Genetic variants in FGFR2 and TNRC9 genes are associated with breast cancer risk in Pakistani women

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Abstract. Single nucleotide polymorphisms (SNPs) lead to genetic differences in breast cancer (BC) susceptibility among women from different ethnicities. The present study aimed at investigating the involvement of SNPs of three genes, including fibroblast growth factor receptor 2 (FGFR2), trinucleotide-repeat-containing 9 (TNRC9) and mitogen-activated protein kinase kinase kinase 1 (MAP3K1), as risk factors for the development of BC. A case-control study (90-100 cases; 90-100 controls) was performed to evaluate five genetic variants of three genes, including FGFR2 (SNPs: rs1219648, rs2981582), TNRC9 (SNPs: rs8051542, rs3803662) and MAP3K1 (SNP: rs889312) as BC risk factors in Pakistani women. Significant associations were observed between BC risk and two SNPs of FGFR2 [rs2981582 (P=0.005), rs1219648 (P=9.08e-006)] and one SNP of TNRC9 [rs3803662) (P=0.012)] in Pakistani women. On examining the different interactions of these SNPs with various clinicopathological characteristics, all three associated genetic variants, rs2981582 rs1219648 and rs3803662, exhibited a greater predisposition to sporadic, in comparison to familial, BC. Furthermore, there was an increased effect of BC risk between haplotype combinations of the two SNPs of FGFR2 (rs2981582 and rs1219648) in Pakistani women. The results of the present study suggest that variants of FGFR2 and TNRC9 may contribute to the genetic susceptibility of BC in Pakistani women.

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Abbreviations: BC, breast cancer; BRCA1/2, breast cancer 1/2; SNP, single nucleotide polymorphism; FGFR2, fibroblast growth factor receptor 2; GWASs, genome-wide association studies; TNRC9, trinucleotide-repeat-containing 9; MAP3K1, mitogen-activated protein kinase kinase kinase 1; ER; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PCR, polymerase chain reaction; ORs, odds ratios; CIs, confidence intervals;

Key words: breast cancer susceptibility, single nucleotide polymorphism, FGFR2, TNRC9, MAP3K1, Pakistani women

Introduction

Breast cancer (BC) is a leading cause of mortality among women with, on average, 400,000 mortalities per year (1). The incidence of BC has been increased in Asian countries (2), including Pakistan [the estimated rate is 38.4%, and every ninth woman is at risk of the disease at certain stages in her life (3)]. BC is caused by complex inherited and environmental factors, and is therefore called a multifactorial disease (4). However, genetics of an individual play a vital role in the development of BC. Tumor suppressor genes, including breast cancer 1 (BRCA1), BRCA2, phosphatase and tensin homolog ('PTEN') and tumor protein 53, have been recognized in inherited BC syndromes. Furthermore, DNA-repair genes, such as checkpoint kinase 2, ataxia telangiectasia mutated ('ATM'), BRCA1-interacting protein and partner and localizer of BRCA2, have been moderately associated with the risk of BC (5). However, all these known common genes account for only 25% of the familial BC cases (5), suggesting that an additive effect of multiple susceptibility alleles with low penetrance may be, in part, responsible for the risk of BC (6). This hypothesis leads to a polygenic model of susceptibility of BC to genetic factors, in which a large number of low-risk variants having high frequencies in populations may determine the overall risk of disease due to their multiplicative

effect (7). Single nucleotide polymorphisms (SNPs) are the genetic factors that have been considered as the key variations leading to BC susceptibility among individuals (8). To date, >40 common low-risk variants have been reported by genome-wide association studies (GWASs) that are associated with BC risk (9). Certain studies have shown that the BC risk is associated with different SNPs in three novel genes: Trinuc leotide-repeat-containing 9 (TNRC9), fibroblast growth factor receptor 2 (FGFR2) and mitogen-activated protein kinase kinase kinase 1 (MAP3K1). It has been previously shown in numerous studies that SNPs in the genes FGFR2, TNRC9 and MAP3K1 [or MEKK1 (MEK kinase 1)] are associated with a high risk of BC in the general population, as well as in BRCA2 mutation carriers (10-12).

FGFR2 is a tumor suppressor gene (located on chromosome 10q26 with 22 exons), which is responsible for 10-15% of breast tumors due to its overexpression (13,14). Common SNPs and numerous mutations within, or neighboring, the FGFR2 gene have been associated with the susceptibility of BC. For example, two intronic polymorphic variants of the FGFR2 gene (rs1219648 and rs2981582, which are located in intron 2) have been closely associated with BC (10,15). In another study, the SNP, rs1219648, was closely linked with the early onset of sporadic BC in African, American and Chinese women, specifically in young women (16), the Azeri population of Iran (17) and the North Indian population (18). GWASs also identified TNRC9 (located on chromosome 16q12.1) as a BC susceptibility locus, and placed it in one of the low-risk BC variants (15). Overexpression of the TNRC9 gene is associated with a poor diagnosis, as it increases BC cell propagation, migration and survival (19). The SNPs rs3803662 and rs8051542, belonging to the TNRC9 gene, have been shown to be clearly associated with BC in women of different ethnicities (10,20-22). However, repeated studies among European, African-American and East Asian individuals reported contradictory results (23-25). MAP3K1 belongs to the MAPK family, and it exerts a pivotal role in multiple normal and tumor cell types through being involved in functions such as apoptosis, cell survival and cell motility/migration (26). Differential expression of MAP3KI has been reported in all BC subtypes (7,10). The variant of rs889312 (MAP3K1) has been demonstrated to be a powerful risk factor for the development of BC in European and Asian ancestry populations (27,28). However, similar SNPs do not show any association with the risk of BC in women of African ancestry (29-31). Taken together, the novel genetic variants of the genes FGFR2, MAP3K1 and TNRC9 have shown a marked association with BC in populations of diverse ethnicity (32), and the variants identified as BC risk factors have revealed a variable impact on the risk of BC associated with different populations. Hence, the replications of previously BC associated loci in multiple populations are required to explore the genetic heterogeneity of BC (18).

Therefore, the genetic association of five genetic variants of the three genes, including FGFR2 (SNPs: rs1219648, rs2981582), TNRC9 [new name: TOX high-mobility group box family member 3 (TOX3), Ser51 variant; SNPs: rs8051542, rs380662), and MAP3K1 (SNP: rs889312) was performed in female patients with BC, as well as age-matched healthy subjects of Pakistan. Furthermore, the association of variants with a BC risk for stratified groups of patients, based on their clinicopathological characteristics, was also determined.

Materials and methods

Sample collection. Blood samples of 100 patients with BC were collected in k3 EDTA vials obtained from the Institute of Nuclear and Medicine Oncology (INMOL), Lahore, and ITTEFAQ Hospital, Lahore. Clinical data of the patients, including their estrogen receptor (ER) and progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status, as well as patients with a family history of BC, were collected from the files of patients. Informed consent according to the declaration of Helsinki was obtained from patients or from their relatives, as well as healthy women who were involved in the study, and the COMSATS Ethics Committee approved the study.

Extraction of DNA. DNA was extracted from the fresh blood samples of patients with BC and healthy controls using either a DNA extraction kit (K0512-Thermo Scientific Genomic DNA Purification kit; Thermo Fisher Scientific, Waltham, MA, USA) or an organic method of DNA extraction, as previously described (33). The DNA quality and quantity was determined using agarose gel electrophoresis, as described below.

Design of primers. The allele-specific amplification primers for allelic variants of FGFR2, TNRC9 and MAP3K1 were designed using Primer 3 (v. 0.4.0) software from the website, http://frodo.wi.mit.edu. Genomic DNA flanking the SNP was amplified with allele-specific primers. Two different pairs of primers were used for SNP amplification: One wild-type allele-specific primer, and the other mutant allele-specific primer. The universal primer was non-allele-specific, and identical in wild and mutant genotypes of each marker. The protocol of Hirotsu *et al* (34) was followed for the design of the allele-specific primers. The primer sequences, along with the optimized annealing temperatures for each variant, are shown in Table I.

Allele-specific polymerase chain reaction (PCR) amplification. Allele-specific PCR was performed in a 20 μl reaction volume containing 10 ng genomic DNA, 0.4 pM each oligonucleotide primer, 1X PCR buffer, 200 μM deoxynucleoside triphosphates, 2 mM $MgCl_2$ and 2 U *Taq* polymerase (all obtained from Thermo Fisher Scientific, Inc.). The reactions were performed using the following PCR cycling conditions: 3 min at 95˚C for one cycle, 35 cycles at 95˚C for 30 sec, with the different annealing temperatures as shown in Table I for 30 sec and 72˚C for 30 sec, followed by one cycle at 72˚C for 7 min.

Agarose gel electrophoresis. Amplified products of SNPs were electrophoresed (80 V) on a 2.5% agarose gel stained with ethidium bromide and visualized on UV trans-illuminator. The allelic variants of FGFR2, TNRC9 and MAP3K1 were genotyped using a gel-based method, as detailed below.

Sanger sequencing. In order to validate the gel-based method of SNP genotyping, Sanger sequencing of purified PCR products

Table I. List of primers for the allele-specific amplification of the SNP regions of the genes, FGFR2, MAP3K1 and TNRC9.

Allele name	Name of primer	Primer sequence (5'-3')	Annealing temperature
MAP3K1 rs889312	Universal forward	5'-GACACAGGCATCAATTATTTCT-3'	57
	C reverse	5'-GTAGTCTCTTAATTTGCACATG-3'	57
	A reverse	5'-GTAGTCTCTTAATTTGCACATT-3'	57
FGFR2 rs1219648	Universal forward	5'-CATGATGTGGCCAAAGTCCA-3'	58
	A reverse	5'-CATGGCCATCCTTGAAGAGT-3'	58
	G reverse	5'-CATGGCCATCCTTGAAGAGC-3'	58
FGFR2 rs2981582	T forward	5'-GCCACTTAATGAACCTGTTTGT-3'	56
	C forward	5'-GCCACTTAATGAACCTGTTTGC-3'	56
	Universal reverse	5'-ACGCAACCCTCCTTCCTAAAC-3'	56
TNRC9 rs8051542	Universal forward	5'-GCCAGAAGTTTTCCATCTCT-3'	54
	T reverse	5'-CTCCAATCATAGTGCTGCA-3'	54
	C reverse	5'-CTCCAATCATAGTGCTGCG-3'	54
TNRC9 rs3803662	T forward	5'-TTAATGCCTCTATAGCTGTCT-3'	53
	C forward	5'-TTAATGCCTCTATAGCTGTCC-3'	53
	Universal reverse	5'-AGGAGACAAAGGTAGTAATGG-3'	53

SNP, single nucleotide polymorphism; MAP3K1, mitogen-activated protein kinase kinase kinase 1; FGFR2, fibroblast growth factor receptor 2; TNRC9, trinucleotide-repeat-containing 9.

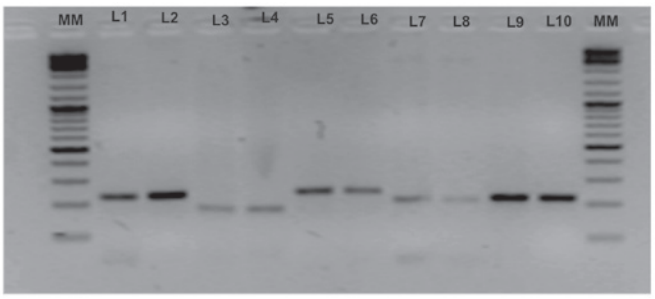

Figure 1. Amplification of allelic variants of five genetic markers and their electrophoresis on a 2% agarose gel. Lanes were loaded as follows: MM (molecular marker; Thermo Fisher_Scientific™ O'GeneRuler™ 100 bp Plus DNA Ladder); L1, variant T of rs2981582 (223 bp); L2, variant C of rs2981582 (223 bp); L3, variant A of rs1219648 (177 bp); L4, variant G of rs1219648 (177 bp); L5, variant T of rs8051542 (242 bp); L6, variant C of rs8051542 (242 bp); L7, variant T of rs3803662 (210 bp); L8, variant C of rs3803662 (210 bp); L9, variant C of rs889312 (219 bp); L10, variant A of rs889312 (219 bp).

of selected samples was performed to confirm the different allelic variants of FGFR2, TNRC9 and MAP3K1. Sequencing of the purified products using universal primer (either reverse or forward, as shown in Table I) was performed with a Big Dye Sequencing kit, according to the manufacturer's protocol (Applied Biosystems Life Technologies, Foster City, CA, USA). The sequencing chromatograms were analyzed using Genious software (version R9.1; www.genious.com).

Statistical analysis. For estimating the association of genetic variants with BC, the Chi-squared test was used, and the odds ratio (OR) and 95% confidence intervals (CIs) were also calculated. Fisher's exact test was performed for determining the association of haplotypes with the risk of BC. Statistical

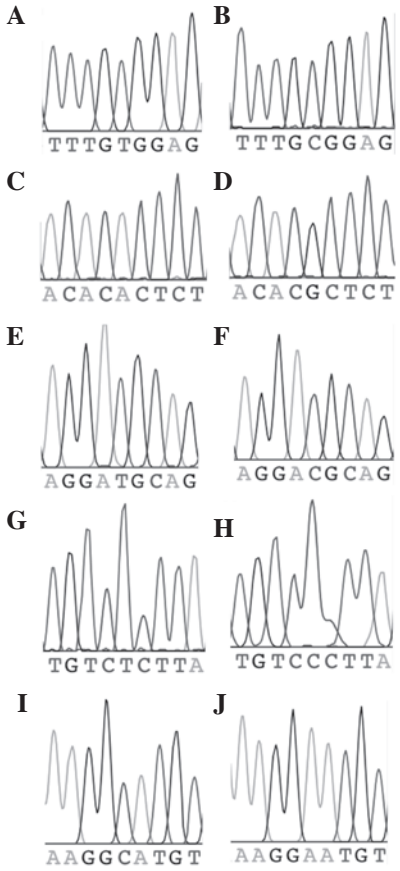

Figure 2. Validation of the allele-specific extension method of genotyping by Sanger sequencing. The chromatogram shows the alternative alleles of each marker. (A and B) FGFR2 rs2981582 (variant T and C), (C and D) FGFR2 rs1219648 (variant A and G), (E and F) TNRC9 rs8051542 (variant T and C), (G and H) TNRC9 rs3803662 (variant T and C), (I and J) MAP3K1 rs889312 (variant C and A). MAP3K1, mitogen-activated protein kinase kinase kinase 1; FGFR2, fibroblast growth factor receptor 2; TNRC9, trinucleotide-repeat-containing 9.

Table II. Baseline characteristics of selected variables in female breast cancer patients of Pakistan (n=100).

Variable	Value
Age, years (mean ± SD)	46.1±11.63 (range 20-67)
History	
Sporadic	76
Familial	24
Consanguineous marriages (out of total samples)	48
Clinical staging of cancer (UICC)[a] (n=100)	
Stage 0 (*in situ*)	0 (0.0%)
Stage 1	6 (6.00%)
Stage 2	30 (30.0%)
Stage 3	39 (39.0%)
Stage 4	11 (11.0%)
Unknown	14 (14.0%)
Receptor status	
Estrogen receptor (n=100)	
Positive	50 (50%)
Negative	33 (33%)
Unknown	17 (17%)
Progesterone receptor (n=100)	
Positive	39 (39%)
Negative	46 (46%)
Unknown	15 (15%)
Human epidermal growth factor receptor 2 (n=100)	
Positive	37 (37%)
Negative	26 (26%)
Unknown	02 (02%)
Triple-negative[b]	27 (27%)
Luminal A[c]	11 (11%)

[a]Union for International Cancer Control (UICC) stages; ER, PR, and HER2 all negative; [c]ER or PR positive, HER2 negative. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

analysis was performed using SHEsis online software (35), with the exception of the Chi-squared test to compare the distribution of SNP genotypes between the cancer group and healthy controls, where SPSS version 17 (SPSS Inc., Chicago, IL, USA) was used. P<0.05 was considered to indicate a statistically significant value.

Results

The allele-specific amplification of five selected low-risk variants of three genes, including FGFR2 (SNPs: rs2981582, rs1219648), TNRC9 (SNPs: rs8051542, rs3803662), and MAP3K1 (SNP: rs889312), revealed the presence of DNA fragments of 223, 177, 242, 210 and 219 bp in length, respectively, on 2% agarose gel electrophoresis (Fig. 1). The sequencing of selected PCR products for each genetic marker revealed 100% concordance with the gel electrophoretic method. The sequencing chromatograms for the two allelic variants of each marker (two chromatograms per marker) are shown in Fig. 2.

Table III. The association of FGFR2 (rs2981582 and rs1219648) and TNRC9 (rs3803662) with breast cancer risk in Pakistani female patients.

Gene	Marker	Case	Control	Fisher's P-value
FGFR2	rs2981582[a]	100	100	0.005
	rs1219648[a]	90	90	9.08e-006
TNRC9	rs8051542	96	90	0.506
	rs3803662[a]	96	90	0.012
MAP3K1	rs889312	100	100	0.245

FGFR2, fibroblast growth factor receptor 2; TNRC9, trinucleotide-repeat-containing 9; MAP3K1, mitogen-activated protein kinase kinase kinase 1. [a]The marker showed significant association with breast cancer (P<0.05).

The baseline characteristics of 100 female patients with BC are shown in Table II. The mean age of all the patients at diagnosis was 46.1±11.63 years (range, 20-67 years). Out of the total of 100 patients with BC, only 24 cases were familial, and the remaining 76 cases were sporadic, according to the medical history written in the patients' files. Consanguineous marriages accounted for ~50% of the patients (n=48). Overall, the most common UICC cancer stages (36) identified in the randomly selected group of patients were stages 2 to 3, which accounted for 69% (30 and 39% respectively) of the cases, whereas stages 1 and 4 accounted for only 6 and 11%, respectively. The stage of the remaining 14% of the cases was unknown. No patient presented at stage 0 (carcinoma *in situ*). The BC patient samples comprised 50 (50%) ER-positive tumors and 33 (33%) ER-negative tumors, whereas the status of the remaining 17 (17%) was unknown; 39% of the patients with BC had PR-positive tumors and 46% had PR-negative tumors, with 15% of the patients unknown; 37% of the patients with BC had HER2-positive tumors and 26% had HER2-negative tumors, with 10% of the patients untested. Triple-negative (i.e. ER-, PR- and HER2-) patients with BC accounted for 27%, whereas 11% of the patients had luminal A tumors (ER or PR+, HER2-) in randomly collected blood samples.

Subsequently, two FGFR2 SNPs (rs1219648, rs2981582), two TNRC9 SNPs (rs8051542, rs3803662) and one MAP3K1 SNP (rs889312) were genotyped in the cases of Pakistani women with BC (90-100), as well as control subjects. The Hardy-Weinberg equilibrium was assessed for all five SNP genotypes in patients with BC and in controls using SHEsis online software (35). The association of each genetic marker with BC risk (P-values), as well as the total number of samples analyzed in both case and control subjects, is shown in Table III. Significant associations were observed between two SNPs of

Table IV. Association of FGFR2, TNRC9 and MAP3K1 genotypes with breast cancer in Pakistani women.

SNP ID	Genotype	Number		OR (95% CI)[b]	P-value[c]
		Control	Case		
rs2981582	CC	20	5	Reference	-
	CT	76	91	1.197 (1.070-1.340)	0.001[a]
	TT	4	4	2.667 (0.840-8.463)	0.097
rs1219648	AA	23	1	Reference	-
	AG	57	76	1.385 (1.202-1.596)	0.000018[a]
	GG	10	13	3.064 (1.790-5.245)	0.000087[a]
rs8051542	CC	35	45	Reference	-
	CT	38	37	0.867 (0.626-1.199)	0.389
	TT	17	14	0.726 (0.398-1.324)	0.294
rs3803662	CC	64	50	Reference	-
	CT	25	46	1.706 (1.152-2.526)	0.006[a]
	TT	1	0	-	0.378
rs889312	AA	41	40	Reference	-
	CA	44	52	1.092 (0.831-1.434)	0.526
	CC	15	8	0.622 (0.289-1.339)	0.215

[a]P<0.05. [b]Crude odd ratio (OR), 95% CI, confidence interval in 95%. [c]P-value calculated from the Chi-squared test for genotype distribution between cases and controls.

FGFR2 [rs2981582 (P=0.005), rs1219648 (P=9.08e-006)], one SNP of TNRC9 [rs3803662 (P=0.012)] and the BC risk, although no significant associations were identified in the second SNP of TNRC9 (rs8051542) and the SNP of MAP3K1 (rs889312). The most significant association with the BC risk was observed for rs1219648 in the FGFR2 gene (P=9.08e-006).

The distribution of SNP genotypes between the cancer group and the healthy controls was compared using the Chi-squared test to see which genotype of a particular marker is associated with BC risk (Table IV). The homozygote GG (P=0.000087), as well as heterozygote AG (P=0.000018), genotypes of SNP rs1219648 in the FGFR2 gene exhibited significant association with the risk of BC. However, only homozygote CT genotype (P=0.001) of SNP rs2981582 in the FGFR2 gene was significantly associated with BC risk, and homozygote TT genotype (P=0.097) did not show any association, as the number of samples of this particular genotype was equal in both the case (4) and control (4) groups. Similarly, only heterozygote CT genotype (P=0.006) of SNP rs3803662 in the TNRC9 gene was significantly associated with BC risk, and homozygote TT genotype (P=0.378) did not reveal any association, as the number of samples for this TT genotype was very low in the control group (1) and no samples were identified with this genotype in the case group. No significant correlation was identified between MAP3K1 SNP (rs889312) and BC risk on an examination of its homozygote or heterozygote genotypes (Table IV).

Furthermore, genotypes were sorted on the basis of various clinicopathological characteristics of BC for each marker, and the association of the genetic marker was assessed in stratified groups. The genetic variants exhibiting statistically significant differences with respect to associations of various clinicopathological characteristics with BC risk are shown in Table V. Analyses stratified by ER status revealed that SNP rs1219648 of the FGFR2 gene remained significantly associated with BC risk in ER-positive (P=0.042) and ER-negative (P=0.003) tumors. However, neither SNP rs2981582 of the FGFR2 gene nor SNP rs3803662 of the TNRC9 gene revealed significant associations with either ER-positive or ER-negative tumors. On considering the PR status, SNP rs1219648 of the FGFR2 gene again remained significantly associated with BC risk in PR-positive (P=0.090) and PR-negative (P=0.0007) tumors. However, neither SNP rs2981582 of the FGFR2 gene nor SNP rs3803662 of the TNRC9 gene revealed a significant association with either PR-positive or PR-negative tumors. In the case of HER2 carriers, SNP rs1219648 of FGFR2 exhibited a significant association with a higher BC risk in HER2-positive (P=0.0009) tumors. However, SNP rs3803662 of the TNRC9 gene revealed differing levels of association, and it was only associated significantly with BC risk in HER2-negative patients (P=0.023). By considering triple-negative cases (ER-negative, PR-negative and HER2-negative), SNP rs1219648 of FGFR2 (P=0.004) and rs3803662 of TNRC9 (P=0.014) revealed a significant association with BC risk, although no significant association was observed in the case of SNP rs2981582 of FGFR2 gene (P=0.058).

The sporadic and familial breast cases were sorted to assess whether the association of markers with BC risk would be significant, based on the family history of the patients. SNP rs1219648 of the FGFR2 gene again remained significantly associated with BC risk in sporadic (P=0.002) and familial (P=0.008) cases, although the other two SNPs (rs2981582 of FGFR2 and rs3803662 of TNRC9) exhibited significant associations with BC risk only in sporadic cases (P=0.002, P=0.035 respectively).

Table V. The association of FGFR2 and TNRC9 variants with breast cancer risk considering various clinicopathological characteristics.

A, ER+/-

		ER-positive cases			ER-negative cases		
SNP	Controls (n)	Cases (n)	P-value	OR (95% CI)	Cases (n)	P-value	OR (95% CI)
rs3803662	72	37	0.139	0.604 (0.298-1.225)	13	0.170	0.489 (0.184-1.301)
rs2981582	66	33	0.111	0.709 (0.360-1.393)	08	0.290	0.650 (0.229-1.839)
rs1219648	32	31	0.042[a]	1.138 (0.539-2.404)	16	0.003[a]	1.476 (0.607-3.584)

B, PR+/-

		PR-positive cases			PR-negative cases		
SNP	Controls (n)	Cases (n)	P-value	OR (95% CI)	Cases (n)	P-value	OR (95% CI)
rs3803662	72	31	0.196	0.618 (0.292-1.307)	20	0.069	0.475 (0.207-1.089)
rs2981582	66	20	0.165	0.718 (0.352-1.464)	11	0.185	0.650 (0.262-1.607)
rs1219648	32	27	0.090[a]	1.095 (0.505-2.372)	21	0.0007[a]	1.476 (0.650-3.352)

C, HER2+/-

		HER2-positive cases			HER2-negative cases		
SNP	Controls (n)	Cases (n)	P-value	OR (95% CI)	Cases (n)	P-value	OR (95% CI)
rs380366272	72	33	0.410	0.735 (0.344-1.568)	12	0.023[a]	0.360 (0.137-0.944)
rs2981582	66	20	0.051	0.650 (0.319-1.323)	08	0.290	0.650 (0.229-1.839)
rs1219648	32	31	0.0009[a]	1.476 (0.701-3.108)	12	0.226	0.885 (0.327-2.395)

D, sporadic and familial

		Sporadic cases			Familial cases		
SNP	Controls (n)	Cases (n)	P-value	OR (95% CI)	Cases (n)	P-value	OR (95% CI)
rs3803662	72	76	0.035[a]	0.582 (0.322-1.051)	21	0.101	0.508 (0.222-1.158)
rs2981582	66	57	0.002[a]	0.650 (0.391-1.078)	10	0.215	0.650 (0.253-1.669)
rs1219648	32	66	0.002[a]	1.181 (0.644-2.164)	21	0.008[a]	1.328 (0.606-2.911)

E, triple-negative

		Triple-negative cases		
SNP	Controls (n)	Cases (n)	P-value	OR (95% CI)
rs3803662	72	25	0.014[a]	0.420 (0.197-0.896)
rs2981582	66	19	0.058	0.650 (0.314-1.342)
rs1219648	32	24	0.004[a]	1.249 (0.565-2.761)

[a]Marker showed significant association with breast cancer (P<0.05). ER, estrogen receptor; PR, progesterone receptor; HER2, HER2, human epidermal growth factor receptor 2; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Another important issue taken into consideration during the study was to assess whether women carrying risk alleles at both loci of the identical marker are at an even greater risk of BC compared with those carrying only one risk allele, and

Table VI. The association of FGFR2 and TNRC9 haplotypes with breast cancer risk.

Gene	Marker	Haplotype[a]	Case frequency	Control frequency	P-value	OR (95% CI)
FGFR2	rs2981582	C A[b]	6.07 (0.034)	82.56 (0.459)	3.22e-015	0.041 (0.017-0.097)
		C G[b]	85.93 (0.477)	22.44 (0.125)	3.46e-013	6.414 (3.775-10.900)
	rs1219648	T A[b]	71.93 (0.400)	20.44 (0.114)	5.60e-010	5.196 (3.002-8.994)
		T G[b]	16.07 (0.089)	54.56 (0.303)	3.38e-007	0.225 (0.123-0.412)
TNRC9	rs8051542	C C	88.86 (0.463)	96.15 (0.534)	0.168778	0.751 (0.500-1.129)
		C T[b]	38.14 (0.199)	11.85 (0.066)	0.000176	3.519 (1.769-6.999)
	rs3803662	T C	57.14 (0.298)	56.85 (0.316)	0.703778	0.918 (0.591-1.427)
		T T	7.86 (0.041)	15.15 (0.084)	0.083435	0.464 (0.191-1.127)

[a]Haplotypes are shown in the physical order: rs2981582, rs1219648 (FGFR2), rs8051542, rs3803662 (TNRC9); [b]Haplotype was significantly associated with breast cancer (P<0.05). OR, odds ratio; CI, confidence interval; FGFR, fibroblast growth factor receptor 2; TNRC9, trinucleotide-repeat-containing 9.

whether any effects would ensue as a consequence of their epistatic interactions. Therefore, combined effects (haplotypes) of the two SNPs of the FGFR2 gene (rs1219648, rs2981582), as well as the two SNPs of the TNRC9 gene (rs8051542, rs3803662), were analyzed (Table VI). The analysis identified four common haplotypes in the SNPs of FGFR2, with frequencies >0.03, and all haplotypes were significantly associated with BC risk, providing evidence that women carrying more alleles associated with risk have a greater chance of developing BC. However, in the case of haplotyping of TNRC9 SNPs, of the four identified haplotypes, the CT haplotype (OR, 3.519; 95% CI, 1.769-6.999; P=0.00017) revealed the most significant association with risk of BC.

Discussion

In this case-control study of BC in Pakistani women, significant associations were observed between two SNPs of FGFR2 [rs1219648: P=9.08e-006 (GG, P=0.000087, AG, P=0.000018) and rs2981582: P=0.005 (CT, P=0.001, TT, P=0.097)]. These results are consistent with results obtained from other Asian regions, including China (16,32,37), Japan (38) and India (18). Furthermore, the SNPs rs1219648 and rs2981582 of FGFR2 have been consistently associated with BC risk in several other ethnic groups, including European (39), Hispanic and non-Hispanic Caucasian women from Southwestern United States (40), African American (29,41) and Tunisian (42) women populations. The two SNPs (rs1219648 and rs2981582) belong to intronic region (intron 2) of the FGFR2 gene, and the precise mechanism that would explain how FGFR2 risk alleles induce upregulation of the expression of FGFR2 has yet to be fully elucidated (32). However, it has been reported in a couple of previous studies that these variants upregulate the expression of FGFR2 in BC tissues by acting as enhancer regions, which may result in tumor formation (18,43). The aberrant expression of nine different isoforms of FGFR2 as a result of alternative splicing has shown the activation of signal transduction and development of BC (14,44). In addition, a high degree of conservation in intron 2 of FGFR2 in mammals, and the presence of several putative transcription-factor binding sites (15) in the proximate regions of the significant SNPs, suggest that these SNPs may exert a significant role in tumor development (18).

The additional classifications of BC based on clinicopathological characteristics, including ER, PR and HER2 status, have been utilized to understand the etiology of the heterogeneous tumor, which may be helpful in terms of elucidating the mechanisms of carcinogenesis and in improving the prevention and treatment of cancer (45). Therefore, the associations between the two variants of FGFR2 and clinicopathological characteristics of BC were further assessed. Analyses stratified by the status of the ER and PR in the present study revealed that SNP rs1219648 of the FGFR2 gene remained significantly associated with BC risk in the two intrinsic subtypes, including ER-positive (P=0.042) and ER-negative (P=0.003) tumors, as well as in PR-positive (P=0.090) and PR negative (P=0.0007) tumors, consistent with the results of certain previous studies (16,18,32,37). The second SNP of the FGFR2 gene, rs2981582, did not show significant associations with either ER- and PR-positive or ER- and PR-negative tumors, although SNP rs2981582 was significantly associated in the whole sample set (non-stratified) of cases. The lack of any association may be the result of a low number of samples in each intrinsic subtype following stratification, considering ER, PR and HER2.

In the case of TNRC9 (TOX3) gene variants, a significant association was observed between SNP rs3803662 and the risk of BC (P=0.012), although no associations were identified in SNP rs8051542 of TNRC9 in Pakistani women, consistent with the results of a meta-analysis study (46). Previously published reports have revealed a positive correlation of SNP rs3803662 of TNRC9 with BC risk in different ethnic groups (10,15). However, repeated studies of the identical SNP among European, African-American and East Asian populations reported contradictory results (23-25,28). Furthermore, the role of these SNPs as BC susceptibility variants in TNRC9 has yet to be determined. However, the overexpression of TNRC9 in BC, particularly in advanced BC, has been reported in a few studies (47,48), and its amplification has been associated with reduced disease-free and metastasis-free survival rates. The inverse correlation of the expression of TNRC9 and BRCA1 provided further evidence for the involvement of TNRC9 in the development of BC (19). Analyses stratified by ER and PR

status revealed that SNP rs3803662 of the TNRC9 gene did not exhibit significant associations with any intrinsic subtype (ER- and PR-positive, as well as ER- and PR-negative tumors) in the present study. Similar observations of the genetic variant rs3803662 in TNRC9 have been reported in a previous study on a Chinese population (49). However, a study that included 12,974 ER-positive and 3,765 ER-negative cases reported the association of rs3803662 with BC risk in the two tumor subtypes (ER+ and ER- tumors), in contrast with our results (50). These controversies in the literature regarding the association of TNRC9 SNPs with risk of BC, as well as with intrinsic subtypes in different ethnic groups, exist due to the following two major reasons: First, genetic factors differ according to ethnicity, and secondly, larger sample sizes are required to assess gene-gene and gene-environment interactions to signify a powerful BC risk in the population (49).

Regarding the variant of MAP3K1, no significant association of SNP rs889312 (P=0.245) with the risk of BC in Pakistani women was observed in the present study. This finding is also relevant to previous studies for women of African ancestry (29-31), demonstrating a poor association of the SNP with BC. However, the rs889312-C allele of MAP3K1 has been reported as a risk factor for the development of BC in European and Asian ancestry populations (27), contrary to the results in the present study.

All three BC-associated genetic variants in the whole sample set (rs2981582, rs1219648, rs3803662) also remained associated significantly with an increased risk of BC in the sporadic group of patients when sorted on the basis of the family history of BC in our population. This finding regarding the involvement of these genetic variants for sporadic BC in our population is consistent with previous observations made in Chinese women (37), in Chinese Han Women (32) and in sporadic post-menopausal women of European ancestry with respect to the SNPs of FGFR2 (rs2981582, rs1219648) (51). Furthermore, the overexpression of the FGFR2 and TNRC9 genes in sporadic patients with BC compared with controls further supports the greater involvement of these susceptibility loci in a predisposition to sporadic BC (19).

In conclusion, the present study has provided evidence revealing a significant association of FGFR2 intron 2 SNPs (rs2981582 and rs1219648) and TNRC9 SNP (rs3803662) with BC among Pakistani women. Along with variable interactions of these SNPs with different clinicopathological characteristics, all three genetic variants (rs2981582, rs1219648, rs3803662) revealed a significant association with increased risk of sporadic BC in this population. In addition, there was an increased effect (stronger significant association) between haplotype combinations of the two SNPs of FGFR2 (rs2981582 and rs1219648) with BC risk in Pakistani women. Further studies of larger data sets, along with subcategorization by clinical parameters, are required to confirm the role of these variants in intrinsic subtypes of BC in Pakistan that may help to improve our understanding of the genetic heterogeneity in this complex disease in our population.

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