Original Article

An investigation of the effects of *FGFR2* and *B7-H4* polymorphisms in breast cancer

ABSTRACT

Introduction: Polymorphisms in *FGFR2* are important markers for breast cancer susceptibility in the general population. *CHEK2* and *FGFR2* polymorphisms with known susceptibility alleles of *BRCA1*, *BRCA2*, *PTEN*, and *TP53*, can be investigated as potential modifiers of high penetrant risk alleles. Although the *B7-H4* gene is highly expressed in many different tumors, there is one published study showing the association of polymorphisms with breast cancer. We aimed to investigate *FGFR2* and *B7-H4* polymorphisms in breast cancer in the Turkish community.

Materials and Methods: In a group of 31 cases diagnosed with breast cancer and 30 healthy women with matched ages, the single-nucleotide polymorphisms (SNPs) rs1219648, rs2981582 in *FGFR2* gene were identified by sequence analysis and the SNPs rs10754339, rs10801935, and rs3738414 in the *B7-H4* gene were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Statistical analysis was performed using SPSS.

Results: Although statistically not significant, the frequency of *FGFR2* heterozygous polymorphisms in the group with breast cancer was detected to be higher. In the *B7-H4* SNP rs10801935, polymorphic AA, and AG genotype distributions were found in higher frequencies in the breast cancer patients. In contrast to the results of a published study, the present study shows that *B7-H4* rs3738414 polymorphism GG genotype was found in higher frequency in the control group than the breast cancer group and the result was statistically significant (*P*=0.018).

Conclusion: Larger scale studies are necessary to determine the prevalence of these polymorphisms and association with breast cancer in Turkish community, as this study is the first study performed.

KEY WORDS: Breast cancer, *FGFR2*, *B7-H4*, polymorphism

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INTRODUCTION

In recent years, a growing number of single-nucleotide polymorphism (SNPs) in genes and chromosomal loci have been identified in genome wide association (GWA) studies that have been linked to breast cancer such as in the genes *FGFR2*,[1,2] *LSP1*,[2] *MAP3K1*,[2] *TGFB1*,[3] *TOX3*,[2,4] and chromosomal loci 2q35[4] and 8q.[2]

The two SNPs (rs2981582 and rs1219648) in the second intron of *FGFR2* gene, which is known to be expressed and amplified in sporadic breast cancer, are associated with a high risk of breast cancer development by two independent GWA SNP studies.[1,2] Huijts *et al.,* further confirmed this association by employing a candidate gene approach.[5] A three phase GWA study among Ashkenazi Jews also supported the association previously established by Hunter *et al.,* and Easton *et al.*[1,2,6]

The biological relevance and functions of many SNPs are not currently understood.[7] Meyer *et al.,*

reported that SNPs identified in the second intron of the *FGFR2* gene resulted in an alteration in the binding affinity of the transcription factors OCT1, RUNX2 and C/EBPβ leading to an increased expression of *FGFR2* and thereby increased susceptibility to breast cancer.[8] The human *FGFR2* gene is located in chromosome 10q26 and consists of 21 exons. The *FGFR2* gene codes the two isoforms Fgfr2 IIIb (fgfr2b) and Fgfr2 IIIc (fgfr2c) due to alternative splicing. While the FGFR2b isoform is dominantly expressed in epithelial cells, the FGFR2c isoform is expressed more in mesenchymal cells.[9]

B7 like molecules belong to the immunoglobulin super family of proteins. These molecules play a critical role in the control and regulation of antigen specific immune response by binding to their receptors in T cells.[10] Interestingly, B7 molecules can both stimulate and suppress T-cell activation.[11,12] The negative signals that suppress T-cell activation are generally provided by the newly identified B7 members, that is, B7-H1 and B7-H4.[13] It has been discovered that B7-H4, which is expressed in many tissue and cells,

can regulate inherent immune response of the host by suppressing the growth of neutrophil progenitors, in addition to its effect on the T-cell response. The human *B7-H4* gene has been mapped to Chromosome 1 (1p13.1), and consists of six exons and five introns within an area of 66 kb. The sixth exon can be alternatively spliced to form two different transcripts.[14] The B7-H4 molecule can functionally inhibit T-cell attachment, growth, cytokine secretion, and the development of cytotoxicity by inhibiting cell cycle progression.[10] It has been found that B7-H4 is expressed in many human cancer types.[15] B7-H4, is over expressed at both mRNA and protein levels in ductal and lobular breast adenocarcinoma.[16] Immunohistochemistry has shown that B7-H4 was over expressed in 95% of primary breast cancers as well as in metastatic breast cancers independent of tumor grade and stage.[17] A statistically significant association was reported between B7-H4 positive cells and a decrease in the number of infiltrating lymphocytes in invasive ductal carcinoma and tumors.[18] It has been noted that the untranslated regions (UTRs) and introns, especially the first intron can regulate gene expression at many levels such as the production of stable mRNA, enhanced translational efficiency and mRNA decay. In a study by Zhang *et al.*, three polymorphisms have been discovered in the UTRs and the first intron of *B7-H4*, which indicated an association with breast cancer risk in the Han community in northeast of China.[19] This is the first polymorphism study to report an association between *B7-H4* polymorphism and the risk of breast cancer.[19]

MATERIALS AND METHODS

Ethical approval for this study was obtained from local ethical committee before the study was commenced. The principles adopted in the Helsinki declaration were conformed and written informed consent from the participants was also obtained.

A total of 31 premenapausal and postmenapausal patients with breast cancer and 30 postmenapausal, older than age of 50, healthy women without breast cancer were included in the study.

Genomic DNA was isolated from 2 ml of blood samples drawn into tubes containing ethylenediaminetetraacetic acid (EDTA) using high pure polymerase chain reaction (PCR) template preparation kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Obtained genomic DNA was amplified by PCR for *FGFR2* rs1219648 and rs2981582 polymorphisms. Sequence PCR for *FGFR2* rs1219648 and rs2981582 was carried out following PCR cleaning by the same forward primers using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, CA, USA). For *FGFR2* rs1219648 and rs2981582 sequence analysis, capillary electrophoresis was carried out on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Obtained genomic DNA was amplified by PCR in order to investigate *B7-H4* gene rs10754339, rs10801935 and rs3738414

polymorphisms, respectively. Control of the PCR products were performed in 2% agarose gel. Later, PCR products were enzyme digested by MscI, SalI, and BtsI enzymes. Control of the enzyme digested products was performed in 2% agarose gel. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS for Windows Release version 12, SPSS Inc. Headquarters, 223 S. Wacker Drive, 11th floor, Chicago, IL) program. All data were compared by *t*-test. Assessment was performed by Fisher's Chi-square test.

RESULTS

The polymorphisms in the SNPs rs2981582 and rs1219648 in the *FGFR2* gene for both control and breast cancer patients are given in Table 1. The frequencies of both polymorphs in breast cancer case group and control groups were found to be

Table 1: Genotype distribution of *FGFR2* gene polymorphisms in the breast cancer patient and control groups

Groups	FGFR2 rs2981582 CC Genotype frequency *n* (%)	FGFR2 rs2981582 CT Genotype frequency *n* (%)	FGFR2 rs2981582 TT Genotype frequency *n* (%)
Patient (*n*=31)	6 (19.4)	16 (51.6)	9 (29)
Control (*n*=30)	8 (26.7)	12 (40)	10 (33.3)
Groups	FGFR2 rs1219648 AA Genotype frequency *n* (%)	FGFR2 rs1219648 AG Genotype frequency *n* (%)	FGFR2 rs1219648 GG Genotype frequency *n* (%)
Patient (*n*=31)	4 (12.9)	18 (58.1)	9 (29)
Control (*n*=30)	9 (30)	10 (33.3)	11 (36.7)

Table 2: Genotype distribution of *B7-H4* gene polymorphisms in the breast cancer patient and control groups

Groups	B7-H4 rs10754339 AA Genotype frequency *n* (%)	B7-H4 rs10754339 AG Genotype frequency *n* (%)	B7-H4 rs10754339 GG Genotype frequency *n* (%)
Patient (*n*=31)	24 (77.4)	6 (19.4)	1 (3.2)
Control (*n*=30)	26 (86.7)	3 (10)	1 (3.3)
Groups	B7-H4 rs10801935 AA Genotype frequency *n* (%)	B7-H4 rs10801935 AC Genotype frequency *n* (%)	B7-H4 rs10801935 CC Genotype frequency *n* (%)
Patient (*n*=31)	11 (35.5)	16 (51.6)	4 (12.9)
Control (*n*=30)	8 (26.7)	13 (43.3)	9 (30)
Groups	B7-H4 rs3738414 AA Genotype frequency *n* (%)	B7-H4 rs3738414 AG Genotype frequency *n* (%)	B7-H4 rs3738414 GG Genotype frequency *n* (%)
Patient (*n*=31)	3 (9.7)	14 (45.2)	14 (45.2)
Control (*n*=30)	1 (3.3)	6 (20)	23 (76.7)

statistically insignificant.

The distribution of polymorphisms in the SNPs rs10754339, rs10801935, and rs3738414 in the *B7-H4* gene in breast cancer patient group and control group are given in Table 2. The frequencies of the first two SNPs were found to be almost similar while the GG genotype in the rs3738414 SNP was found to be statistically significantly lower in the breast cancer group than in the control group (P=0,018). The breast cancer group consisted of 23 patients in the age group of over 50 years (74.19%) and 8 patients (25.81%) who were aged below 50 years. No statistically significant difference was found between the percentages of *FGFR2* and *B7-H4* genotypes in the group of patients above 50 years and below 50 years of age (P>0.05) [Table 3].

Moreover, no statistically significant difference in the frequency of *FGFR2* and *B7-H4* genotypes was found in the breast cancer patients with and without a family history of breast cancer, patients with estrogen receptor (ER) positive or negative cancers; patients with progesterone receptor (PR) positive or negative cancers, patients with c-erb-B2 (HER-2/neu) positive and negative cancers, patients with lymph node-positive and negative tumors, as well as tumor sizes (P>0.05) [Table 3].

DISCUSSION

Five SNPs, which show the association of breast cancer risk, have been localized to intron 2 of the *FGFR2* gene. However, in patients with these SNPs, the mechanism of mammalian carcinogenesis could not be elucidated.

Low-penetrance susceptibility genes coupled with environmental and genetic factors have been found to be important for carcinogenesis. Recently, the *FGFR2* gene, one of the low-penetrance genes, has been identified as a potential susceptible gene common to breast cancer.[20]

The *FGFR2* gene product functions as FGF receptors where the FGFR2b and FGFR2c isoforms have different expression domains and ligand specificities.[21] FGFR2 is a member of the FGFR family of receptor tyrosine kinases (RTK). FGF receptors play an important role in control of many cellular processes such as cell proliferation, differentiation, migration, and cellular homeostasis.[22] Dysregulated FGFR2 signaling may enhance the risk of the development of carcinogenesis.[21]

Recently, two independent GWA studies showed that many polymorphic loci were associated with the risk of breast cancer. One of the most powerful associations was found in the *FGFR2* gene.[1,2]

While the SNP rs2981582, which is located in the upstream border of intron 2, showed the most significant association in the study of Easton *et al.*,[2,23] Hunter *et al.*, reported the highest association with the SNP rs1219648 for breast cancer risk.[1]

According to our data, *FGFR2* rs2981582 and rs1219648 SNPs do not appear to be associated within the breast cancer patients (P>0.05). However, for rs2981582 the breast cancer case group showed a high frequency of 58.1% AG and 43.55% G alleles

Table 3: The relationship of B7-H4 and FGFR2 genotypes with clinical properties

Gene	FGFR2						B7-H4								
SNP	rs2981582			rs1219648			rs10754339			rs10801935			rs3738414		
Genotype	TT	CC	CT	GG	AA	AG	GG	AA	AG	AA	CC	AC	GG	AA	AG
n (lymph node +)	7	5	10	8	3	11	1	15	6	8	3	11	GG	AA	AG
%	31.8	22.7	45.5	36.36	13.6	50	4.55	68.2	27.3	36.4	13.6	50	11	2	9
n (lymph node -)	2	1	6	1	1	7	0	9	0	3	1	5	50	9.1	40.9
%	22.2	11.1	66.7	11.11	11.1	77.8	0	100	0	33.3	11.1	55.6	3	1	5
n (breast CA + in family)	1	3	4	3	1	4	0	7	1	4	0	4	33.3	11.1	55.6
%	12.5	37.5	50	37.5	12.5	50	0	87.5	12.5	50	0	50	4	1	3
n (breast CA - in family)	8	3	12	8	1	14	1	17	5	7	4	12	50	12.5	37.5
%	34.8	13	52.2	34.8	4.35	60.9	4.35	73.9	21.7	30.4	17.4	52.2	10	2	11
n (premenopausal)	5	1	5	4	2	5	1	7	3	4	3	4	43.5	8.7	47.8
%	45.5	9.1	45.5	36.36	18.2	45.5	9.1	63.6	27.3	36.4	27.3	36.4	5	1	5
n (postmenopausal)	4	5	11	6	1	13	0	17	3	7	1	12	45.5	9.1	45.5
%	20	25	55	30	5	65	0	85	15	35	5	60	10	2	8
n (ER +)	4	4	12	5	2	13	1	18	1	9	2	9	50	10	40
%	20	20	60	25	10	65	5	90	5	45	10	45	8	2	10
n (ER -)	5	2	4	4	2	5	0	7	4	2	2	7	40	10	50
%	45.5	18.2	36.4	36.36	18.2	45.5	0	63.6	36.4	18.2	18.2	63.6	6	1	4
n (PR +)	5	5	11	5	3	13	1	18	2	9	2	10	54.6	9.1	36.4
%	23.8	23.8	52.4	23.81	14.3	61.9	4.76	85.7	9.52	42.9	9.52	47.6	8	2	11
n (PR -)	4	1	5	4	1	5	0	7	3	2	2	6	38.1	9.52	52.4
%	40	10	50	40	10	50	0	70	30	20	20	60	6	1	3
n (c-erb-B2+)	4	4	5	4	2	7	1	10	2	3	0	10	60	10	30
%	30.8	30.8	38.5	30.77	15.4	53.9	7.69	76.9	15.4	23.1	0	76.9	9	0	4
n (c-erb-B2-)	5	2	11	5	2	11	0	14	4	7	4	7	69.2	0	30.8
%	27.8	11.1	61.1	27.78	11.1	61.1	0	77.8	22.2	38.9	22.2	38.9	6	2	10

n: number, +: positive, -: negative, CA: cancer

compared with the control group (33.3% AG; 36.67% G allele). For the SNP rs2981582, the breast cancer case group showed a high frequency of 51.6% CT genotype and 40.32% T allele compared with the control group (40% CT and 36.67% T allele). In the breast cancer group of the current study, the *FGFR2* rs2981582 CT genotype and T allele frequency and rs 1219648 AG genotype and G allele frequency may be associated with breast cancer [Table 1]. The fact that the data are statistically insignificant can be due to the low number of individuals employed in this study.

Liang *et al.,* reported that the rs2981582 C/T and the rs1219648 A/G variant genotypes in the Chinese population may be associated with a high risk of breast cancer.[24] In line with the results of the Liang *et al.,* study, our data show that the *FGFR2* rs2981582 CT genotype and T allele frequency and rs1219648 AG genotype and G allele frequency are higher in the breast cancer group than in the control group.

Samson *et al.*, investigated the relationship between *FGFR2* rs2981582 polymorphism and cancer risk in south Indian women, however, their results did not support the results from the studies of Hunter *et al.*,[1] and Easton *et al.*[25]

In a study by Boyarskikh *et al.*, the frequency for rs2981582 in breast cancer patients and control groups from western Siberia, Russian Federation, was reported to be in the middle, between the frequencies observed in the Asian and European populations.[23] Our results are in line with the study of Boyarskikh *et al.*, because rs2981582 CT allele was found to be high in breast cancer group than in the control group.

Raskin *et al.*, evaluated the association of rs1219648 in various ethnic groups in Israel. *FGFR2* was noted as a breast cancer susceptibility gene among Ashkenazi and Sephardi Jews but it poses less risk in the Arab community.[26] Our study showed a higher frequency in the AG genotype and G allele frequency for rs1219648 in the breast cancer group; therefore, from this point of view, is in line with their Arab–Israeli population study.

In a meta-analysis for rs1219648 and rs2420946 revealed a significantly higher risk of breast cancer in postmenopausal women. In addition, a study by Kawase *et al.*, also reported the risk in premenopausal women.[27] However, we have also observed that the rs2981582 TT genotype in premenopausal breast cancer patients had a higher frequency than postmenopausal patients; on the contrary, AG genotype of rs1219648 in postmenopausal patients with breast cancer was at higher frequencies than premenopausal patients. This shows that our study is in line with the study of Raskin *et al.*, [Table 3].[26]

In our study, the rs2981582 TT genotype frequency in patients with lymph node-positive tumors was higher than in the lymph-node negative patients, while the rs1219648 GG genotype frequency in patients with lymph node-positive tumors was also higher than lymph node-negative patients [Table 3].

In a study of breast cancer patients of European and Asian origin, Garcia-Closas *et al.*,[28] reported a stronger association of *FGFR2* rs2981582 in patients with ER positive (ER+) tumors than in ER negative (ER-) tumors ($P < 0.001$). This SNP was also highly associated with PR positive (PR+), low grade node positive tumors.[29]

Liang *et al.*, reported that postmenopausal and elderly women were more likely to be ER-/PR- breast cancer patients.[24]

Samson *et al.*, reported that the rs2981582 polymorphism showed a significant association with PR status but not with ER status in the patients.[25]

In the current study, although not statistically significant, both rs2981582 and rs1219648 polymorphisms were associated at higher frequencies in patients with ER and PR negative breast cancer, rather than positive patients, and there was no association with c-erb-B2 (HER-2/neu) [Table 3].

In the study by Kawase *et al.*, an association between *FGFR2* polymorphisms in breast cancer and familial history of breast cancer ($P = 0.003$) was reported.[27] However, in our study, a statistically significant difference was not found between the percentages of the *FGFR2* gene genotypes in individuals with a family history of breast cancer and in individuals without family history of breast cancer ($P > 0.05$) [Table 3].

B7-H4 was recently reported as a member of the B7 family, which negatively affected T-cell activation, cytokine secretion, and cytotoxicity.[10,30] It was reported that B7-H4 was expressed by most professional antigen-presenting cells as shown by both flow cytometric analysis, as well as tissue localization studies by immunoreactivity.[29,31] Moreover, B7-H4 seems to bind only to activated T-lymphocytes but not resting T-lymphocytes.[10,31] *In vivo* experiments support the function of B7-H4 in inhibiting the host cell-mediated immunity.[10] In addition to its immunomodulatory functions, B7-H4 protein was found to be over-expressed in several different tumors such as breast carcinoma,[17] ovarian carcinoma,[30,32] and lung carcinoma.[30] There is, however, very little expression or no expression in normal nontransformed tissues.[10,17,30]

In our study, the association of *B7-H4* rs10754339, rs10801935 and rs3738414 SNPs with breast cancer was evaluated. These SNPs were initially reported to be associated with breast cancer by Zhang *et al.*[19] Zhang *et al.*, selected three potential SNPs rs10754339, rs10801935, and rs3738414 of *B-H4* and showed statistically significant differences in genotype and allele distributions of these SNPs.[19] These authors, for the first time, found an association of these SNPs with breast cancer risk and prognosis in Han Chinese women. To the best of our knowledge, this is the only study related to this subject in the literature. Our study is therefore the second study related to *B7-H4* gene

polymorphisms and their association with breast cancer.

The results of the study by Zhang *et al.*, revealed that some of the alleles, genotypes and haplotypes of these three SNPs could be associated with breast cancer risk and prognosis. According to their results, the possibility of a high risk of breast cancer in women with AG genotype and G allele of rs10754339 (OR = 1.455, OR = 1.325) suggests that rs10754339 G allele may play a role in breast cancer risk. rs10754339 may also play a role in the formation of different types of transcripts and expression of *B7-H4*.[19] In line with the results of Zhang *et al.*,[19] our study showed that, although not statistically significant (*P* > 0.05), the AG genotype of rs10754339 (19.4%) and G allele frequency (22.58%) was higher in the breast cancer case group than the control group (10% and 13.33%, respectively). Our study showed no association with the GG genotype.

Again, in line with the data from Zhang *et al.*, although statistically not significant, the AA and AG genotype frequency in the breast cancer group was higher than in the control group. The CC genotype frequency in breast cancer case group was lower than in the control group [Table 2]. Zhang *et al.*, reported a lower risk of breast cancer development (OR = 0.328) in women with the CC genotype for rs10801935, which shows that that rs10801935 CC genotype in intron 1 of *B7-H4* may be a protective factor in breast cancer.[19]

A potential protective effect on breast cancer development in women with AA genotype and A allele of rs3738414 (OR = 0.412) may implicate the A allele of rs3738414 in a potential protective effect in breast cancer.[19] In our study, contrary to the results reported by Zhang *et al.*,[19] the AA and AG genotype frequency (9.7% and 45.2%, respectively) for rs3738414 in the breast cancer patient group was higher than in the control group (3.3% and 20%, respectively). Again, in contrast to the results reported by Zhang *et al.*,[19] the frequency of GG genotype in the breast cancer group (45.2%) was lower than in the control group (76.7%). This may be explained by the assumption that the Turkish population may display a different pattern for this SNP. New studies are necessary to verify this condition in different societies, as well as in the Turkish population.

According to the association analysis between *B7-H4* polymorphisms and clinical features reported by Zhang *et al.*,[19] the rs10754339 GG genotype was found to associate significantly with lymph node metastases and PR status, whereas the G allele and AG genotype was found to associate with lymph node metastasis and ER status, respectively. In our study, the GG and AG genotypes showed association only for lymph node metastasis in accordance with the study by Zhang *et al.*[19] However, in the study by Zhang *et al.*, the other two SNPs were not connected to clinicopathological features.[19] In our study, although not statistically significant, rs10801935 shows an association with ER+ and PR+ breast cancers; while the frequency of AA genotype in ER+ patients were higher

than in ER- patients. The AA genotype frequency in patients with PR+ tumors was again higher than in PR- patients. Although the GG genotype for rs3738414 has a higher frequency in patients with positive lymph node involvement, PR and c-erb-B2, however, this association was not statistically significant. As the GG genotype, was found to be higher in control group than in the breast cancer group in our study, this may be regarded as meaningless. In our study group, the AC genotype frequency for *B7H4* rs10801935 was lower in premenopausal patients with breast cancer compared with the postmenopausal cases [Table 3]. Zhang *et al.*, did not report such similar data.[19]

In this study, polymorphisms in the *FGFR2* and *B7H4* genes, which might be associated with breast cancer risk, have been evaluated for the first time in Turkish women with breast cancer.

The clinical benefit of *FGFR2* gene polymorphisms in breast cancer should be further confirmed in new studies. *B-H4* gene polymorphisms also should be studied in cancer types with high expression of this gene. Breast cancer is one such cancer where this gene is highly expressed, and being one of the most common cancers that women are afflicted with, the potential effects of these polymorphisms in breast cancer should be brought out by further studies.

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