0.03
e3	c.538C>T	R180C	0	1 (0.2%)	–[e]		
e3	c.541C>T[g]	R181C	2 (0.3%)	0	–[e]		
i1	IVS1-5T>A[g]	?	1 (0.2%)	0	–[e]		
i2	IVS2+1G>A	fs154X	2 (0.3%)	0	–[e]		
i2	IVS2+24C>T[g]	?	3 (0.5%)	1 (0.2%)	3.4	0.4–32.4	0.4
i2	IVS2-55C>T[f]	?	1 (0.2%)	0	–[e]		
All alterations within coding sequence			33 (5.3%)	18 (2.6%)	2.1	1.2–3.7	0.02
Alterations excluding I157T			10 (1.6%)	2 (0.3%)	5.6	1.2–25.7	0.02
All alterations			39 (6.2%)[h]	19 (2.8%)	2.3	1.3–4.1	0.003

Note: Patients and controls were categorised as follows: (1) carriers of any alteration within coding sequence (R145Q, I157T, R180C, R181C); (2) carriers of any alteration excluding I157T and (3) carriers of any alteration.

a The frequency of all alterations within analysed fragment in the control subgroups of hospital-based controls and blood donors was 2.9% (15/524) and 2.5% (4/169), respectively ($p = 0.8$; ANOVA test for difference). The frequency of I157T mutation in the control subgroups of hospital-based controls and blood donors was 2.6% (14/524) and 1.9% (3/169), respectively ($p = 0.6$; ANOVA test for difference).

b Mantel–Haenszel common odds ratio (OR) estimate.

c 95% confidence interval (CI).

d Fisher's exact test, *p* (2-sided).

e Not performed due to the presence of 0 value in one group.

f Novel mutation.

g Alterations characterised in Czech breast cancer patients.[22]

h One patient carrier of both I157T and IVS2+24C>T was found.

2.5. *Analysis of large deletion of 5395 bp*

For the assessment of the large deletion (del5395), method previously published by Walsh and colleagues was used with minor modifications.[19] Briefly, two primers flanking the deletion (CHEK2delUSF primer located in intron 7 and CHEK2delUSR primer located in intron 9) were used for PCR identification of 1325 bp fragment indicating the large deletion in *CHEK2*. Separate PCR with primers CHEK2delUSF and CHEK2delUSR2 (annealed to the sequence in intron 7 lost in the case of deletion) amplified the wild-type *CHEK2* sequence, and served as a positive control of PCR (1195 bp fragment). Horizontal 1% agarose gel electrophoresis stained with ethidium bromide was used for visualisation of fragments. Samples with deletion were verified by DNA sequencing.

2.6. *Statistical analysis*

Crude odds ratios (ORs) were calculated from 2×2 tables by Mantel–Haenszel statistics (unconditional, df = 1). Two-sided Fisher's Exact Test was used for the evaluation of significance of results. The differences in clinical and histopathological characteristics between mutation carriers and non-carriers were calculated using Pearson's chi-square test and ANOVA. The *p* value lower than 0.05 was considered significant. Analyses were performed by Win SPSS v 13.0 program (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. *Analysis of CHEK2 gene fragment containing coding sequence for FHA domain*

Seven different *CHEK2* alterations (Table 1) were found in 39 of 631 CRC patients (6.2%) contrary to only two alterations found within the same gene fragment in 19 of 683 controls (2.8%) analysed previously using the same method.[22] The presence of any alteration elevated the risk of CRC in the group of patients more than twofold (OR = 2.3; 95% confidence interval (CI = 1.3–4.0; $p = 0.003$). Alongside the most frequent c.470T>C (I157T) mutation, and four alterations described previously (R181C, IVS1-5T>A, IVS2+1G>A and IVS2+24C>T) we characterised two novel gene variants – the missense variant c.434G>A (R145Q) and the intronic variant IVS2-55C>T (Fig. 1). The missense variant R180C was detected in one of 683 control samples only. The prevalence of I157T mutation was significantly higher in CRC patients – 30/631 (4.8%) than in controls [17/683 (2.5%); $p = 0.03$]. The inheritance of I157T mutation enhanced the risk of CRC twofold (OR = 2.0; 95% CI = 1.1–3.6; Table 1). The prevalence of other alterations detected in the *CHEK2* gene region containing FHA domain-coding sequence was also found to differ significantly between CRC patients and controls (10/631 versus 2/683; $p = 0.02$), and the risk of CRC associated with the inheritance of these allelic variants was enhanced accordingly (OR = 5.6; 95% CI = 1.2–25.7; Table 1). The inheritance of any *CHEK2* missense variant within FHA-coding sequence enhanced the risk of CRC more than twofold (OR = 2.1; 95% CI = 1.2–3.7; $p = 0.02$; Table 1). Both I157T and IVS2+24T>C variants were detected in one CRC patient.

3.2. *Analysis of c.1100delC mutation*

Truncating mutation 1100delC was found in four of 631 CRC patients (0.6%). Compared to previously analysed controls (2/730; 0.3%), the difference in frequency of 1100delC was not statistically significant (OR = 2.3; 95% CI = 0.4–12.8; $p = 0.4$).[21] The average age of CRC diagnosis in 1100delC carriers was 60.5 ± 8.5 years (mean ± SD). Positive familial history of cancer was scored in one of the four patients carrying

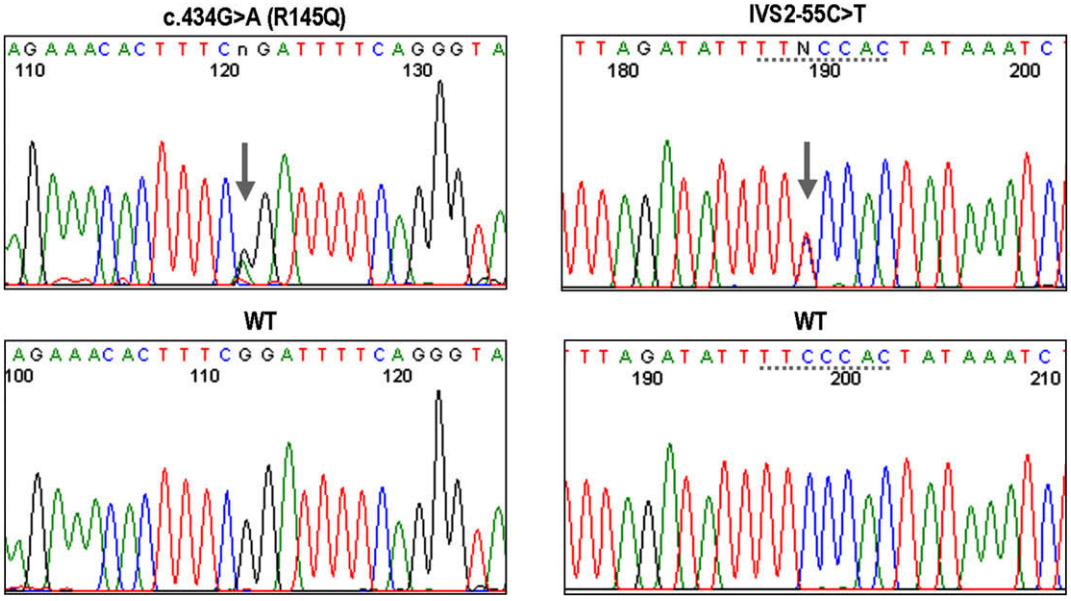

Fig. 1 – Sequencing chromatograms show two novel alterations in *CHEK2* gene (R145Q and IVS2-55C>T, marked by arrow) and corresponding wild-type (WT) sequences. Position of predicted branch-site (5′-TTCCCAC; Supplementary Fig. 2) affected by IVS2-55C>T transition is underlined.

1100delC (father with gastric cancer diagnosed at the age of 50).

3.3. *Analysis of 5395 bp deletion*

The large deletion of 5395 bp was successfully screened in 522 of 631 CRC patients and 565 of 683 controls. Analysis failed in 17% of the samples due to poor DNA quality or due to the lack of DNA sample. We have found no carrier of this mutation in the group of CRC patients. One heterozygote carrier was identified in the control group (1/565; 0.2%).[g]

3.4. *Association of CHEK2 gene I157T mutation and other alterations in its proximity with clinico-pathological characteristics of CRC patients*

To analyse the impact of various *CHEK2* allelic variants on clinical and histopathological characteristics of colorectal tumours, the group of CRC patients was categorised into subgroups containing (i) patients carrying any alteration within fragment containing coding sequence for FHA domain, (ii) carriers of I157T mutation and (iii) subjects with wild-type alleles (Table 2). Age at the diagnosis in 39 mutation carriers of any *CHEK2* mutation (59.4 ± 12.6 years; mean ± SD) and in 30 carriers of I157T mutation (60.1 ± 11.8) was similar to patients without mutation (61.0 ± 10.6; $p = 0.4$ compared to any mutation within analysed fragment). We did not note any relationship between the presence of *CHEK2* alteration and localisation of primary tumour or clinical stage (AJCC). However, statistically significant difference between mutation carriers and patients without mutation was associated with tumour grade ($p = 0.0495$ compared to any mutation carriers and wild-type patients; Table 2).

The frequency of positive family cancer history (defined as any cancer in the first or second degree relatives and index case) did not differ between CRC patients carrying any *CHEK2* alteration in analysed fragment containing FHA-coding sequence (18 of 39; 46.2%) and CRC patients with wild-type *CHEK2* alleles (261 of 576; 45.3%; Table 3). The most frequent cancer diagnoses in 18 families of *CHEK2* alteration carriers were colorectal and lung cancers (both in six families) and breast cancer (in three families). No association was observed between the presence of *CHEK2* alterations and hereditary CRC. The increased frequency of patients carrying *CHEK2* alterations was apparent only in the group of patients from colorectal and lung cancer families (defined as lung cancer in the first or second degree relatives and index case with CRC). Six CRC and lung cancer families were identified among 39 carriers of any *CHEK2* alteration and in 37 of 537 *CHEK2* wild-type CRC patients (15.4% and 6.9%, respectively; $p = 0.051$).

4. Discussion

We studied the impact of four *CHEK2* founder mutations and other sequence variants on the development of CRC in Czech patients. The I157T mutation found in 30 of 631 CRC patients (4.8%) was the most prevalent *CHEK2* alteration. The occurrence of truncating mutations 1100delC and IVS2+1 G>A was higher in analysed CRC patients (0.6% and 0.3%) than in controls (0.3% and 0%); however, due to the low prevalence of these alterations in the Czech population their role in CRC

[g] Contrary to previously published description of 5395 bp deletion, we assume that the deletion of 5395 bp [c.909-2028_1095+330del5395; (Supplementary Fig. 1)] affects coding exons 8 and 9 (not 9 and 10) and causes synthesis of protein with truncated kinase domain (p.Met304Leufs15X).

Table 2 – Selected clinico-pathological characteristics of colorectal tumours in patients analysed for the presence of mutations in the CHEK2 gene region covering coding sequence of FHA domain.

	Wild-type *N* (%)	Any CHEK2 alteration *N* (%)	*p* Value[a]	I157T *N* (%)	*p* Value[a]
Location of primary tumour			0.6		0.6
Ascending colon	76 (91.6%)	7 (8.4%)		6 (7.2%)	
Transverse colon	31 (100.0%)	0 (0.0%)		0 (0.0%)	
Descending colon	43 (93.5%)	3 (6.5%)		2 (4.3%)	
Sigmoid rectum	232 (93.2%)	17 (6.8%)		14 (5.6%)	
Rectum	177 (93.7%)	12 (6.3%)		8 (4.2%)	
Staging (AJCC)			0.4		0.6
Stage I	17 (94.4%)	1 (5.6%)		1 (5.6%)	
Stage IIA-B	253 (95.1%)	13 (4.9%)		10 (3.8%)	
Stage IIIA-C	139 (90.8%)	14 (9.2%)		10 (6.5%)	
Stage IV	110 (92.4%)	9 (7.6%)		7 (5.9%)	
Tumour grade[b]			0.0495		0.06
G1	86 (90.5%)	9 (9.5%)		7 (7.4%)	
G2	298 (95.5%)	14 (4.5%)		10 (3.2%)	
G3	74 (89.4%)	9 (10.6%)		7 (8.3%)	

Note: Patients carrying any alteration within analysed *CHEK2* fragment covering exons 2 and 3, and patients carrying I157T were analysed separately against patients with wild-type sequence.

a Chi-square test.

b Two grade 4 tumours (wild-type in analysed sequence) were excluded from the statistics.

Table 3 – Selected characteristics of colorectal cancer patients analysed for the presence of allelic variants in the CHEK2 gene region covering coding sequence of FHA domain.

	Wild-type *N* (%)	Any *CHEK2* alteration *N* (%)	*p* Value	I157T *N* (%)	*p* Value
CRC patients (*N* = 631)	592 (93.8%)	39 (6.2%)	–	30 (4.8%)	–
Males; *N* (%)	345 (94.0%)	22 (6.0%)	–	16 (4.4%)	–
Females; *N* (%)	247 (93.6%)	17 (6.4%)	–	14 (5.3%)	–
Age at diagnosis (range) in years	61.0 (23–86)	59.4 (28–78)	–	60.1 (28–76)	–
Family cancer history (N = 576)[c]					
Positive	261 (93.5)	18 (6.5%)	–	14 (5.0%)	–
Age at diagnosis (range) in years	60.3 (23–83)	58.3 (28–78)	–	57.9 (28–75)	–
Negative	276 (92.9)	21 (7.1%)	–	16 (5.4%)	–
Age at diagnosis (range) in years	61.3 (26–86)	60.4 (28–76)	–	62.1 (42–76)	–
HCC	94 (94.0%)	6 (6.0%)	–	5 (5.0%)	–
Age at diagnosis (range) in years	60.4 (23–83)	56.5 (50–65)	0.4[a]	57.2 (50–65)	–
CC&LC	37 (86.0%)	6 (14.0%)	0.051[b]	4 (9.3%)	0.2[b]
Age at diagnosis (range) in years	59.7 (35–74)	57.5 (41–78)	–	56.5 (43–75)	–

HCC – hereditary CRC (defined as CRC in the first or second degree relatives and index case); HBCC – hereditary breast cancer and CRC (defined as breast cancer in the first or second degree relatives and index case); CC&LC – CRC and lung cancer (defined as lung cancer in the first or second degree relatives and index case).

Note: patients carrying any alteration within analysed *CHEK2* fragment covering exons 2 and 3, and patients carrying I157T were analysed separately against patients with wild-type sequence.

a ANOVA test.

b Chi-square test.

c Cases, where family history of cancer was available.

development is of limited clinical importance. The lack of the 5395 bp deletion in analysed CRC patients suggests that the effect of this mutation may be limited to an increase in breast cancer risk only. Moreover, recent studies showed limited relevance of *CHEK2* truncating mutations to CRC development.[4,23]

According to our data, the I157T mutation associates with an increased risk of CRC in the Czech population (OR = 2.0). The frequency of I157T mutation in both CRC and control groups of Czech origin (4.8% and 2.5%, respectively; OR = 2.0) was lower compared to that reported by Kilpivaara and colleagues in Finland (7.8% and 5.3%, respectively; OR = 1.5) and Cybulski and colleagues in Poland (7.1% and 4.8%, respectively; OR = 1.5).[24,4] The frequency of I157T mutation in control population similar to our observation was reported by Brennan and colleagues in different control groups of Czech origin (2.5%; 16/636) contributing to analysis of I157T prevalence in patients with tobacco-related cancers.[11] Contrary to the above-mentioned studies from Finland and Poland, we did not find association of I157T with family history of CRC.

In its place we observed increased frequency of lung cancer in relatives of I157T carriers with CRC [4/30 (13.3%) cases with I157T versus 37/537 (6.9%) wild-type cases; $p = 0.2$]. This trend turned even stronger when all alterations detected in the gene fragment containing exons 2 and 3 were included [6/39 (14.0%) cases with *CHEK2* alteration; $p = 0.051$]. However, we are aware that interpretation of this association is limited by the small sample size but it remains interesting, as the I157T mutation was recently demonstrated to associate negatively with sporadic lung cancer development.[11,25] Our results indicate that I157T moderately increases the risk of CRC, but the alteration is not linked to familial CRC development in the Czech Republic. Several genetic aspects can contribute to this effect: (i) Genetic origin of CRC in patients not carrying disease-causing mutations in high-penetrant genes is multifactorial. Recently, Cybulski and colleagues reported the cooperative increase of breast cancer and CRC risk in patients carrying both c.326T>G (V109G) allele in p27 and one of I157T, IVS2+1G>A, 1100delC or del5395 mutations in *CHEK2*.[26] (ii) The penetrance of these (so far poorly characterised or undisclosed) multifactorial genetic loci varies in broad scale below the threshold, in which it turns into the *sine qua non* condition for cancerogenesis initiation. Because of usually low penetrance of contributing alleles (maximally ~OR 2.0), their frequencies could be quite high in population. However, they may vary substantially among diverse populations (e.g. the occurrence of 1100delC allele has been shown to decrease in European countries in North-to-South direction). (iii) Current evidences have shown that carriage of low penetrant alleles influences the risk of particular cancer type. The I157T could serve as an example, increasing the risk of CRC but protecting against tobacco-related lung cancer.[11] (iv) The individual genetic cancer risk in cancer patients is probably driven by the mutual interplay of risk factors. The multifactorial interplay of numerous 'low penetrant' or 'modifying' alleles with diverse population frequencies could explain the association to sporadic but not to hereditary CRC. CRC develops in a subset of CRC patients that inherited 'cancer-promoting collection' of alleles (e.g. including I157T) from their parents. This 'collection' is assembled from two allele pools that were alone incapable to evoke cancer in their parents (the cumulative OR for CRC in each parent is lower than the cumulative OR of combined genotype in their CRC-affected child). It should also be considered that siblings of such CRC patient might be at an increased risk. However, it is probable that the composition of 'cancer-promoting collection' will be diluted in descent of patient. We hypothesise that numerous (however limited) such 'cancer-promoting collections' may exist, and at least some of them may have a population-specific character. We speculate that the I157T mutation (and possibly other alterations within FHA domain-coding sequence) participates as one of several genetic contributors to CRC development in our population.

Alongside the I157T and IVS2+1G>A mutations, the analysis of *CHEK2* gene fragment containing the FHA-coding exons 2 and 3 with adjacent intronic sequences revealed the presence of five another alterations in eight CRC patients. The novel c.434G>A transition (R145Q) found in one CRC patient leads to the replacement of highly conservative Arg to Glu. Other missense variants affecting Arg145 [c.433C>T (R145W) and c.434G>C (R145P)] were described elsewhere in patients with Li-Fraumeni syndrome, breast and prostate cancer and in CRC cell line HCT15.[20,27–29] The R145W variant has been shown to cause reduced ATM-dependent CHK2 phosphorylation and CHK2 kinase activity, and thus affecting the association of CHK2 with other cellular proteins in response to DNA damage.[7,8,30] Therefore, it is possible that R145Q may also alter CHK2 activation. The c.541C>T transition (R181C) detected in two CRC patients affects non-conservative amino acid residue located in proximity to C-end of FHA domain. This alteration was earlier described by Dong and colleagues in one of 178 prostate cancer tumour samples. It was not found in any of 298 men with familial prostate cancer, 400 men with sporadic prostate cancer or 423 unaffected men.[20] We identified recently R181C in one breast cancer patient from the Czech Republic.[22] The occurrence of intronic variants IVS1-5T>A (identified in one CRC patient) and IVS2+24C>T (found in three CRC patients and one control sample) was described in previously analysed population of breast cancer patients. Based on computer prediction, we deduced that both variants might interfere with the binding of splicing factors.[22] In this study, we characterised another intronic variant IVS2-55C>T in one CRC patient. The IVS2-55C>T transition alters the most probable branching-site (based on software prediction in ESE finder algorithm; Supplementary Fig. 2) in intron 2, and hence may lead to aberrant mRNA splicing. However, this assumption needs to be confirmed by analysis at mRNA level.

Clinical and histopathological characteristics in CRC patients with *CHEK2* alterations and wild-type alleles were similar, except for tumour grading in carriers of I157T. However, instead of the clear trend showing increased mutation frequency with higher grading, we detected uneven distribution of grading with increased mutation frequencies in both grade 1 and 3 tumours. Thus, this observation may be a result of limited size of analysed groups or due to multiple comparisons.

In conclusion, the analysis of a gene fragment containing coding sequence of *CHEK2* FHA domain in CRC population supports our previous observation in breast cancer patients that exons 2 and 3 and flanking intronic sequences are subject to numerous population-specific genetic alterations.[22] Alterations in this region enhanced the effect of I157T and together contributed to an increased risk of sporadic CRC development (OR = 2.3) in the Czech population. Prevalence of truncating mutation 1100delC is low in CRC patients, and play clinically less important role in CRC tumourigenesis.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.09.022.

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APPENDIX V

Mohelnikova-Duchonova B, **Havranek O**, Hlavata I, Foretova L, Kleibl Z, Pohlreich P, Soucek P.

***CHEK2* gene alterations in the forkhead-associated domain, 1100delC and del5395 do not modify the risk of sporadic pancreatic cancer.**

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CHEK2 gene alterations in the forkhead-associated domain, 1100delC and del5395 do not modify the risk of sporadic pancreatic cancer

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ABSTRACT

Checkpoint kinase 2 gene (*CHEK2*) alterations increase risk of several cancer types. We analyzed selected *CHEK2* alterations in 270 Czech pancreatic cancer patients and in 683 healthy controls. The pancreatic cancer risk was higher in individuals who inherited rare alterations in *CHEK2* region involving forkhead-associated domain other than I157T (OR = 5.14; 95% CI = 0.94–28.23) but the observed association was non-significant ($p = 0.057$). The most frequent I157T mutation did not alter the pancreatic cancer risk and neither the followed deletion of 5395 bp nor c.1100delC were found in any of pancreatic cases. We conclude that the I157T, other alterations in its proximity, del5395 and c.1100delC in *CHEK2* do not predispose to pancreatic cancer risk in the Czech population.

1. Introduction

Pancreatic carcinoma (OMIM: 260350) is the fourth leading cause of cancer-related deaths in the Czech Republic with a 5-year survival rate less than 5% [1,2]. The majority of genetic changes, identified in ductal pancreatic adenocarcinoma target the core intracellular signaling pathways including apoptosis, cell cycle and also DNA repair [3].

Checkpoint kinase 2 (*CHEK2*, CHK2, OMIM 604373) is a nuclear phosphoprotein involved primarily in DNA repair signaling and hence genome integrity maintenance, however, its activities also contribute to cell cycle regulation, apoptosis and senescence [4]. Mutation analyses have been indicating that *CHEK2* acts as a multiorgan cancer susceptibility low or moderate penetrant gene modifying the risk of sporadic and/or familial breast, colorectal, prostate, ovarian, thyroid, kidney and lung cancers [5–8]. The role of *CHEK2* in sporadic pancreatic cancer development has not been studied so far, however, it has been shown that the risk of pancreatic cancer development is increased in several hereditary cancer syndromes resulting from inherited mutations in genes directly involved in DNA repair pathways (e.g. *BRCA1*, *BRCA2*) [9]. Our previous studies on the Czech population have shown significant associations of alterations flanking to or localized within the *CHEK2* forkhead-associated (FHA) domain-coding region (residues 112–175; containing the most prevalent *CHEK2* mutation – I157T) with the increased risk of sporadic colorectal but not breast cancer [10,11].

We aimed to assess the relevance of the previously identified cancer risk-modifying *CHEK2* alterations including alterations encompassing the FHA domain-coding region, c.1100delC mutation, and the large genomic deletion of 5395 bp (del5395) in exons 8 and 9 [12] for pancreatic cancer risk in the Czech population.

2. Patients and methods

2.1. Subjects

A total of 953 individuals of Czech Caucasian ancestry were included into the study in the period between January 2003 and February 2009. The cases included 270 incident pancreatic cancer patients. The design, eligibility criteria, and characteristics of the cases were described in detail previously [13].

Randomly selected controls represented 683 unrelated non-cancer individuals from the same catchments area as the cases. Characteristics and recruitment criteria of the control group

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Table 1
Identified alterations in sequence surrounding the *CHEK2* FHA domain.

	Cases, *N* (%)	Controls, *N* (%)	OR[a]	95% CI[a]	*p*[a]
No alterations[b]	259 (96.3)	664 (97.2)	Reference (1.00)		
c.470T>C (I157T)	6 (2.2)	17 (2.5)	0.89	0.35–2.29	0.815
c.538C>T (R180C)	0	1 (0.1)	–	–	–
IVS1-5T>A	1 (0.4)	0	–	–	–
IVS2+24C>T	1 (0.4)	1 (0.1)	–	–	–
IVS2+1G>A (fs154X)	1 (0.4)	0	–	–	–
IVS2-55C>T	1 (0.4)	0	–	–	–
Alterations excluding I157T	4 (1.5)	2 (0.3)	5.14	0.94–28.23	0.057
All alterations	10 (3.7)	19 (2.8)	1.35	0.62–2.94	0.451

[a] Common odds ratio (OR) estimate with 95% confidence interval (CI) and significance p by 2-sided Fisher's Exact Test.
[b] Alterations were not determined in one case due to the absence of PCR product in the sample.

including the results of mutation analysis of *CHEK2* FHA domain, del5395, and c.1100delC were previously described [11,12,14].

All participants gave their informed written consent to participate in the study approved by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague and Masaryk Memorial Cancer Institute, Brno, Czech Republic.

2.2. Mutation analyses

Genomic DNA was isolated from peripheral blood lymphocytes by published protocols [13,14]. The analyses of *CHEK2* alterations in FHA domain-coding exons 2 and 3 (with adjacent intronic sequences of introns 1 and 3 and whole sequence of intron 2) and the c.1100delC mutation were based on denaturing high-performance liquid chromatography (DHPLC WAVE system 3500) and sequencing of samples with aberrant chromatograms, whereas the del5395 mutation was resolved by fragment analysis of long-range PCR products on agarose gel electrophoresis as we described in details recently [11,12,14].

2.3. Statistical analyses

Two-sided Fisher's Exact Test was used for evaluation of significance of results. Crude odds ratios (OR) were calculated from 2×2 tables by unconditional Mantel–Haenszel statistics using Win SPSS v 15.0 program (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

We analyzed the impact of *CHEK2* alterations to the development of sporadic pancreatic cancer in 270 Czech patients. The c.470T>C (p.I157T) mutation affecting the FHA-coding domain was the most prevalent *CHEK2* alteration in pancreatic cancer cases found in six out of 269 analyzed cases (2.2%). However, this frequency was similar to that noted in controls (2.5%) resulting in non-significant association with the pancreatic cancer risk ($p = 0.815$; Table 1). The occurrence of other alterations within the *CHEK2* FHA domain-coding sequence (IVS2+1G>A) or those localized in its proximity (IVS1-5T>A, IVS2+24C>T, IVS2-55C>T, c.538C>T) was in our study higher among cases (4/269; 1.5%) compared to controls (2/683; 0.3%) but the association with the pancreatic cancer risk was non-significant ($p = 0.057$; Table 1). All four intronic alterations characterized in this *CHEK2* fragment were described previously in breast and/or colorectal cancer cases from the Czech Republic [10,11]. Except the mutation IVS2+1G>A demonstrably altering the CHK2 protein structure (fs154X), the biological relevance of the others remains unknown *in vivo*. Based on computer prediction, we previously deduced that intronic variants IVS1-5T>A and IVS2+24C>T may interfere with binding of splicing factors [11], whereas IVS2-55C>T may affect the most probable branching site [10]. The c.538C>T (R180C) found in one control individual occurs in the less conservative coding sequence flanking to C-terminal proportion of the FHA domain and it has been described previously in several cancer patients representing rare, probably neutral polymorphism [10]. Analyses of other *CHEK2* hot-spot regions in our set of pancreatic cancer patients revealed no carrier of either *CHEK2* c.1100delC mutation or the del5395 (described as Czech founder mutation in breast cancer cases) [12]. The frequency of c.1100delC in the Czech population is low (0.3%) and our results indicate that this alteration unlikely contributes to the sporadic pancreatic cancer development [14]. One heterozygote carrier of del5395 was previously identified in the control group only (1/565; 0.2%) and thus the del5395 remains relevant exclusively for breast cancer families [11,12]. However, the overall number of detected variants in our study was relatively small, and some associations may have been missed as a result of limited study power.

The role of *CHEK2* alterations in sporadic pancreatic cancer has not been studied so far. Bartsch et al. identified one 1100delC mutation carrier among 35 German familial cancer patients and suggested a possible contribution of this alteration to onset of familial pancreatic cancer [15]. Miyasaka et al. indicated that DNA damage checkpoint activation occurs at an early stage of intraductal papillary mucinous neoplasms of pancreas (IPMNs) and prevents their progression [16]. Disturbance of this pathway due to CHK2 inactivation or *TP53* mutation was suggested to contribute to carcinogenesis of IPMNs.

Due to the limited sample size, the effect of familial etiology could not have been discerned but it remains an interesting task for future studies as the I157T was recently demonstrated to positively associate (OR = 2.1; $p = 0.0004$) with mismatch repair-negative hereditary non-polyposis colorectal cancer in Polish population [17].

In conclusion, our data suggest that in contrary to breast and colorectal cancers, alterations in the *CHEK2* FHA domain-coding region, c.1100delC and del5395 do not significantly modify the risk in sporadic pancreatic cancer.

Conflict of interest statement

None declared.

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APPENDIX VI

Kleibl Z, **Havránek O**, Novotný J, Kohoutová M, Štekrová J, Matouš M.

Analýza nutace c.1100delC genu *CHEK2* v populaci pacientů se sporadickým karcinomem kolorekta a familiární adenomatózní polypózou.

Klinická onkologie 2007; 20:224-226

ANALÝZA MUTACE C.1100DELC GENU CHEK2 V POPULACI PACIENTŮ SE SPORADICKÝM KARCINOMEM KOLOREKTA A FAMILIÁRNÍ ADENOMATÓZNÍ POLYPÓZOU

ANALYSIS OF THE C.1100DELC MUTATION OF THE CHEK2 GENE IN SPORADIC COLORECTAL CARCINOMA AND FAMILIAL ADENOMATOUS POLYPOSIS PATIENT POPULATION

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Souhrn

Východisko: Česká republika patří mezi státy s nejvyšší incidencí kolorektálního karcinomu. Přestože je většina kolorektálních karcinomů sporadického původu, významnou část pacientů, především z hlediska prediktivní onkologie, tvoří nemocní s výskytem hereditárních nádorů. Do současné doby byla charakterizována řada genů, jejichž mutace zvyšují riziko vzniku kolorektálního karcinomu (*APC*, *MLH1*, *MSH2*, *MSH6*, *SMAD4*, *BMPR1a*) a způsobují vznik hereditárních nádorových syndromů, jako je například familiární adenomatózní polypóza (FAP). Intenzivní pozornost je věnována rovněž genetické modifikaci nádorového rizika na základě mutací v tzv. „genech s nízkou penetrancí". Mezi tyto geny patří i gen *CHEK2*, u kterého byla popsána patogenní mutace 1100delC podílející se na vzniku syndromu hereditárního výskytu kolorektálního karcinomu a karcinomu prsu (HBCC). **Metody:** Provedli jsme detekci patogenní mutace *CHEK2* c.1100delC u 433 pacientů s kolorektálním karcinomem a 113 pacientů s FAP a její atenuovanou formou (AFAP). Mutace byly analyzovány pomocí denaturační vysokoúčinné kapalinové chromatografie (DHPLC) produktu PCR zahrnujícího exon 10 genu *CHEK2*. Nalezené mutace byly ověřeny sekvenováním. **Výsledky:** V souboru 433 pacientů jsme identifikovali celkem 3 nosiče sledované mutace (0,7%); v souboru pacientů s diagnózou FAP nebyla mutace nalezena. **Závěry:** Výskyt alely CHEK2*1100delC je v České republice nízký. Ačkoliv četnost nálezu mutace v populaci pacientů s kolorektálním karcinomem je vyšší než v nenádorové populace, je pravděpodobné, že nosiči mutace představují méně než 1% všech nemocných.

Klíčová slova: karcinom kolorekta, familiární střevní polypóza, *CHEK2*, dědičná dispozice, zárodečná mutace

Summary

Backgrounds: Czech republic belongs to the countries with the highest incidence of colorectal cancer in both male and female population. The vast majority of colorectal cancer diagnoses arise in a form of sporadic disease. The hereditary predisposition for colorectal cancer development could be found in about 5%. Along with mutations in the major predisposing genes (*APC*, *MLH1*, *MSH2, MSH6*, *SMAD4*, *BMPR1a*), the role of mutations in low penetrance genes is intensively studied. Recently it has been found that the frame-shifting *CHEK2* c.1100delC mutation contributes to the development of hereditary breast and colorectal cancer syndrome (HBCC). **Methods:** We have performed mutation analysis of pathogenic *CHEK2* allele c.1100delC in 546 patients including 433 colorectal cancer patients and 113 patients with familial adenomatous polyposis (FAP). Mutation analysis was based on DHPLC prescreening, mutations were confirmed by sequencing. **Results:** We have characterized 3 mutation carriers, all in the colorectal cancer patients' cohort (0.7%). **Conclusion:** Based on our data we can speculate that Czech Republic belongs to countries with low occurrence of *CHEK2* c.1100delC allele, which therefore rarely contributes to the development of colorectal cancer and plays an insignificant role in cancer development in patients with FAP/AFAP.

Key words: colorectal cancer, CHEK2, familial adenomatous polyposis, hereditary disposition, germline mutation

Úvod

Česká republika patří mezi přední země s ohledem na výskyt karcinomu kolorekta (1). Incidence jeví trvale rostoucí charakter a v roce 2001 dosáhla u mužů 61,8/100 000, u žen pak 46,1/100 000 (UZIS ČR, NOR ČR 2001). Okolnosti vzniku kolorektálního karcinomu ovlivňuje řada faktorů, mezi které počítáme vlivy prostředí, dietní návyky i dědičné vlohy. Doposud charakterizované genetické rizikové faktory (především zárodečné mutace v genu *APC* a genech MMR systému) dávající vzniknout syndromům familiární adenomatózní polypózy (FAP), hereditárního nepolypózního kolorektálního karcinomu (HNPCC - Lynchův syndrom) a Lynchova syndromu. Základní charakteristikou FAP je výskyt mnohočetných polypů, které s téměř 100% pravděpodobností progredují ke vzniku kolorektálního karcinomu v průběhu pacientova života. Kromě syndromu FAP se vyskytují i pacienti s atenuovanou formou FAP (AFAP), u kterých je nacházen nižší počet polypů a pozdější nástup

onemocnění . Hereditární formy kolorektálního karcinomu způsobují necelých 5% karcinomů kolorekta. Prevalence FAP se v ČR pohybuje kolem 1 na 5000-7000 obyvatel (2). Vznik kolorektálního karcinomu je kromě mutací v hlavních predispozičních genech ovlivněn i alteracemi v řadě dalších genů. Jedním z možných kandidátních genů je *CHEK2* (checkpoint kinase 2, OMIM 604373) - proteinkináza modulující odpověď na genotoxická poškození. Stěžejní role proteinu CHEK2 spočívá v aktivaci (fosforylaci) proteinu p53. Delece cytosinu v pozici 1100 (*CHEK2* c.-1100delC) způsobuje inaktivaci proteinu CHEK2. Díky posunu čtecího rámce dochází k předčasné terminaci translace se zkrácením proteinového produktu v místě kinázové domény. *CHEK2* c.1100delC je nejstudovanější mutací s prokázaným vztahem k řadě karcinomů (3). Na základě rozsáhlých populačních studií v různých zemích je tato mutace uváděna v souvislosti se zvýšeným rizikem vzniku karcinomu prsu (RR = 2) a výskytem HBCC syndromu - rodinného výskytu karcinomu prsu a kolorekta (4,5). V genu *CHEK2* bylo doposud popsáno několik dalších inaktivujících mutací v souvislosti se vznikem jak sporadických maligních nádorů (karcinom prsu, kolorekta, prostaty, štítné žlázy, osteosarkom) tak i hereditárních nádorových syndromů (Li-Fraumeni syndrom). Například mutace c.470T>C [I157T] se vyskytuje signifikantně častěji u pacientek s karcinomem prsu a u pacientů s karcinomem prostaty (6,7).

Cíl studie

Provedli jsme vyšetření četnosti alely *CHEK2**1100delC u neselektované populace pacientů s karcinomem kolorekta a v populaci pacientů s FAP a AFAP s cílem zhodnocení vlivu této alely na vznik hereditární a sporadické formy karcinomu kolorekta.

Metody

Pacientkám zařazeným do studie bylo odebráno 5 ml nesrážlivé žilní krve po podpisu informovaného souhlasu schváleného etickou komisí. Genomová DNA byla izolována pomocí JetStar 96 blood kitu (Genomed, Löhne, BRD) dle protokolu výrobce. Z důvodů homologních sekvencí v lidském genomu byla oblast, zahrnující exon 10 genu CHEK2 amplifikována dvoukrokově pomocí nested PCR na základě publikovaných postupů (8,9). Výsledný PCR produkt byl analyzován pomocí denaturační vysokoúčinné kapalinové chromatografie (DHPLC) systému WAVE (Transgenomic, Omaha, NE) na DNASep koloně při teplotě 55°C v gradientu acetonitrilu 50,4-59,4% (Buffer B, Transgenomic). Vzorky s aberantním elučním profilem na DHPLC byly charakterizovány sekvenováním (BigDye Terminator ver. 3.1, Applied Biosystems, Foster City, CA) na sekvenátoru ABI310 (Applied Biosystems).

Výsledky

Celkem bylo analyzováno 433 vzorků od pacientů s kolorektálním karcinomem a 113 vzorků od pacientů s FAP/AFAP. Mutační analýza v tomto souboru odhalila přítomnost tří nosičů patogenní alely 1100delC v souboru 433 nemocných s kolorektálním karcinomem (0, 69%). Klinicko-patologické charakteristiky nosičů nalezené mutace jsou uvedeny **v tabulce č.1.** Pouze u jedné ze tří pozitivně testovaných osob ve skupině karcinomu kolorekta byl zaznamenán rodinný výskyt karcinomu prsu (u matky vyšetřované osoby).

Ve skupině 113 nemocných s FAP/AFAP nebyla studovaná mutace *CHEK2* c.1100delC nalezena.

Frekvence výskytu mutace *CHEK2* c.1100delC v neselektované obecné populaci (2/720; 0,27%) byla stanovena v předchozí studii (10) .

Tabulka 1.: Klinické a histopatologické charakteristiky pacientů s karcinomem kolorekta a mutací *CHEK2* c.1100delC. Ve všech třech případech se jednalo o pacienty mužského pohlaví.

Pacient	Věk při dg.	Charakteristiky nádoru		Sekundární malignita	Kouření	Maligní onemocnění v rodině (věk dg)
		Histologie nádoru	Stádium			
R72	57	středně diferencovaný adenokarcinom	Dukes D	0	Ano	?
No340	60	Tubulárně uspořádaný ložiskově povrchně ulcerovaný adenokarcinom	Dukes B	0	Ano	0
No366	52	Tubulární adenokarcinom	Dukes C	0	Ano	Matka: karcinom prsu (45)

Diskuse

Od roku 2002 bylo provedeno testování několika rozsáhlých souborů pacientů s hereditárními i sporadickými karcinomy prsu na přítomnost mutace *CHEK2* c.1100delC u nás i v zahraničí. Z výsledků těchto šetření vyplývá, že gen *CHEK2* patří mezi geny s nízkou penetrancí a specificky delece cytosinu v pozici 1100 kódující sekvence může modifikovat riziko vzniku nádorů prsu, kolorekta, nebo prostaty. Výsledky rovněž naznačují, že výskyt nosičů této mutace se v evropském regionu snižuje směrem od severu ke středomoří. Menší počet prací, ve srovnání s pracemi věnovanými karcinomu prsu, je věnován výskytu mutace c.1100delC u pacientů s karcinomem kolorekta a pacientů s HNPCC (11,12,13). I výsledky těchto analýz ukazují na podobný trend daný geografickým rozložením četnosti výskytu alely CHEK2*1100delC, jako je tomu v případě karcinomu prsu. Zatímco studie pacientů s kolorektálním karcinomem v Nizozemí prokázala zvýšení relativního rizika vzniku kolorektálního karcinomu u nemocných s kolorektálním karcinomem (RR=1,5-2,0), podobná práce ve Španělsku nenalezla žádného nosiče mutace c.1100delC u 182 pacientů s HNPCC/HNPCC-like/HBCC. Četnost mutace c.1100delC u pacientů s kolorektálním karcinomem v naší populaci dosahuje 0,69%. Třebaže je tato hodnota

vyšší, než četnost v populaci pacientek s karcinomem prsu (4 ze 1046 pacientek s hereditárním a sporadickým karcinomem prsu; 0,38%), i vyšší než u nenádorových kontrol (0,27%), k potvrzení nálezu by bylo nezbytné vyšetřit velmi rozsáhlou populaci nemocných (10).

Závěr

Výskyt alely CHEK2*1100delC je v České republice nízký. Ačkoliv četnost nálezu mutace v populaci pacientů s kolorektálním karcinomem je vyšší, je pravděpodobné, že nosiči mutace představují méně než 1% všech nemocných. Ze zahraničních údajů vyplývá, že zvýšení rizika vzniku onemocnění je mírné (RR = 1,5-2,0). To spolu s nízkou penetrancí alely omezuje klinickou použitelnost jako predispozičního faktoru pro vznik kolorektálního karcinomu. Na druhé straně, vzhledem k rychlosti stanovení je možné uvažovat o zařazení tohoto vyšetření do bloku genetické analýzy predispozičních genů u osob se zvýšeným rizikem vzniku onemocnění, kde je prováděno genetické testování. Získání dalších výsledků by umožnilo relevantní statistické zpracování četnosti výskytu i stanovení rizika v české populaci.

Seznam zkratek

AFAP - atenuated familial adenomatous polyposis (OMIM 175100), HBCC - hereditary breast and colorectal cancer syndrome; FAP - familial adenomatous polyposis (OMIM 175100), FHA - fork head associated; HNPCC - hereditary nonpolyposis colorectal cancer (OMIM 120435), *CHEK2* - checkpoint kinase 2 (OMIM 604373); *p53* (OMIM 191170); PCR - polymerase chain reaction; DHPLC - denaturant high performance liquid chromatography

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