doi: 10.4149/gpb_2017033

Association of single nucleotide polymorphisms in FGF-RAS/MAP signalling cascade with breast cancer susceptibility

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Abstract. The fibroblast growth factor receptors (FGFRs) and Ras/mitogen activated protein (RAS/MAP) signalling cascades are the main molecular pathways involved in breast carcinogenesis. This study aims to determine the association between *FGF10* (rs4415084 *C>T*), *FGFR2* (rs2981582 *C>T*) and *MAP3K1* (rs889312 *A>C*) gene polymorphisms and breast cancer, to analyse the discriminative ability of each SNP and to test the accuracy of the predictive breast cancer risk model which includes all SNPs. We conducted a case-control study of 170 women (57.06 ± 11.60 years) with histologically confirmed breast cancer and 146 controls (50.24 ± 10.69 years). High resolution melting (HRM) method with Sanger sequencing validation was used in analyses. We have revealed significant association of *FGFR2* and *MAP3K1* polymorphisms with breast cancer. The odds ratio of *FGFR2 T* allele was 1.897 (95% CI 1.231–2.936, $p = 0.004$) and *MAP3K1 C* allele 1.804 (95% CI 1.151–2.845, $p = 0.012$). *FGFR2* polymorphism achieved the best discriminative ability (41.95%). The Random Forest algorithm selected *FGFR2*, *MAP3K1* and age as important breast cancer predictors. The accuracy of this prediction model approached moderate accuracy (70%), with 35.9% sensitivity and 88.6% specificity.

Key words: ROC curve — HRM method — Carcinogenesis — Discriminative ability

Introduction

Breast cancer is a heterogeneous disease. On the molecular level, it develops and progresses from alterations in genes regulating biological processes such as cell growth, proliferation and differentiation (Harlid et al. 2012; Pham et al. 2013; Chen et al. 2016a; Toss et al. 2017). Large studies of genomic modifications and protein expression involved in breast tumorigenic pathways identified several differently penetrating polymorphisms associated with this disease and provided an increasing number of targets for drugs which significantly improve patient prognosis (Easton et al. 2007; Fachal and Dunning 2015; Michailidou et al. 2015). Emerging evidence from clinical trials has proven also that specific genetic background and molecular landscapes significantly influence the sensitivity and resistance profile (Wilson et al. 2016; Zardavas and Piccart-Gebhart 2016; Miller et al. 2017; Toss et al. 2017). Moreover, identification of molecular signatures and gene expression profiling is increasingly inevitable for full understanding of the tumorigenesis, especially in personalised targeted medicine (Ellsworth et al. 2010; Pereira et al. 2016).

Breast carcinogenesis is a complex of several mutually intertwined signalling pathways, such as 1) the oestrogen

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signalling pathway, 2) PI3K/AKT/mTOR pathway, 3) RAS/MAPK signalling pathway, 4) angiogenic pathway and 5) the FGFR signalling or p53 pathway (Eroles et al. 2012; Pham et al. 2013; Suman et al. 2016). Genetic alterations at any level induce aberrant signalling, thus leading to increased cancer risk (Guille et al. 2013; Sullivan et al. 2016; Ng et al. 2017).

Fibroblast growth factor receptor 2 (FGFR2) is transmembrane tyrosine kinase receptor for FGF family members with high affinity for FGF10. Their binding induces functional FGFR dimerization and kinase activation by trans-autophoshorylation which activates multiple downstream cellular cascades and responses (Wesche et al. 2011; Tiong et al. 2013). The cascade involving mitogen-activated protein kinase 1 (MAP3K1), a serine-threonine kinase in the MAP3K family and the STE superfamily, comprises of several steps: activated FGFR kinase trigger stimulation of its intracellular substrates. Major FGFR substrate 2α (FRS2α) binds the adaptor protein growth factor receptor-bound 2 (GRB2) which recruits the guanine nucleotide exchange factor son of sevenless (SOS). It activates RAS GTPase which initiates activation of the whole RAS-RAF-MEK-MAPK cascade (Goetz and Mohammadi 2013). MAPK translocates from the cytoplasm to the nucleus, where it phosphorylates and triggers early gene transcription factors which mediate the expression of oncogenes involved in proliferation, cell differentiation, cell migration and survival (McCubrey et al. 2007; Jara et al. 2013; Pham et al. 2013; Zheng et al. 2014). The activation of this receptor tyrosine kinase signalling is thus one of the mechanisms underlying tumour development and growth and genetic alterations of genes involved in this cascade contribute to aberrant cell biology.

Diverse *FGF10*, *FGFR2* and *MAP3K1* gene polymorphisms have already been identified as breast cancer susceptible in various populations (Rebbeck et al. 2009; Ripperger 2009; Harlid et al. 2012; Jara et al. 2013; Murillo-Zamora et al. 2013; Pritchard and Hayward 2013; Siddiqui et al. 2014; Zheng et al. 2014; Campbell et al. 2016), and studies on breast cancer genetic background have also been performed in Slovakia (Franeková et al. 2007; Zubor et al. 2007, 2008, 2014; Kasajová et al. 2016). Results from these studies emphasised the relevance of several polymorphisms in breast cancer susceptibility and pointed out the importance of an inter-population genetic variability.

The aim of our case-control study was to determine the association of single-nucleotide polymorphisms in *FGF10*, *FGFR2* and *MAP3K1* genes with breast cancer in a sample of Slovak women. Furthermore, we focused on the discriminative ability of each SNP to determine the risk of disease status in individual patients and tested the accuracy of the disease prediction model including all SNPs by unconditional linear regression and the random forest classification algorithm.

Materials and Methods

Subjects

We conducted the case-control study with 170 women (57.06 ± 11.60 years) with histopathologically diagnosed breast cancer and the control group comprised 146 healthy females (50.24 ± 10.69 years) without previous history of breast carcinoma or other malignancies. Women were of Slavic Caucasian origin, and samples were collected at the Clinic of Gynaecology and Obstetrics at the Jessenius Faculty of Medicine in Martin, the Comenius University in Bratislava and University Hospital in Martin. All subjects gave written informed consent and study was approved by the Ethical Committee (EC 1269/2013) and implemented in accordance with the Declaration of Helsinki.

Histopathological analyses

Immunohistopathology parameters of breast cancer tissue samples were provided by the pathologists. Tumour and lymph node specimens were fixed in formalin and embedded in paraffin. The basic histological examination was performed on 4–5-μm-thick slides stained with haematoxylin and eosin. In selected cases, lymph nodes were stained immunohistochemically (cytokeratin 19) to detect potential isolated tumour cells or micrometastases. Tumour type and histological grade were evaluated according to the WHO criteria and Nottingham grading modification (Elston and Ellis 1991; Lakhani et al. 2012). Briefly, immunohistochemistry for oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) was performed concurrently on serial sections with ready to use reagents using an automated immunostainer Autosteiner Link 48 (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Primary ER antibody (FLEX Monoclonal Rabbit, ER alpha, clone EP1, RTU, IR08461) and PR antibody (FLEX Monoclonal Mouse, clone PgR636, RTU, IS0683) were supplied by Dako; Agilent Technologies, Inc. (Santa Clara, CA, USA). Antigen retrieval was performed using EnVision ^{TM} FLEX Target Retrieval Solution High pH (pH 9.0) for 20 min at 97–98°C in PT Link instrument (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Endogenous peroxidase activity was blocked by 10 min incubation in 3% hydrogen peroxide, followed by antibody incubation for 20 min at room temperature. EnVision^{TM} FLEX/HRP, High pH kit (K8000, Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used as detection system according to the manufacturer's instructions. The immunohistochemistry for HER2 was performed using a HercepTest^{TM} Breast+Gastric kit (SK001, Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Antigens were retrieved in HercepTest ^{TM} Epitop Retrieval Solution (pH

6.0), using PT Link for 40 min at 97–98°C. Sections were blocked for endogenous peroxidase in 3% hydrogen peroxide for 10 min, then incubated with primary antibody for 30 min at room temperature. HercepTest™ Visualization Reagents were used 30 min at room temperature according to the manufacturer's instructions.

ER, PR and HER2 status were interpreted following the American Society of Clinical Oncology/College of American Pathologists criteria from 2010 and 2013 (Hammond et al. 2010; Wolff et al. 2013). Tumours were considered as ER and PR positive if ≥1% of neoplastic cells stained positively. Positive HER2 status was considered for cases exhibiting a 3+ reaction in ≥10% of neoplastic cells. Cases with 2+ reaction of HER2 staining were considered as equivocal and were analysed by fluorescent *in situ* hybridization to confirm or exclude *HER2* gene amplification. Table 1 provides information on histological type, grade, receptor status and molecular subtypes of the studies samples.

Table 1. Histopathological characteristics of breast cancer patients

	Frequency (%)
Histological type	
DIC	76.0
LIC	7.8
DCIS	1.9
Other	14.3
Histological grade	
Grade 1	16.7
Grade 2	38.3
Grade 3	45.0
ER status	
ER-positive	85.8
ER-negative	14.2
PR status	
PR-positive	79.8
PR-negative	20.2
HER2 status	
PR-positive	16.5
PR-negative	83.5
Molecular subtype	
Luminal A	79.8
Luminal B	13.8
TN BC	3.7
HER2	2.8

DIC, ducal invasive cancer; LIC, lobular invasive cancer; DCIS, ductal cancer in situ; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

DNA extraction and HRM assays

DNA was isolated from the peripheral blood using the commercially available isolation kit based on silica gel membranes, and sample concentrations were analysed by Qubit fluorometer. All samples were diluted in sterile distilled water to 20 ng/µl final concentration.

Genotyping was performed by high resolution melting (HRM) method which is based on the different disassociation characteristics of double-stranded DNA amplicons and fluorescence measurement. Validated positive controls of all genotypes and negative controls were included in each analysis which ran in a 96-well plate in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics). The single-tube mixture was prepared in a total volume of 20 µl (Light Cycler Master Mix 10.0 µl, 25 mM $MgCl_2$ 2.4 µl, 10 pmol P1 0.4 µl, 10 mol P2 0.4 µl, H_2O 4.8 µl, 20 ng DNA 2.0 µl). We designed all primers in accordance with HRM method requirements and using Primer3 online application. The specificity was checked by Primer-BLAST application and formation of dimers by OligoAnalyzer tool 3.1. Primer sequences were as follows: rs4415084 (*FGF10*): forward 5′-TAGCCCTGTTGTATTCCTGATGAC-3′and reverse 5′-AAGATTGCTGTATGTGTGGCAGGT-3′; rs2981582 (*FGFR2*) forward 5′-CGAGAATAAAACGGCAGATC-CC-3′, reverse 5′-GACTGCTGCGGGTTCCTAAAG-3′

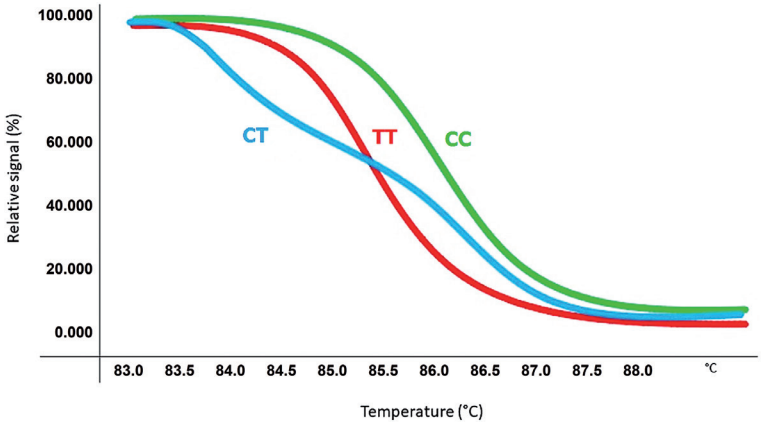

Figure 1. Schematic HRM (high resolution melting) curves determining *CT*, *TT* and *CC FGFR2* genotypes. Different melting curves represent three genotypes. The curve course and temperature of the melting depends on the amplicon sequence. At the beginning of the HRM analysis the fluorescence is high as the dye is bound to dsDNA amplicons. By heating, dsDNA decreases as the DNA is melting and the fluorescence is reduced.

Table 2. Genotype and allele frequencies of polymorphisms in *FGF10, FGFR2* and *MAP3K1* genes

	Genotype *n* (%)			Allele		
FGF10 C>T	CC	CT	TT	*C*	*T*	
Cases (170)	49 (28.7)	77 (45.0)	45 (26.3)	0.51	0.49	$\chi^2 = 1.895$ $p = 0.388$
Controls (146)	52 (35.6)	57 (39.0)	37 (25.3)	0.55	0.45	
FGFR2 C>T	CC	CT	TT	*C*	*T*	
Cases (170)	57 (33.5)	81 (47.6)	32 (18.9)	0.57	0.43	$\chi^2 = 6.879$ $p = 0.032$*
Controls (146)	70 (47.9)	56 (38.4)	20 (13.7)	0.67	0.33	
MAP3K1 A>C	AA	AC	CC	*A*	*C*	
Cases (170)	79 (46.2)	79 (46.2)	13 (7.6)	0.69	0.31	$\chi^2 = 8.383$ $p = 0.015$*
Controls (146)	90 (61.6)	51 (34.9)	5 (3.4)	0.79	0.21	

Frequencies of genotypes are shown as number (*n*) and percent (%); * $p < 0.05$.

and rs889312 (*MAP3K1*) forward 5′- ACACAAGTCAGGCCCCATTA-3′, reverse 5′-TGGGAAGGAGTCGTTGAGTT-3′. The genotypes were determined by the different shapes of the melting curve (Figure 1). Several samples from each SNP were validated by Sanger sequencing. The PCR samples were purificated by Nucleo Spin Gel and PCR Clean-up kit and by SigmaSpin™ Sequencing Reaction Clean-Up. The analyses were carried out on the 3500 Genetic Analyzer (Applied Biosystems™). The sequences were read with free trace viewer Chromas program v.2.6.2.

Statistical analyses

The conformity of genotypes distribution with Hardy-Weinberg Equilibrium was tested by a goodness-of-fit χ^2 test in both controls and cases groups. The comparison of genotype distribution between cases and controls was tested by the χ^2 test with Monte-Carlo *p*-values. Odds ratios and their 95% confidence intervals were obtained in logistic regression, with adjustment for age. The predictive accuracy of the model was measured by the area under the ROC (receiver operating characteristic) curve. The random forest classification algorithm with the nested cross-validation feature selection was used to obtain a realistic estimate of the predictive performance of the breast cancer risk model.

All data were analysed using R software (R Core Team, 2015) and IBM SPSS program version 21. *p* values below 0.05 were considered statistically significant.

Table 3. Odds ratios of the *FGF10, FGFR2* and *MAP3K1* genotypes

	OR	95% CI		*p*
		Lower bound	Upper bound	
FGF10 C>T				
CT	1.43	0.85	2.42	0.188
TT	1.29	0.72	2.32	0.458
FGFR2 C>T				
CT	1.77	1.09	2.89	0.026*
TT	1.95	1.01	3.83	0.049*
MAP3K1 A>C				
AC	1.76	1.11	2.81	0.019*
CC	2.89	1.03	9.57	0.048*

OR, odds ratio; CI, confidence interval; * $p < 0.05$.

Results

The distribution of genotypes and allele frequencies of all three SNPs according to health status are shown in Table 2. Genotypes with risk minor alleles occurred more often in all three SNPs in the breast cancer case group than in the control group. However, statistical analyses revealed significant association only between the *FGFR2* ($p = 0.032$) and *MAP3K1* ($p = 0.015$) gene polymorphisms and breast cancer susceptibility. The genotype distribution of all SNPs showed conformity with Hardy-Weinberg Equilibrium criteria in both study groups ($p > 0.05$) except for *FGF10* genotype distribution in the control group ($p = 0.011$).

Table 3 shows the odds ratios of heterozygotes and homozygotes with two minor risk alleles of the three studied polymorphisms. Individuals with two minor alleles in homozygous form had higher risk than heterozygotes in *FGFR2* (OR 1.95 *vs.* 1.77) and in *MAP3K1* (OR 2.89 *vs.* 1.76) polymorphisms. The range of confidence intervals and *p*-values for rs4415084 *FGF10* polymorphism confirmed that neither homozygosity nor heterozygosity for the *T* variant is associated with increased breast cancer risk.

We also analysed association of these polymorphisms with histopathological characteristics of the samples displayed in the Table 1. However, neither the association of three polymorphisms and tumour type, histological grade, receptor status nor molecular subtype was statistically significant ($p < 0.05$).

To assess the predictive capability of single SNPs, we calculated classification error of each SNP. Although the best value was found for *FGFR2* polymorphism (41.95%), its discriminative ability was low (Table 4).

ROC curves and the area under the ROC curve (AUC) were used to quantify the predictive accuracy of the multivariate logistic regression model of all studied SNPs. *FGFR2, MAP3K1* polymorphisms and age were statistically significant predictors of breast cancer in the model. The accuracy of this prediction model approached 70%, with moderate accuracy (Figure 2).

The Random Forest algorithm was applied to the data as an alternate predictive algorithm in order to obtain a realistic estimate of the predictive performance of the three SNPs and age by nested cross-validation feature selection. This algorithm also determined *FGFR2*, *MAP3K1* and age as the important breast cancer predictors. The predictive accuracy of this model was 67.3% with 35.9% sensitivity and 88.6% specificity.

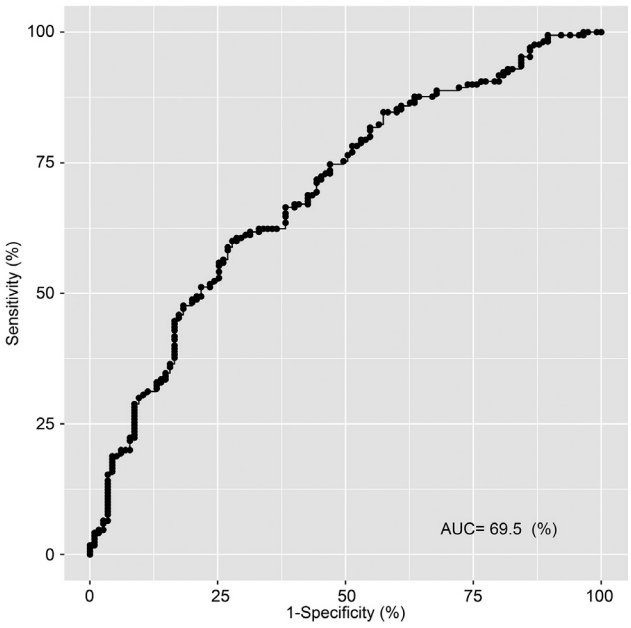

Figure 2. ROC (receiver operating characteristic) curve with the area under the ROC curve (AUC) for the multivariate logistic regression model. The position of the ROC on the graph reflects the accuracy of the diagnostic test. Closer to the upper left corner, the better, as the area under the curve is higher, which means better prediction power of the model based on higher sensitivity and specificity.

Discussion

Our study confirmed significant associations of low-penetrant *FGFR2* and *MAP3K1* polymorphisms with breast cancer risk in Slovak women. However, we found no evidence to implicate the *FGF10 T* allele polymorphism as a breast cancer risk factor even though this had been previously identified by large-scale and genome-wide association breast cancer studies (Easton et al. 2007; Hunter et al. 2007; Stacey et al. 2008). The odds ratios for *FGFR2* heterozygous and homozygous form (1.77 and 1.95) and for *MAP3K1* (1.76 and 2.89) were higher than reported in the literature (1.2 and 1.6 for *FGFR2* and 1.1 and 1.3 for *MAP3K1*) (Easton et al. 2007; Ripperger et al. 2009). These differences can be explained by inter-population variability but also by sample size.

Although, the *MAP3K1* mutations were more frequently observed in HER2+ breast cancer and *FGFR2* variants are reported relevant in ER+ and PR+ breast cancer (Pham et al. 2013; Campbell et al. 2016), we found no significant association between genetic polymorphisms and histopathological characteristics of the cancer samples. Further studies of other polymorphisms in these genes and the expression profile could elicit essential information because *FGFR2* is a tumour suppressor gene amplified and overexpressed in 10–15% of breast tumours (Rebbeck et al. 2009; Ahmad et al. 2012; Tiong et al. 2013) and enhanced FGFR expression may not only be due to genetic alterations but also to epigenetic deregulation at transcriptional and post-translational levels.

Multiple studies have already examined and confirmed the association of these low-penetrant genes with breast cancer susceptibility in several populations (Easton et al. 2007; Rebbeck et al. 2009; Murillo-Zamora et al. 2013; Chen et al. 2016b). However, most of these did not assess SNP's predictive capability, and this is one of the most important factors for precision medicine (Wu et al. 2016). To understand the discriminative ability of studied SNPs, individually, we computed the classification error. Our results indicate that overall accuracy was weak using classification error; ranging from 41.95% to 45.11%. With regard to low-penetrating SNPs, it is assumed that more SNPs have greater cumulative power, and therefore, we generated ROC curve of the multivariate logistic regression model which includes all three studied genotypes. Based on the AUC value of 0.695, the model's predictive performance approached moderate accuracy.

Table 4. Discriminative ability of three SNPs according to the classification error

	Classification error (%)	Discriminative ability
FGF10 C>T	45.11	low
FGFR2 C>T	41.95	low
MAP3K1 A>C	42.59	low

Wu et al. (2016) analysed more SNPs and the AUCs for the models with 10, 22 and 77 SNPs were 0.591, 0.622 and 0.684, respectively, and this indicates that more SNPs the prediction model includes, the better discriminative ability the model attains. However, their last model with 153 SNPs demonstrated lower predictive performance in terms of AUC 0.650. It appears that the common genetic variants attained the upper limit of their predictive power. Higher AUC value of our model might be explained by age, one of the main determinants in breast carcinogenesis, which we included in our model. Further, because the logistic regression overfits data and provides upward-biased estimates of accuracy, sensitivity and specificity, we employed the Random Forest algorithm with nested cross-validation selection to obtain a realistic estimate of the SNP's predictive ability with age. This provided us with lower, but realistic estimate of model´s accuracy (67.3%). We assume that inclusion of other clinical risk factors could increase the predictive power of the models.

In conclusion, we confirmed *FGFR2* and *MAP3K1* as breast cancer susceptible genes with *T* allele (*FGFR2* rs2981582) and *C* allele (*MAP3K1* rs889312) elevating the disease risk. The discriminative ability of single SNP models was low. Based on the AUC value of 0.695, the model's predictive performance approached moderate accuracy.

Further studies examining more low-penetrant genes and combination of highly-, moderate- and low-penetrant polymorphisms involved in another main signalling pathways, together with clinical characteristics would enhance assessment of the cumulative role of genetic variants in breast carcinogenesis and determine their diagnostic accuracy.

Acknowledgement. This work was supported by the Slovak Research and Development Agency grant under the contract No. APVV-14-0815 and by KEGA grant No. 015UK-4/2015 and by VEGA 1/0199/17 and VEGA 1/0124/17.

Conflict of interest. There is no conflict of interest, and no competing financial interests exist.

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Received: March 31, 2017
Final version accepted: July 27, 2017