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Autoreferát dizertační práce

GENETIC FACTORS IN LYMPHOPROLIFERATIVE MALIGNANCIES

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

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Dizertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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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.

ABSTRACT

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.

1. INTRODUCTION

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. The annual incidence rate of NHLs and HL in the Czech Republic is 11.6 and 2.4 cases per 100 000 inhabitants, respectively.[1]

Alongside environmental and lifestyle risk factors (reviewed recently by Alexander et al.[2]), lymphoma development is influenced by so far poorly understood genetic factors. 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 considerable higher risk of NHL development (OR range from 1.5 to 2.9).[3] This association was shown for different types of NHL[4] being even more apparent in aggressive NHL subtypes.[5] The hypothesis, that risk of HL development is also modified by a genetic background is supported by reported increased incidence of HL in monozygotic twins[6] and first degree relatives of lymphoma patients.[7] The genetic susceptibility to NHL in relation to the germline variation in various genes have been reviewed recently.[8]

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.[9]

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.[10]

The ***CHEK2*** (check point kinase 2, CHK2, OMIM 604373) gene 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). 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. 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.[11]

The initial report of Bell et al.[12] 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 performed on large cohorts of cancer patients have shown that these *CHEK2* alterations act as a low penetrance alleles increasing the risk of different cancer types including breast, colorectal, ovarian, prostate, thyroid, or kidney cancers.[13–15] However, the role of *CHEK2* alterations in lymphomas is not clear.

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.[16] 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.[17]

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.[18] 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[19] or cell cycle arrest in G1 phase[20] and in p53-dependent DNA repair efficiency.[21] 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.[22–28]

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. 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*

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. Samples were collected at three Pragues hematological departments between the years 2000 and 2010. Individual control groups for consequent analyses were selected from two populations – non-cancer controls and blood donors.

3.2. *Mutation analysis of CHEK2 gene*

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). 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.[29,30] 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. PCR-amplified fragments were consequently analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE3500; Transgenomic) and samples with aberrant elution profiles on DHPLC sequenced from independent amplifications (ABI 3130; Applied Biosystems). 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.

3.3. *Analysis of copy number variants by 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 according to the manufacturer's instructions with consequent software evaluation in Coffalyser *v*.8 software (MRC-Holland). Large deletion involving exon 8 and 9 of *CHEK2* (c.909-2028_1095+330del5395; Met304Leufs15X) was confirmed by method previously published by Walsh et al. with minor modifications.[31] Other gains or losses identified by MLPA were confirmed by array-based comparative genomic hybridization (aCGH).

3.4. *Genotyping of TP53 R72P polymorphism*

The exon 4 of *TP53* gene (where R72P polymorphism is located) was amplified in 363 bp long fragment and consequently analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE3500; Transgenomic). DHPLC elution profiles in heterozygotic samples were confirmed by bidirectional sequencing on ABI3130 (Applied Biosystems).

3.5. *In silico analyses*

Biological significance of missense variants was evaluated using freely available web-based program Align-GVGD (http://agvgd.iarc.fr),[32,33] intronic variants were evaluated using another web-based program, ESE Finder 3.0 (http://rulai.cshl.edu/tools/ESE).[34,35]

3.6. *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 All analyses were performed using SW Statistica *v*.9.0 (StatSoft) or NCSS *v*.2007 (NCSS).

4. RESULTS

4.1. *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. Overall, 26 different alterations of *CHEK2* were identified. Frequencies of individual alterations in NHL patients and non-cancer controls are summarized in Table 1. 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 1). 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). 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%). Fourteen of *CHEK2* alterations characterized in NHL patients were not described previously (Table 1).

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 (p = 0.02).

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) 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 1). 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 2). Only borderline association was found between I157T mutation and worse OS in DLBCL subgroup (p = 0.055; Figure 2).

Table 1 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.

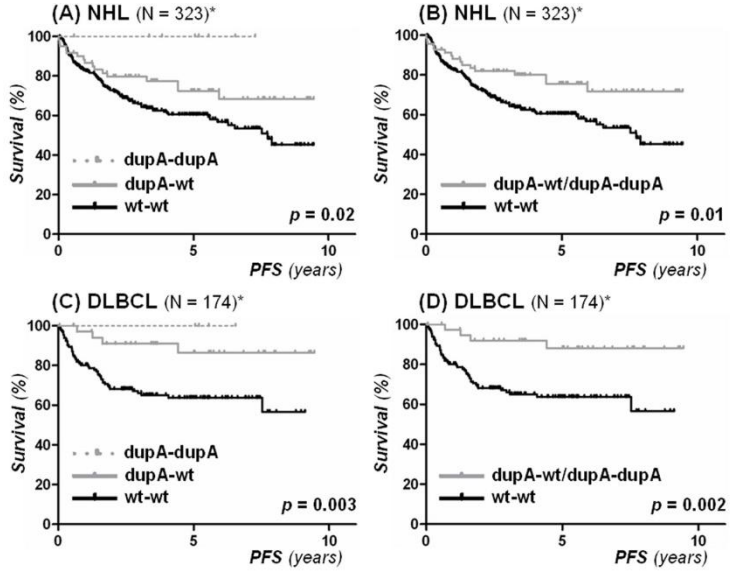

Figure 1
Progression free survival (PFS) curves of non-Hodgkin lymphoma patients (A, B) and patients with diffuse large B-cell lymphoma (C, D) divided according to the presence of **IVS1+43dupA** alteration (in graph legends abbreviated as dupA). Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

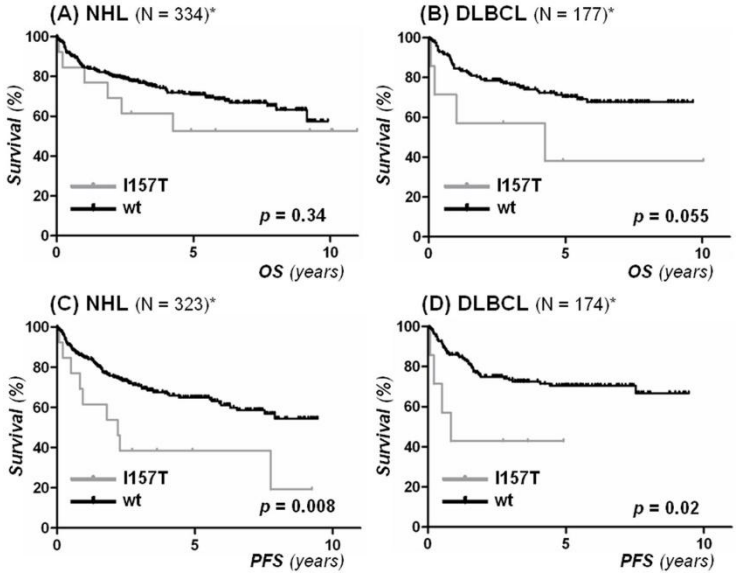

Figure 2
Overall survival (OS) and progression free survival (PFS) curves of non-Hodgkin lymphoma (NHL) patients (A, C) and patients with diffuse large B-cell lymphoma (B, D) divided according to the presence of **I157T** mutation. Log-rank test *p*-values are displayed in each graph.
* Number of patients with available survival data.

4.2. *Copy number alterations*

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[31] were identified (Figure 3). 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. 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. 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. 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. 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.

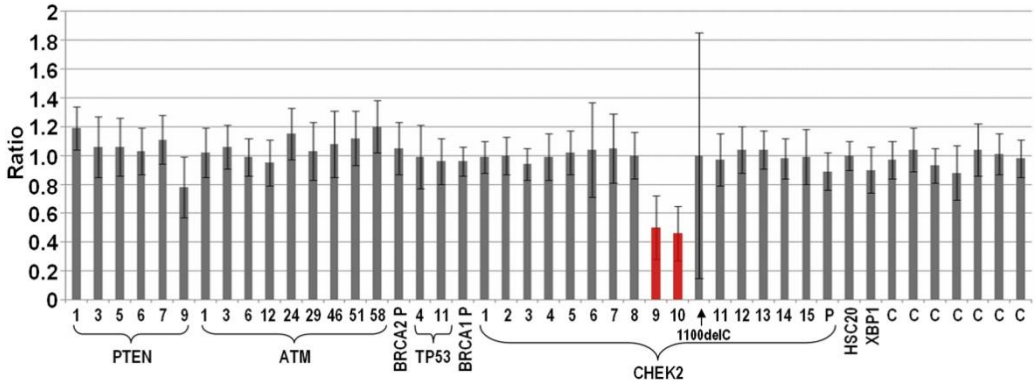

Figure 3 Result of MLPA analysis (kit P190) of L026 sample with del5395 bp of *CHEK2* (coding exons 8+9; in MLPA mix designated as 9+10).

4.3. *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 2). 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 2).

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

Table 2 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.

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. 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 and had no impact on PFS or OS 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.

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.[36]

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

We performed the largest study analyzing the entire *CHEK2* gene in lymphoma patients. 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.[37] 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.[13] 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 alteration within FHA-coding region excluding the most frequent I157T mutation (OR = 5.8; 95% CI 1.1 - 30.1). Polled analysis of all lymphoma cases revealed even stronger association of alterations within *CHEK2* FHA-coding region with higher risk of lymphoma (OR = 2.1; 95% CI 1.2 - 3.7; $p = 0.01$).

The most frequent alteration of FHA-coding region identified among lymphoma patients was c.470T>C (I157T) variant. 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 mutated I157T CHK2 protein is defective in ability to bind some of its protein targets including the p53 protein[38] or the BRCA1 protein[39] *in vitro* and due to the retained dimerization capacity the I157T heterozygotes exerts impaired substrate binding *in vivo*.[40] 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.[41] 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, however, these hypotheses have not been confirmed using functional *in vitro* analyses so far.

No *CHEK2* alteration was ever evaluated as factor influencing lymphoma survival. Alterations within the *CHEK2* FHA-coding region and especially the I157T alteration was 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 represent 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 ($p = 0.002$). 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.

5.2. *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. We have found even more significant association of these alterations with colorectal cancer risk (OR = 2.3; $p = 0.003$).[42] 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.[43] 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).[44,45] Contrary to the sporadic colorectal cancer cases, we did not identify any association of *CHEK2* alterations with the risk of breast and pancreatic cancers.[41,46]

5.3. *Copy number variants*

Deletion of 5395 bp affecting exons 8 and 9 of the *CHEK2* gene was firstly identified by Walsh et al.[31] 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.[47] Association of *CHEK2* del5395 with increased cancer risk has been reported also for prostate cancer[48] but was not found in hereditary non-polyposis colorectal cancer[49] or melanoma.[50] 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[42,46] and thus the role of this alteration could be restricted only to several other cancer types.

5.4. *TP53 R72P polymorphism in lymphomas*

The first study evaluating R72P polymorphism in NHL patients was performed by Hishida at al.[22] 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.[23] 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.[24] 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[25] and the USA,[26] respectively, no correlation of R72P with NHL risk was found. 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. 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 unlikely 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 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 PUBLICATIONS

1. publikace *in extenso,* které jsou podkladem dizertace

   a) s IF

- **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 journal Neoplasma (**IF_{2009}= 1.192**)
- **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**)
- 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)**
- 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)**
- 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.** Cancer Epidemiol 2010; 34(5):656-8 (former name Cancer Detect Prev **IF_{2009}= 2,056; first two authors contributed equally)**

   b) bez IF

- Kleibl Z, **Havránek O**, Novotný J, Kohoutová M, Štekrová J, Matouš B: **Analýza mutace c.1100delC genu *CHEK2* v populaci pacientů se sporadickým karcinomem kolorekta a familiární adenomatózní polypózou.** Klinická onkologie 2007; 20:224-226

2. publikace *in extenso* bez vztahu k tématu dizertace

   a) s IF

- Kleibl Z, **Havranek O**, Kormunda S, Novotny J, Foretova L, Machackova E, Soukupova J, Janatova M, Tavandzis S, Pohlreich P. **The *AIB1* gene polyglutamine repeat length polymorphism and the risk of breast cancer development.** J Cancer Res Clin Oncol 2011; 137:331-338 (**IF_{2009}= 2,216; first two authors contributed equally**)
- Kleibl Z, **Havranek O**, Prokopcova J. **Rapid detection of CAA/CAG repeat polymorphism in the *AIB1* gene using DHPLC.** J Biochem Biophys Methods 2007; 70:511-513 (**IF_{2007}=1,338**)

   b) bez IF