HER-2 [Ile655Val] polymorphism in association with breast cancer risk: a population-based case-control study in Slovakia*

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Breast cancer belongs to the most frequent types of cancer affecting women and it occurs at any age. Around 1600–1800 women are getting ill annually in the Slovak republic. One of the most important factors in connection with cancer genesis refers to changes in specific genes.

HER-2 proto-oncogene belongs to low penetrating genes, which increase susceptibility to breast cancer genesis. Clinical studies demonstrated an association between polymorphism at codon 655 of this gene and increased risk for breast cancer development.

The aim of this case-control based prospective study was to determine the distribution of HER-2 genotype and its association with risk factors of breast cancer in the population of women in Slovak republic. HER-2 genotypes were determined with PCR-RFLP method. The DNA was isolated from white blood cell nuclei.

The frequency of Val allele in the cancer group was 29.79% and was higher than in the control group 15.84% ($p<0.05$). The presence of the heterozygote (Ile/Val) genotype was identified in 46.81% of patients in the case group and in 28.33% in healthy individuals, and the homozygote (Val/Val) genotype in 6.38% and 1.67, respectively ($p<0.01$). The risk of breast cancer development for carriers of one valine (Val) allele in genotype was two-times lower (OR=2.47) than for carriers of two Val alleles (OR=5.73) ($p<0.05$). Risk of cancer genesis for Val allele carriers was higher in multiparas (OR=2.90), among women with positive family history of breast cancer (OR=5.0), BMI>24 (kg/m^2), and late menopause (OR=1.5). Contraceptives in anamnesis contrariwise showed tend to decrease the risk in Val allele carriers (OR=0.3).

In conclusion, this study revealed relatively high frequency of the Val allele among the women population of the Slovak republic. Ile655Val polymorphism of HER-2 gene was associated with a statistically significantly increased risk of breast cancer all above in homozygotes for Val allele.

Key words: breast cancer, HER-2, polymorphism, allele, risk, ethnic distribution

Breast cancer belongs to the most frequent types of cancer affecting women and it occurs at any age [1]. Around 1600–1800 women are getting ill annually in the Slovak Republic, and more than 750 out of them die. Predominantly, this disease affects women between ages of 45 to 65, but there is still an increasing rate of young women affected by this disease. Following several meta-analytic studies it is known, that the incidence of breast cancer is different in individual populations, ethnic and geographic locations [2–5]. Development of cancer is affected by risk factors (modifiable, or non-modifiable). One of the most important ones in connection with cancer genesis that can not be affected are changes in specific genes [6–8].

Hereditary occurrence of breast cancer is most often caused by mutation of well defined, high penetrance genes like BRCA1 and BRCA2 [9–11]. Apart from these genes there are also low penetrance genes, which increase susceptibility to breast cancer genesis, such as some proto-oncogenes and genes involved in estrogen metabolism or immunomodulatory cell pathways [12]. Genes of low penetrance are defined as genes in which subtle sequence variants or polymorphisms may be associated with a small to moderate

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increased relative risk for breast cancer. Such variants are relatively common in the population and as such may confer a much higher attributable risk in the general population than rare mutations in high penetrance cancer susceptibility genes such as BRCA1 and BRCA2 [13]. One of such low penetrance genes is HER-2 (human epidermal growth factor receptor 2) proto-oncogene. Changes in HER-2 gene have been observed in several studies in association with the prognosis or treatment prediction. Determination of HER-2 status is already a normal part of the diagnostic protocol.

The proto-oncogene HER-2 (synonyms HER2/neu, c-neu, c-erbB-2, ERBB2) is located on the long arm of chromosome 17, exactly on 17q21.1 and encodes a p185-kDa integral type I glycoprotein with an extracellular domain rich in cysteine, transmembrane domain and intracellular domain with tyrosine-kinase activity [14–17]. The extracellular domain may be shed into the blood stream from the surface of the breast cancer cells and serve like tumor marker in patients serum [18].

HER-2 protein serves as a receptor for growth factors [19, 20] and belongs to the HER cell growth factors receptor family. The growth factors binding upon these receptors regulate cell growth, proliferation rate, differentiation, adherence and motility through dimerization of various HER receptor combinations. Activation of the HER-2 proto-oncogene leads to loss of its regulation functions, which may evoke uncontrolled cell growth, as an ingredient of oncogenous transformation [21]. Several clinical studies demonstrated association among estrogen and progesterone receptor absence, high rate of cell proliferation, tumor aneuploidy, bad grading, higher tumor aggressiveness, large tumor size, reduced response to chemo- and hormonal therapy, rapid metastasis spreading into lymph nodes, cancer appearance in young women and HER-2 gene amplification with HER-2 protein overexpression [22, 23]. The alterations of this gene are found in 20–30% of all human breast cancers [24, 25]. Amplification of HER-2 proto-oncogene is the most frequent gene amplification found in breast tumors, and it looks to be the first change in the initiation of oncogenous progression to invasive cancer [26].

Mutations in the human HER-2 gene have not been identified, but sequence analysis of cDNA determined a polymorphism in codon 655 in the DNA region coding a transmembrane domain [27]. A polymorphism of a nucleotide in this codon results in the transition ATC/isoleucine to GTC/valine [Ile(655)Val]. The frequency of Val allele of this polymorphism varies between populations and is present in up to 24% with significant increase of frequency among women affected by breast cancer [28]. In some studies [4, 29] this polymorphism was associated with increased risk of breast cancer, especially in young women or in those at increased risk of localized cancer [16], but subsequent larger studies showed disappointing results, that support the findings that there is no association between breast cancer and this single nucleotide polymorphism [30–33]. Based on this, and the fact that up to present only patients from Asia, America and Western Europe countries were investigated, we decided to determine the distribution of HER-2 genotype and its association with risk factors of breast cancer in the population of the Slovak republic.

Material and methods

Human subjects. This project was a case-control based prospective study. Case patients and control subjects included 110 Slovak women at the age ranging from 20–81 years and were divided into two groups. Written informed consent created an inevitable condition to enter the study. The committee of ethics for the use of human subjects in research approved the study protocol. Subjects in the control group were randomly selected from female population, while patients in the case group were women with diagnosed breast cancer, being followed-up or those who previously underwent an operation because of breast cancer. The disease (n=47) was verified by histological examination. The control group (n=60) was represented by healthy, clinically asymptomatic women without a past history of breast disease (benign, malignant) and without any history of breast disease in their family. These women underwent special gynecological investigation of the breast including inspection and palpation (BCE), ultrasonography or mammography, which confirmed negative finding of the breast. All women in this trial took a structured questionnaire to obtain patient history and risk factors in relation to breast cancer development. The questionnaire was concentrated on demographic and lifestyle characteristics, reproductive and menstrual history, physical activities, alcohol and/or tobacco dependencies, estrogen exposure, anthropometrical variables and family history. Three women were excluded from the study because of incomplete questionnaire information. All patients and controls belonged to the Slovak population and were residents of the same geographic area (Slovak republic).

DNA extraction and PCR. Patients eligible for the study subsequently underwent DNA testing. From each woman 10 ml of peripheral venous blood sample from vena cubitalis was taken and stored into tubes containing 0.5 M EDTA. The DNA was isolated from white blood cell nuclei by NaCl precipitation method. Appropriate HER-2 genotypes were determined with polymerase chain reaction (PCR)-restriction fragment length polymorphism-based (RFLP) method. Sequence containing examined polymorphism of HER-2 gene was amplified by PCR with forward primer (5'-AGAGCGCCAGCCCTCTGACGTCCAT-3') and a reverse primer (5'-TCCGTTTCCTGCAGCAGTCTCCGCA-3'). The PCR products were digested by restriction endonuclease *Bsm*A1 (MBI Fermentas, Vilnius, Lithuania) during 2 hours at 55 °C, subsequently separated by electrophoresis in 3% agarose gel stained with ethidium-bromide. We registered the presence of [Ile(655)Val] polymorphism (the presence of G at the 655 codon; 116 and 32 bp fragments) of HER-2 gene in

Table 1. HER-2 allele and genotypes frequency distribution in case and control group with Odds ratios (ORs) and 95% confidence intervals (CIs) for breast cancer

Genotype / Group	Case (n=47)	Control (n=60)		P value	c2
Frequency distribution					
Allele (%)					
Ile	70.21	84.16		P = 0.0289	4.771
Val	29.79	15.84			
Genotype (%)					
Ile / Ile	46.81	70.00		P = 0.0035	11.335
Ile / Val	46.81	28.33			
Val / Val	6.38	1.67			
Ile/Val+Val/Val	53.19	30.00			
Risk for disease					
All genotypes			Ods	95% CI	
Ile / Ile	22	42	1.0	(Ref.)	
Ile / Val	22	17	2.47	1.09 – 5.59	
Ile/Val + Val/Val	25	18	2.65	1.19 – 5.88	
				trend test p = 0.0115	
Age ≤ 45 years					
Ile / Ile	3	21	1.0	(Ref.)	
Ile/Val + Val/Val	2	8	1.75	0.25 – 12.49	
Age > 45 years					
Ile / Ile	19	21	1.0	(Ref.)	
Ile/Val + Val/Val	23	9	2.82	1.05 – 7.6	
				trend test p = 0.0467	

Table 2. The breast cancer risk related to parity, lactation and family history of cancer

Genotype / Group	Case (n=47)	Control (n=60)	ORs	95% CI
Nuli or primiparous				
Ile / Ile	4	9	1.0	(Ref.)
Ile / Val	5	6	1.87	0.35 – 9.98
Ile/Val + Val/Val	6	6	2.25	0.44 – 11.5
Parous 2 ≥				
Ile / Ile	18	33	1.0	(Ref.)
Ile / Val	17	11	2.83	1.09 – 7.34
Ile/Val + Val/Val	19	12	2.90	1.15 – 7.31
				trend test p = 0.0255
Lactation period ≤ 6 months				
Ile / Ile	15	28	1.0	(Ref.)
Ile / Val	18	11	3.05	1.15 – 8.12
Ile/Val + Val/Val	20	12	3.11	1.20 – 8.06
				trend test p = 0.0222
Lactation period > 6 months				
Ile / Ile	7	14	1.0	(Ref.)
Ile / Val	4	6	1.33	0.28 – 6.32
Ile/Val + Val/Val	5	6	1.66	0.37 – 7.42
Family history of breast cancer				
Ile / Ile	4	8	1.0	(Ref.)
Ile/Val + Val/Val	5	2	5.00	0.66 – 38.15
History of other malignancy				
Ile / Ile	9	13	1.0	(Ref.)
Ile/Val + Val/Val	6	5	1.73	0.40 – 7.46

both groups of women and the achieved results were compared to specific observed parameters.

Statistical analysis. For statistical analysis of HER-2 genotypes and alleles distribution in the population we applied the Chi-squared test. For risk designation of breast cancer genesis in control group and in study group we had evaluated odds ratio (OR) and 95% confidence interval (95% CI) obtained from unconditional logistic regression analysis. P value less then 0.05 was considered as statistically significant. Statistics were performed using SPSS® 12.0 (SPSS Inc., Chicago, IL) and MedCalc® 7.2.1. (MedCalc, Belgium) for Windows.

Results

In total we have completed a genotypization of 107 women, 47 out of them represented a group with breast cancer, and 60 patients belonged to the control (healthy) group. The mean age of women with breast cancer was 60.2 years (36–81) against 42.7 years (20–71) in the control group. The frequency of the Val allele was higher in the cancer group (29.79%), than in the control group (15.84%) ($p<0.05$). The presence of the Ile/Val genotype was identified in 46.81% of patients in the case group and in 28.33% in healthy individuals, and the Val/Val genotype in 6.38% and 1.67%, respectively ($p<0.01$). For the distribution of alleles and genotypes see Table 1. The risk of cancer genesis for the Ile/Val (OR=2.47; CI=1.09–5.59) and Val/Val genotype (OR=5.73; CI=0.56–58.35) was increased compared to women with Ile/Ile genotype ($p<0.05$). The risk adjusted for altered genotype regardless of heterozygote or homozygote form (Ile/Val + Val/Val) for carriers of Val allele in genotype was 2.65 (CI=1.19–5.88). It was also increased in women >45 years of age with Val allele in genotype compared to women younger than 45 years with Val allele (OR=2.82; CI=1.05–7.6, OR=1.75; CI=0.25–12.49, respectively) (Tab. 1). The presence of breast cancer with a simultaneously finding of Val allele in genotype in the cancer group (n=47) was 8.0% (n=2) in patients with ≤45 years, and 92.0% (n=23) in patients more than 45 years old.

Table 2 expresses correlation between

factors, like e.g. parity, length of lactation, positive family history of breast cancer or other malignancy and their relation to the risk of cancer genesis in patients with Val allele in genotype. The risk of cancerogenesis was higher in multiparas (OR=2.90; CI=1.15–7.31) (p=0.0255) than in nulli- or primiparas. We have concluded a protective effect of lactation above 6 months to the risk of cancer genesis (OR=1.66; CI=0.37–7.42), than below 6 month (OR=3.11; CI=1.2–8.06) (p=0.0222), however, the risk of breast cancer was still higher than in women with Ile/Ile genotype. If we take into account positive family history for breast cancer, it is connected with 5-times higher chance of the risk of cancer genesis if Val allele in genotype is present simultaneously (OR=5.0; CI=0.66–38.15). Positive family history for another type of cancer than breast cancer was connected with a 1.73 times higher risk of genesis.

Taking into account the relationship between the risk of breast cancer and the abnormal endogeneous and/or exogeneous exposition to steroid hormones, as well as some modifiable risk factors (HRT, OC, BMI, regular exercise) we have examined also these parameters in the cancer group. All of the women who's BMI was above 24 and in whom the beginning of the menopause occurred after 45 years of age, had an elevated risk of breast cancer development, meanwhile Val allele carriers had this risk more than two times higher (OR=2.2 vs. 4.9; OR=1.1 vs. 1.5, respectively). Long term application of hormone replacement therapy (HRT) was associated with elevated risk of malignancy development, but that risk was somewhat lower in Val allele carriers. In these patients application of oral contraceptives (OC) revealed an insignificant risk decreasing effect (OR=0.3), while in patients with Ile/Ile genotype a slightly elevated risk of breast cancer development was found in relation to the application of OC (OR=1.2) (Tab. 3).

In relation to epidemiologic features the mean BMI (kg/m^2) in group of patients with breast cancer was 28.2, compared to 25.6 in healthy controls. There has not been observed significant difference in mean BMI among case patients and healthy controls in relation to HER-2 gene genotype. (Case: $BMI_{HER\text{-}2\ Val\ allele} = 28.7$, $BMI_{HER\text{-}2\ Ile/Ile} = 27.7$; Controls: $BMI_{HER\text{-}2\ Val\ allele} = 25.5$, $BMI_{HER\text{-}2\ Ile/Ile} = 26.5$). Positive history of fibroadenoma was in 12.75% of all patients with breast cancer. In group with Val allele in genotype that was 16%. In group with breast cancer we have recognized 36.2% women with history for abortion, 57.5% of women with history of chest X-ray examinations more than four times in their life and 87.2% of patients with breast cancer was of middle or low education status.

Table 3. Breast cancer risk for carriers of Val allele associated with estrogen exposure

Genotype		HER-2Ile / Ile				HER-2Ile / Val + Val / Val			
Risk factor		Case	Control	ORs	95% CI	Case	Control	ORs	95% CI
Menarche	> 13 y.	13	21	1.0	(Ref.)	13	10	1.0	(Ref.)
	< 13 y.	9	21	0.7	0.24–1.96	12	8	1.1	0.34–3.89
BMI (kg/m^2)	≤ 24	6	19	1.0	(Ref.)	6	11	1.0	(Ref.)
	> 24	16	23	2.2	0.78–8.52	19	7	4.9	1.33–18.62
Menopause	≤ 45 y.	5	2	1.0	(Ref.)	5	1	1.0	(Ref.)
	> 45 y.	11	5	1.1	0.15–8.13	15	2	1.5	0.11–20.3
Exercise	Regular	7	34	1.0	(Ref.)	8	13	1.0	(Ref.)
	Iregular	15	8	9.1	2.79–29.71	17	5	5.6	1.46–20.9
OC	No	15	30	1.0	(Ref.)	19	8	1.0	(Ref.)
	Yes	7	12	1.2	0.38–3.57	6	10	0.3	0.07–1.0
HRT	No	18	41	1.0	(Ref.)	18	16	1.0	(Ref.)
	Yes	4	1	9.1	0.9–87.34	7	2	3.1	0.56–17.2

Discussion

Breast cancer is the most widespread malignant disease affecting women. Its incidence has a slightly growing potential, however thanks to higher information awareness, earlier diagnosis and improvements in therapeutic effort mortality began to have a gradually decreasing trend. In long term the contribution of lower clinical stages of the disease is growing, higher clinical stages occur more rarely. Significant increase of chemotherapy and hormonal therapy can be observed. Actually, a promising possibility of gene therapy of this malignancy is rising up, as well.

According to our results we can state that women with Val allele in genotype have an increased risk of breast cancer development. Distribution of the homozygote and heterozygote genotypes is specific for the women population in Slovakia. The frequency of the Val allele in our population is relatively high and varies from 15.84% in healthy individuals up to 30% in women affected with breast cancer. The presence of the Val allele in the population of healthy women in our study is nearly half times lower than in women with developed breast cancer. The frequentative distribution of the allelic forms in the group of healthy women is very similar to its presence in the population of Japanese or Chinese women for the Ile (84.16% vs. 85.1% vs. 88.9%) and for the Val (15.84% vs. 14.9% vs. 11.1%) allele, respectively [31, 29]. Our results for the Val allele distribution are little lower than that of AMEYAW et al (2000) established for the healthy Caucasian ethnicity (20%) [34]. On the other hand, Afro-American women belong to the ethnic group with the highest frequency of the Val allele (24%) while in populations of Kenyan, Ghanaian and in the Philippine ethnicity the lowest incidence of valine allele (0–9%) was found out [28, 35]. This finding could serve as one of the numerous explanations to the large geographical and population variability of breast cancer incidence [36]. Concerning the breast cancer group, the distribution of the Val allele in our study was twice that esti-

mated by XIE et al. [29] and HISHIDA et al. [31] (29.79% vs.15.8% and 12.1%). This confirmed high variability in occurrence and presence of the Val allele among not only various ethnicities but also breast cancer groups.

XIE et al (2000) [29] observed, that women with Val allele in genotype have increased risk of breast cancer development (OR= 1.4) and for women younger than 45 years this risk is as much as 1.7 times higher. The risk was significantly higher in women with Val/Val genotype (OR=14.1). In comparison with results from XIE et al (2000) we have registered a higher risk for Ile/Val genotype (OR=2.47), but for the Val/Val genotype nearly 3 times lower risk (OR=5.73 vs. 14.1) [29]. In the group of younger women under 45 years there was a similar finding (OR=1.75 vs. 1.7). We can suggest that in spite of the higher prevalence of the Val allele in the Caucasian against the Chinese population, the risk of breast cancer development in women with Val/Val genotype is lower. The character of such a relation of risk of breast cancer development with the frequency of Val allele represents some space for a possible modulating effect of other factors affecting carcinogenesis of the breast (e.g. endogenous or exogenous estrogen stimulation, late menopause, age at menarche, body mass index, age). RUTTER et al. (2003) [4] observed among Ashkenazim population that the estimated cumulative risk of breast cancer to age 70 was about 30% higher among Val allele carriers (RR=1.33), and that the effect of the Val allele was stronger at younger ages and in women with a positive family history of the disease. MONTGOMERY et al [37] present similar results in Australian women with breast cancer diagnosed before the age of 40 years. The Ile655Val polymorphism was more common between cases (p=0.01) and in recessive models, homozygotes for Val allele were associated with an odds ratio of 2.8 for breast cancer development. The high frequency of the Val allele in population together with cumulative life risk under modulating affect of environmental and demographic factors may be responsible for the high incidence of breast cancer among women in the Slovak population. Findings of these studies together with our results do not support some recent reports [30–33], that there is no association among patients with breast cancer and Ile655Val polymorphism. In our study, the low number of observed cases and healthy controls that could partially alter the true ethnic variation in the penetrance risk of Val allele and different genetic background of observed populations may explain this difference in findings.

Based on our finding of high risk of breast cancer development among women with Val allele genotype and a positive family history of breast cancer (OR=5.0) in comparison to women with other malignancy in their family (OR=1.73), let us to presume that, presence of Val allele in women with positive family history of breast malignancy elevates the risk of breast cancer development more than the risk of others malignancies. This finding evokes to think that presence of Val allele in HER-2 gene participates in tissue specific carcinogenesis predominantly than in general cell malignant transformation. However, such a hypothesis has to be proven in larger studies.

Sufficient evidence indicates that a number of genetic, environmental and lifestyle risk exposures during life may play important roles in the etiology of this disease. However, it is known that the risk decreases with early childbearing, high parity and breastfeeding [38], in our study we recorded higher risk of breast cancer development for Val allele carriers in spite of the protective effect of these factors. The risk of breast cancer development was increased in both groups of observed patients with breastfeeding period shorter as well as longer than six months. The risk decreased with prolonged lactation period.

Current knowledge on the application of adequate therapy with monoclonal antibodies (trastuzumab) against HER-2 receptor is offering significant improvement in breast cancer therapy, but not sufficiently effective in every case [39, 40]. The fact that only 20–40% of the patients indicated to trastuzumab therapy benefits from it, is a stimulus to uncover others mechanisms which modify the effectiveness of therapy [40]. A finding that some cases with immunohistochemistry intensity membrane staining of grade 3+ must not have HER-2 gene amplification or in other cases where strong gene amplification is accompanied with weak protein overexpression is considered together with other factors to be the reason of insufficient therapeutic response. The presence of Val allele with possibility that isoleucine to valine changes might alter the hydrofobicity of proteins responsible for the conformational stability of the hydrophobic transmembrane domains [41] might be of interest to explain the potential reason of altered function of the HER-2 receptor on the cell surface (ligand binding, dimerization, signal transducing, receptor degradation). This might be another factor affecting the effectivity of trastuzumab therapy. Possible impact of this polymorphism at this therapy is gaining importance, especially because MILLIKAN et al (2003) [35] found a trend for HER-2 gene overexpression in homozygote women for Val allele. KAMALI-SARVESTANI et al (2004) [32] also found an insignificant association at the level of 17% between HER-2 protein expression positivity and the heterozygote form (Ile/Val) of genotype in patients affected with breast cancer. It is important to confirm this hypothesis in studies with a larger number of cases with HER-2 receptor overexpression in breast cancer and Val allele in genotype.

In conclusion, this study showed that it is of interest to search for low penetrance gene polymorphisms that can serve as susceptibility biomarkers for breast cancer development. Determination of HER-2 gene polymorphism as well as other low penetrance gene polymorphisms in patients at risk (based on demographic, environmental and lifestyle criteria) could be promising for better management with ambition to improve the prevention of breast cancer development. For the HER-2 polymorphism we found that the presence of the Val allele of HER-2 gene was associated with significantly increased risk of breast cancer genesis. Except the previous re-

ports [4, 29], our results are in the opposite position to those studies that do not confirm this finding [26, 30–32]. Therefore to confirm the benefits of our findings studies on sufficiently larger populations have to be performed.

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