CHEK2 gene alterations independently increase the risk of death from breast cancer in Bulgarian patients

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Checkpoint kinase 2 (*CHEK2*) is a DNA damage-activated protein kinase implicated in cell cycle checkpoint control. The significance of *CHEK2* alterations for breast cancer incidence and clinical behavior is not clear. In this study we determined the mutational spectrum and the level of promoter hypermethylation of *CHEK2* gene in a group of 145 Bulgarian patients with breast cancer. A special emphasis was put on the clinical impact of *CHEK2* alterations for breast cancerogenesis.

PCR-SSCP-sequencing analysis of the entire coding sequence of *CHEK2* gene was performed to estimate the mutational profile of tumor samples. Methylation-sensitive SSCP was applied to determine the methylation status in CpG clusters implicated in *CHEK2* silencing. Clinical significance of *CHEK2* alterations was evaluated using standard statistical methods.

Mutations in *CHEK2* were identified in 9.65 % of the patients. Two novel missense substitutions Thr476Met (C >T) and Ala507Gly (C>G), and a novel silent variant Glu79Glu (A>G) were registered. However, hypermethylation was not found in any of the studied cases. Comparison with clinical characteristics showed that *CHEK2* positive women have predominantly lobular type of breast carcinoma (p=0.04) and PR⁺ status (p=0.092). *CHEK2* mutations correlated significantly with *ATM*⁺ status (p=0.046). All patients with the Glu79Glu variant were progesterone receptor positive (p=0.004). A decrease in overall survival (p = 0.6301) and a threefold increased independent risk of death (HR = 3.295, 95%CI 0.850-12.778, p = 0.085) in *CHEK2*⁺patients was found.

Our data indicate the significance of *CHEK2* gene alterations in contrast to promoter hypermethylation in breast cancerogenesis. Specificity of *CHEK2* mutational profile for the Bulgarian population was found. Though *CHEK2* mutational status correlated with more favorable clinical characteristics, including positive progesterone receptor and lobular histological type, it independently increased the risk of death in these patients.

Key words: breast cancer, CHEK2, mutation, hypermethylation, clinico-pathological characteristics, overall survival

Breast cancer (BC) is the most common female malignancy and the most frequent cause of female cancer mortality. The incidence of BC in Bulgaria amounts to 23% of all women cancers, with about 3800 new cases diagnosed each year [1]. BC tumorigenesis is a complex, multi-step process, in which each step is thought to correlate with one or more abnormal genetic as well as epigenetic changes in genes involved in cell regulation [2, 3].

Cell cycle checkpoint kinase 2 (*CHEK2*, also known as "*CHK2*" [MIM 604373]) is a multifunctional kinase with a key significance for cell cycle regulation in response to DNA double strand breaks thus contributing to maintenance of genome stability [4, 5]. The human *CHEK2* gene consists of 14 coding exons and spans approximately 50 kb of genomic DNA. It encodes a 543 amino acids nuclear serine-threonine kinase with three characteristic domains: an N-terminal SQ/TQ cluster domain (amino-acid residues 20–75), a fork head-associated (FHA) domain (residues 112–175) and a serine/threonine kinase domain (residues 225–490) [6]. CHEK2 kinase is expressed in the vast majority of normal human tissues including many nonproliferating, terminally

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Abbreviations: WT – wild type; T – tumor size; N – nodal status; G – grade of malignancy; HT – histological type; ER – estrogen receptor; PR – progesterone receptor. Significant p values are in bold.

differentiated cells [7]. In response to DNA damage induced by ionizing radiation, CHEK2 is activated through phosphorylation of Thr68 by ATM protein [5, 8-10]. When double strand breaks are caused by UV irradiation or hydroxyurea treatment, CHEK2 is likely to be activated by ATR kinase [10, 11]. After oligomerization and auto-phosphorylation [12], CHEK2 kinase moves throughout the nucleoplasm to transmit the damage signal by phosphorylating several essential cell-cycle proteins including: p53 [11], Cdc25A [12] and Cdc25C phosphatases [9] and BRCA1 [13]. This triggers cellular pathways are involved either in cell-cycle arrest at G1/S, S, and G2/M phases [14, 15] and DNA repair, or apoptosis [5, 16].

The first evidence suggesting implication of *CHEK2* in cancer development was the germline mutation *1100delC* found in several Li-Fraumeni and Li-Fraumeni–like families [17]. Importantly, germ-line mutations within the *CHEK2* gene have been reported in a subset of Li-Fraumeni families who have a wild-type *p53*, suggesting that *CHEK2* may function as a tumor suppressor. The consideration of *CHEK2* as a new tumor suppressor gene has been supported by the identification of germline and somatic mutations in a range of human tumors including breast, colon, ovarian, vulva, prostate and others [18-26]. On that basis *CHEK2* is proposed as a multiorgan cancer susceptibility gene [27]. *CHEK2* frequency and spectrum of mutations displayed considerable differences between populations and between familial and sporadic cases. Lately the *1100delC* variant is evidently associated with a threefold increased risk of breast cancer in heterozygous women from the general population and may also increase the risk of other cancers [28]. In contrast to germ-line mutations in family cases, which are extensively studied, limited data on *CHEK2* mutational frequency and spectrum in sporadic breast cancer is available. The *CHEK2* somatic mutations in sporadic breast cancer are relatively rare [29], and some studies assumed the possibility that, except genetic alterations, alternative mechanisms like epigenetic promoter hypermethylation could silence *CHEK2* expression [20]. Promoter hypermethylation may serve as a basis for cancer development and has been described in several tumor suppressor genes like the *RB* gene in retinoblastoma [30], the *VHL* gene in clear cell renal carcinoma [31], the *ATM* gene in head and neck squamous cell carcinoma [32], the *BRCA1* gene in sporadic breast cancer [33] and others. Limited studies investigated the hypermethylation status of *CHEK2* gene in breast [20, 29], lung [34, 35], lymphoid [36], non Hodgkin lymphomas [37], vulval [22], ovarian [20], colorectal tumors [38], gliomas [39].

So far, a general clinical impact of *CHEK2* cannot be established due to the limited populations studied and the specificity in type and frequency of the found alterations. This study represents a combined genetic and epigenetic analysis of *CHEK2* gene in sporadic breast cancer with a special emphasis on evaluation of *CHEK2* clinical significance. The frequency and spectrum of genetic alterations and the level of epigenetic promoter hypermethylation in *CHEK2* were determined in a group of 145 breast cancer patients. Specific mutational profile of *CHEK2* gene for the Bulgarian population was found. Statistically significant clinical correlations were registered.

Patients and methods

Breast tumors were obtained from 145 Bulgarian female unrelated patients who were treated at the Thoracic Surgery Clinic of the National Oncological Centre (Sofia, Bulgaria) between 2000 and 2003. Cases were staged according to the TNM classification of Union International Contre le Cancer (UICC). The age of breast cancer women was in the range of 29–88 years, with a mean age of 54.4 years. Histologically, 130 invasive ductal carcinomas and 15 invasive lobular carcinomas were identified. Six patients had highly differentiated tumors (G1), 103 – moderately differentiated (G2) and 36 – poorly differentiated (G3). Ninety-two patients had negative nodal status and 53 – positive. Sixty-four patients were ER-negative, 81 – ER-positive. PR-negative were 64 patients, PR-positive – 81. Tumor samples were previously characterized according to *p53*, *BRCA1*, *ATM*, *PIK3CA* mutational status and *HER2* level of expression [40]. Patients underwent adjuvant therapy according to the accepted practice guidelines at that time. The women were followed for a median period of 69 months (range from 11 to 96 months). The follow-up time began at the time of surgery and ended in November 2008 or at the date of death. Blood samples of clinically healthy persons were used as negative controls. Written consent was taken from all participants in the study. Clinical information was obtained from the existing medical records and is presented in a way preventing patients' identification.

DNA isolation. Tumor DNA was isolated from fresh frozen breast tumors by a standard Proteinase K/Phenol procedure including tissue homogenization in lysis buffer (10mM Tris/HCl pH 8.3, 400mM NaCl, 2mM EDTA-Na, 0.14mg/ml Proteinase K, 1% SDS) at 37°C for 48 h, phenol/chloroform/isoamyl alcohol purification and ethanol precipitation. DNA was isolated from blood sample of healthy controls by a standard procedure. DNA concentration and purity was determined using BioSpec-nano Spectrophotometer (Shimadzu Biotech).

Single-strand conformational polymorphism analysis. The 14 coding exons of the *CHEK2* were amplified using 15 primer sets according to Liu Wei-Dong et al [41] (Table 1). PCR reactions were done in a total volume of 25 µl containing 50 ng of template DNA, 1x PCR buffer, 2.25 mM $MgCl_2$, 0.2 mM of each dNTP, 100 nM of each primer and 0.5 units of Taq DNA polymerase (Netzyme). The cycling reactions included 35 cycles of denaturation at 94°C for 30 sec (first cycle – 5 min), annealing at the appropriate temperature (see Table 1) for 30 sec and elongation at 72°C for 30 sec (last cycle – 12 min.).

The amplified products were denatured in 95% formamide, 0.01 N NaOH (1:1.4) at 95°C for 10 min and separated on

Table 1. *CHEK2* **primers used for SSCP and for methylation-sensitive SSCP analysis**

Exon/ Primer	Sequence (5´-3´)	Annealing temperature (°C)
PCR primers used for SSCP analysis		
1a	**F** TAAGTCTTGTGCCTTGAAACTCACC **R** AGAGCTGGAGTGAGAGGACTG	**64**
1b	**F** CCAGCACGATGCCAAACTCCA **R** CGTGATACTATACAACAAAGGGTC	**64**
2	**F** TCAACAGCCCTCTGATGCATGC **R** CAGCTCTCCTAGATACATGGGTAT	**64**
3	**F** GGAGAGCTGGTAATTTGGTC **R** TTTCCTCCTATGAGAGAGTGG	**64**
4	**F** GAATCAGTGATCGCCTCTTGTG **R** GAGAAACCACCAATCACAAATGTATAGTG	**65**
5	**F** ACCCAGAGTGGTAGGTCTC **R** TGATCAGCCTTTTATTGGTACTTA	**63**
6	**F** GGCAGCCTTGAGTCAACTGAG **R** TACTGAAAGGCTTTATACTCTTCTC	**62**
7	**F** GGGAGTTTCTCACTACTTTCCC **R** CAGGATGAGAAAGGCAAGCCTACA	**64**
8	**F** CCCTTGCCTTGCCTTTGTGTTTC **R** GGCAGCTGTCAAAAGAATTGAGG	**64**
9	**F** ACGGCTTACGGTTTCACC **R** CAAGAATCTACAGGAATAGCC	**64**
10	**F** CTTGGACTGGCAGACTATG **R** CTCCTACCAGTCTGTGCAG	**62**
11	**F** GCCACTGAGAATGCCACTTGATTTC **R** CCACCACAGCACATACACATT	**64**
12	**F** ACTGTGATTTGCCCAATTGTTGC **R** ATGCTAGCAGGCACTGTCCC	**64**
13	**F** ATGCTAGCCCTGTCATTCTAGGA **R** CTACATTTAGTGATCATCAGGAATACG	**63**
14	**F** CTTTACTGGAAGCATATTGAGG **R** TTAATTGTACATCAGTGACTGT	**55**
PCR primers used for methylation-sensitive SSCP analysis		
MIP1	**F** TTGTTGTTTAGGTTGGAGTGTAGTG **R** AAAAACTCATAAAAACTTACATTTATCCA	**56**
MIP2	**F** GAGTGGGGTTAGGTGTTTGTAG **R** CCAACCAATCAACAACATTAATCTA	**59**
MIP3	**F** GGTTTTTTTATTTTTTTTAATTTTAG **R** AACAAATTCTTCTACCCACAATACC	**52**
MIP4	**F** TTTTTGAGAAATTAAGATATTTTTTTTA **R** AACAAATTCTTCTACCCACAATACC	**50**
MIP5	**F** GTTATTTTGTTGGTTGAGGTTG **R** TCTTCTTATCCTATATATTCAAACCTATTT	**50**

native polyacrylamide gels in 1xTBE buffer at 15 mA, 4°C. The polyacrylamide gel concentration varied between 6 to 14% depending on the exon. Modified silver staining method was applied for visualization. Briefly, the gels were soaked in 50% methanol for 1 h, then transferred to ammonium–silver solution, containing 0.02 N NaOH, 0.375% NH_4OH, and 0.8% $AgNO_3$ for 15 min and washed with 500 ml dH_2O for 10 min. The staining reaction was developed by the use of 0.01% citric

acid, 0.035% formaldehyde, and the gels were fixed in 0.5% acetic acid.

Amplified products with altered electrophoretic mobility were purified (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences) and sequenced on ABI PrismTM 310 fluorescent sequencing analyzer (DYEnamicTM ET Terminator Cycle Sequencing Kit, Amersham Biosciences) following the manufacturers' instructions. To confirm the mutation all mutant samples were subsequently sequenced in the reverse direction. The sequence data of *CHEK2* gene was obtained from GenBank.

Methylation-sensitive SSCP. The methylation status of *CHEK2* promoter was determined by Methylation-sensitive PCR- SSCP analysis of sodium bisulfite-converted DNA. To convert DNA, EZ DNA Methylation KitTM was used following the manufacturer's recommendations (Zymo Research Corporation). One µg of DNA of each sample was subjected to conversion. The obtained pure modified DNA was further PCR analyzed using four sets of primers (MIP2, MIP3, MIP4, and MIP5) covering a total of CpGs in the distal CpG island (located 6,000-8,000 bp upstream of the translation initiation site), and one set of primers (MIP1) targeting the proximal CpG region (300-600 bp upstream of the translation initiation site) (Table 1). Primer sequences and amplification conditions were according to Louise H. Williams [20]. The PCR products were SSCP analyzed as described above. We included in each assay a totally methylated *in vitro* DNA (Zymo Research) as a positive control, and a normal blood sample DNA as a negative control. The methylation status of representative methylation-sensitive SSCP products was confirmed by bisulfite sequencing (ABI PrismTM 310).

Statistical analysis. The relationship between *CHEK2* alterations and clinico-pathological characteristics of patients was evaluated by Fisher's exact test and the Chi-squared test. Overall survival (OS) was estimated by the Kaplan-Meier method, and differences between survival curves were assessed for statistical significance using the log-rank test. Cox proportional hazards regression model was used to calculate the hazard ratios (HR) and their 95% confidence intervals (95%CI) for each variable in the univariate and multivariate analyses. All p-values were two-sided, and results were considered statistically significant at p less than 0.05. Analyses were done with the SPSS software package (SPSS Inc., Chicago, IL, USA).

Results

Mutational analysis of *CHEK2* gene. The group of 145 BC patients was assessed for *CHEK2* mutations by PCR-SSCP-Sequencing analysis. Mutations in *CHEK2* were found in 14 separate patients who had no family relationship (14/145 i.e. 9.65 %). None of the patients carried more than one *CHEK2* mutation. All mutations were visualized as extra bands with altered electrophoretic mobility in the mutant samples
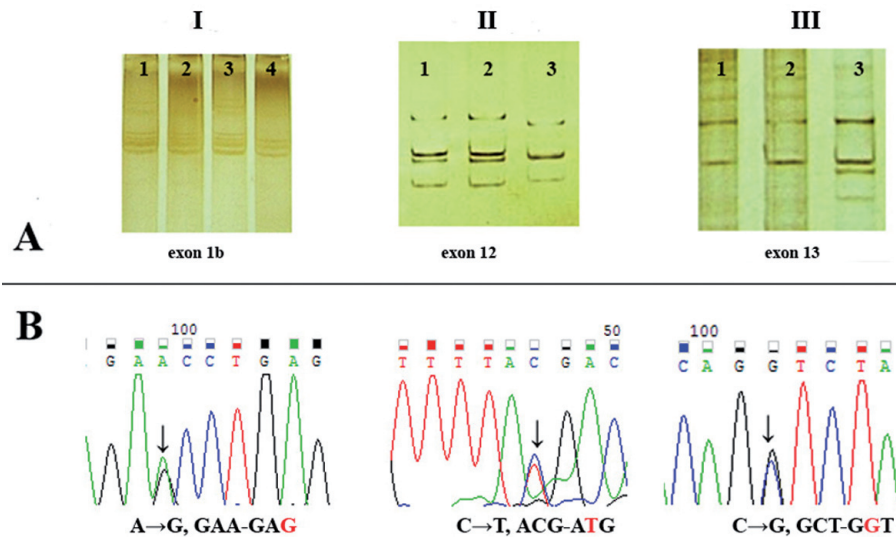
Figure 1 A – SSCP analysis of *CHEK2* gene of I – exon 1b, aberrant profile (lanes 1, 2, 3) and normal profile (lane 4); II – exon 12, aberrant profile (lanes 1, 2) and normal profile (lane 3); III-exon 13 aberrant profile (lane 3) and normal profile (lanes 1, 2). The normal profile comes from healthy controls; B – Sequence analysis with the forward primer of the samples with the respective mutant pattern

(Fig. 1, I A – lanes 1, 2, 3; II A – lane 1 and 2; III A – lane 3). Healthy persons were used as negative controls in the PCR-SSCP analysis (Fig. 1, I A – lane 4; II A – lane 3; III A – lanes 1 and 2). The PCR products with aberrant migration on the SSCP gel were purified and sequenced to determine the type of mutation (Table 2). *CHEK2* mutations were mapped to exons 1b, 12 and 13. All of the found alterations were novel and were either missense or silent. We found nine identical silent variants A to G at a position 237 bp (Glu79Glu) (Fig. 1, I B), three C to T base substitutions at a position 1427 bp, leading to Thr476Met amino acid change (Fig. 1, II B), and two C to G substitutions at a position 1520 bp altering the amino acid composition of the protein (Ala507Gly) (Fig. 1, III B).

Methylation analysis of the *CHEK2* promoter. Methylation-sensitive SSCP was used to determine the methylation status of two distinct CpG clusters located upstream of the ATG. To assess the methylation status two controls – negative (blood DNA isolated from healthy persons) and positive (totally methylated *in vitro* DNA) were used in each electrophoresis. In all tumor samples the distal CpG cluster is not methylated and showed migration profile identical with the

negative control. Reversely, the proximal CpG cluster is totally methylated and had migration profile identical with the positive control in all of the cases.

Mutational gene status in relation to clinico-pathological and molecular characteristics. To estimate the clinical impact of altered *CHEK2* by itself, independent of the type of alteration, we compared the *CHEK2* genetic status of the patients with standard prognostic factors including age of diagnosis, tumor size (T), nodal (N) status, grade of malignancy (G), histological type (HT), estrogen (ER) and progesterone receptor (PR) status (Table 3; Column "*CHEK2* status"). In addition to the clinico-pathological characteristics of the patients, several tumor molecular characteristics previously found as *p53*, *ATM*, *PIK3CA, HER2* status were included in the analysis [40] (Table 3). We found that *CHEK2* mutational status correlated significantly with lobular histological type (p=0.04). A tendency for higher frequency of the PR+ status in *CHEK2*+ patients was found (p=0.092).

To identify if the specific type of the found *CHEK2* variants has a specific clinical impact we further divided the *CHEK2* positive patients into two groups: one with missense variants and the other – with silent variants. Patients with the missense

Table 2. Spectrum of *CHEK2* mutations found in the studied Bulgarian BC patients.

Number of patients	Exon	Position (bp)	Codon	Nucleotide change	Amino acid change	Type of mutation
9	1b	237	79	A→G; GA**A**-GA**G**	Glu – Glu	Silent Glu79Glu (A>G)
3	12	1427	476	C→T; A**C**G-A**T**G	Thr – Met	Missense Thr476Met (C>T)
2	13	1520	507	C→G;G**C**T-G**G**T	Ala507Gly	Missense Ala-Gly (C>G)

Table 3. *CHEK2* **status in relation to clinico-pathological and molecular characteristics**

Variable		CHEK2 status				CHEK2 missense variants			CHEK2 silent variants		
		Total (n = 145)	Positive (n = 14)	Negative (n = 131)	*p value*	Positive (n = 5)	Negative (n = 140)	*p value*	Positive (n = 9)	Negative (n = 136)	*p value*
Age	Years (mean)	54.4	54.71	54.37	1	53.8	54.42	0.655	55.2		0.722
	Range	29-88	32-74	29-88		32-74	29-88		35-70	29-88	
	<50	52	5	47		1	51		4	48	
	>=50	93	9	84		4	89		5	88	
Tumor size	T1	65	8	57	0.401	3	62	0.657	5	60	0.514
	T2-T4	80	6	74		2	78		4	76	
N status	N0	92	8	84	0.771	3	89	1	5	87	0.724
	N+	53	6	47		2	51		4	49	
G	G1	6	1	5	0.473	1	5	0.193		6	0.332
	G2	103	8	95		3	100		5	98	
	G3	36	5	31		1	35		4	32	
Histological type	Lobular	15	4	11	**0.04**	2	13	0.083	2	13	0.234
	Ductal	130	10	120		3	127		7	123	
ER expression	Positive	81	8	73	1	5	76	0.067	3	78	0.183
	Negative	64	6	58		-	64		6	58	
PR expression	Positive	81	11	70	0.092	2	79	0.654	9	72	**0.004**
	Negative	64	3	61		3	61			64	
p53 mutation	Positive	16	1	15	1	1	15	1		16	0.597
	Negative	129	13	116		4	125		9	120	
ATM mutation	Positive	11	2	9	0.287	2	9	**0.046**		11	1
	Negative	134	12	122		3	131		9	125	
PIK3CA mutation	Positive	45	5	40	0.764	2	43	1	3	42	1
	Negative	99	9	90		3	96		6	93	
HER2	Overexpressed	21	1	20	0.679	1	20	1	1	20	1
	Normal	78	8	70		3	75		4	74	
	Unknown	46	Unknown	Unknown		Unknown	Unknown		Unknown	Unknown	

types Thr476Met (three patients) and Ala507Gly (two patients) were combined in a common group due to their limited number. The results showed that *CHEK2* missense variants were significantly associated with *ATM*+ status (p=0.046) (Table 3; Column "Missense variants"). All patients with *CHEK2* missense mutations were ER positive (p=0.067). Statistically significant correlation (p=0.004) was found between the silent variants and PR status (Table 3; Column "Silent variants"). All patients with silent variants were progesterone receptor positive.

Mutational gene status and overall survival. Overall survival of the studied group was estimated to 75.86% (110/145). Kaplan-Meier analysis showed a decrease in OS of patients with *CHEK2*+ status compared to *CHEK2*- though not statistically significant (p = 0.6301).

To estimate the hazard ratio (HR) according to the *CHEK2* status, univariate and multivariate Cox proportional-hazards regression model was used (Table 4). The multivariate model included the clinico-pathological and molecular characteristics of the patients discussed above. A threefold independent increase in the risk of death (HR = 3.295, 95%CI 0.850-12.778, p = 0.085) in *CHEK2*+ patients was found.

Another independent marker which strongly correlated with increased mortality rate was the tumor size (HR = 4.701, 95% CI 1.536–1.392, p = 0.007). Negative, though not independent effect on survival had p53 mutations (HR = 2.28, 95% CI 0.999-5.239, p = 0.05).

Discussion

Breast cancer is rather too heterogeneous and no general molecular phenotype related of the disease could be outlined. Breast carcinogenesis is a result of disruption of cellular pathways, in which key tumor suppressor genes and proto-oncogenes are involved: *BRCA1* and *BRCA2* genes, *p53*, *ATM*, *PIK3CA, HER2, CHEK2, PTEN* and others. Of the BC related genes, *CHEK2* is a key kinase implicated in the regulation of the cell cycle. Despite the intensive studies during the recent years, the complete mutational spectrum and the clinical significance of *CHEK2* have not yet been fully elucidated. The established *CHEK2* mutational profile shows considerable heterogeneity, both with respect to spectrum and frequency of mutations, and their presence (or absence) in the different populations [6, 42]. Several moderately penetrant *CHEK2*

Table 4. Multivariate and Univariate survival analysis

Factor	Multivariate			Univariate		
	HR	95% CI	p	HR	95% CI	p
CHEK2	3.295	0.85-12.778	0.085	1.290	0.455-3.654	0.632
p53	1.310	0.456-3.757	0.616	2.288	0.999-5.239	**0.050**
PIK3CA	0.480	0.179-1.287	0.145	0.502	0.219-1.149	0.103
ATM	1.958	0.391-9.802	0.414	1.084	0.331-3.543	0.894
HER2	0.874	0.300-2.547	0.805	1.266	0.505-3.174	0.616
Age	0.716	0.286-1.793	0.476	1.283	0.629-2.620	0.493
Grade of malignancy	1.332	0.539-3.293	0.535	1.469	0.764-2.824	0.249
Tumor size	4.701	1.536-1.392	**0.007**	1.957	0.958-3.996	0.065
Nodal status	0.962	0.381-2.425	0.934	0.984	0.495-1.956	0.963
Estrogen receptor	0.953	0.375-2.424	0.920	1.440	0.725-2.859	0.297
Progesterone receptor	0.427	0.174-1.048	0.063	0.525	0.268-1.027	0.060

Abbreviations: HR, hazard ratio; CI, confidence interval; Significant *p* values are in bold

cancer susceptibility mutations, increasing the risk of breast cancer have been detected: *1100delC, I157T, S428F* and *del5395* [18, 28, 43-45]. These variants seem not to be present in all populations; their frequency varies in the general population, and is higher in breast cancer patients. In addition, some rare, missense mutations, small deletions and nonsense mutations have been identified [21, 46-48]. Some of these missense CHEK2 mutants have been shown to be unstable or to affect the activation of the encoded protein, indicating that they are likely to be pathogenic [49-50]. No data on *CHEK2* mutational spectrum in Bulgarian patients with breast cancer was so far available. Our results showed specificity in the spectrum of *CHEK2* mutations. Interestingly, the *CHEK2* variants discussed above are not present in the studied group of Bulgarian patients. However, novel alterations were found: Glu79Glu (A to G), Thr476Met (C to T), Ala507Gly (C to G). The mutation Thr476Met (C to T) maps to the kinase domain of CHEK2 and is likely to disrupt the main function of the protein. The Glu79Glu (A to G) variant is located between SCD and FNA domains, and the mutation Ala507Gly (C to G) is close to the C – terminal. Although these two variants do not affect the functional domains of the protein, they may alter the stability of mRNA and protein as well as its interactions with other proteins.

Our results showed that mutational *CHEK2* status significantly correlated with several clinico-pathological parameters including lobular histological type and positive progesterone receptor status. Statistically significant correlation with the lobular histological type is visible when mutated *CHEK2* is considered independent of the type of mutation. The observation was not registered when separate variants were taken into account, probably due to the smaller size of the excerpt. However, the tendency that patients with a *CHEK2* alteration had lobular type of breast carcinoma remained. Invasive lobular carcinoma is the second most common type of breast cancer (10-20%). It is considered to have a better prognosis than ductal carcinoma [51] with better survival rates of patients

[52]. Similar to our findings Huzarski et al. [53] suggested a strong association of *CHEK2 I157T* allele with lobular breast carcinoma among BC patients in Poland. Progesterone and estrogen receptors are considered as breast cancer biomarkers with implications in prognosis and therapy. Over 70% of breast cancers are ER and PR positive [54], and often respond to selective ER modulators such as Tamoxifen, which acts to inhibit estrogen by blocking the ER [55]. When hormone therapy is applied in ER positive patients, good therapeutic outcome is to be expected. Several studies found a correlation between *1100delC* mutation and hormone receptor positive status [56, 57].

In addition to clinico-pathological parameters, the genetic status of several BC related genes was investigated in relation to *CHEK2* status. Most of these genes are involved in pathways which regulate the cellular response to genotoxic stress [58]. CHEK2 kinase directly interacts with some of these proteins, including ATM kinase and p53 transcription factor. In this study we found an interesting association between *CHEK2* missense mutation and *ATM* positive status. *ATM* gene is a sensor of DNA damage and upon ATM inactivation the signal transduction to the target genes is disrupted. The impossibility of effective DNA repair leads to accumulation of mutations, thus the dysfunction of ATM and CHEK2 can further facilitate tumorigenesis [59, 60]. We also found a relationship between *CHEK2* and *p53* mutational gene status. Of a total of sixteen *p53* positive tumors [40] only one had in addition a mutation in *CHEK2*. It seems like *p53* and *CHEK2* mutations somehow are mutually incompatible. In support to this is the observation that germ-line *CHEK2* mutations are present in Li-Fraumeni families with a wild-type *p53*.

Although mutated *CHEK2* correlated with more favorable clinical characteristics (lobular histological type and PR+ status), it increased the risk of death in positive patients. A possible explanation of this observation is that *CHEK2* mutational status has by itself a negative prognostic charge, which is reduced under the influence of the positive prognostic indica-

tors. The positive PR status by itself, as well as the lobular type of tumors, presumes a good therapeutic response and better outcome of patients. In combination with *CHEK2* mutations, however, a slight increase in the risk of death (Univariate Cox proportional hazard regression model) and decrease in overall survival (Kaplan-Meier analysis) were registered. When the influence of all studied tumors and molecular characteristics are taken into account and their effect is neglected (in the Multivariate Cox proportional hazard regression model), the independent impact of mutated *CHEK2* can be observed. Thus, the independent *CHEK2+* considerably increased the risk of death in mutation carriers.

Several studies presume the implication of epigenetic (in addition to genetic) mechanisms in loss of *CHEK2* function [20]. A possible epigenetic cancer related event is the promoter hypermethylation. Hypermethylation in *CHEK2* promoter was found in lung [34], colorectal cancer [38] and Hodgkin's lymphoma cell lines [36]. Higher *CHEK2* hypermethylation frequency was detected in non-small-cell lung carcinoma – 28.1%, and in colorectal cancer – 10% [34, 38]. In gliomas *CHEK2* promoter was hypermethylated only in its proximal high CpG dinucleotides region [39]. With respect to BC, limited data available showed lack of abnormal *CHEK2* hypermethylation [20, 29, 61]. This was also supported by our results, which are similar to the results of Williams LH et al. [20]. The data indicated that the proximal CpG cluster is totally methylated in all tumor samples. Such methylation profile is also present in the studied normal tissue samples, which indicates that it is probably not related to breast tumorigenesis. Reversely, the distal CpG cluster is not methylated either in tumor samples, or in the controls. This data suggest that the distal CpG region may not be significant in regulation of gene transcription and therefore in tumorigenesis.

Conclusion

This study provided the first data on the genetic profile and promoter methylation status of *CHEK2* gene in Bulgarian breast cancer patients. Our data indicate the significance of genetic inactivation of *CHEK2* in sporadic breast carcinogenesis. However, *CHEK2* hypermethylation is probably less essential in this cancer type. Several novel *CHEK2* variants were registered presuming specificity of *CHEK2* mutational profile for the Bulgarian population. Though *CHEK2* mutational status correlated with more favorable clinical characteristics, including positive progesterone receptor and lobular histological type, it independently increased the risk of death in these patients.

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