A rare FANCA gene variation as a breast cancer susceptibility allele in an Iranian population

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Abstract. Fanconi Anemia (FA) is an autosomal recessive syndrome characterized by congenital abnormalities, progressive bone marrow failure and Fanconi anemia complementation group A (*FANCA*) is also a potential breast and ovarian cancer susceptibility gene. A novel allele with tandem duplication of 13 base pair sequence in promoter region was identified. To investigate whether the 13 base pair sequence of tandem duplication in promoter region of the *FANCA* gene is of high penetrance in patients with breast cancer and to determine if the presence of the duplicated allele was associated with an altered risk of breast cancer, the present study screened DNA in blood samples from 304 breast cancer patients and 295 normal individuals as controls. The duplication allele had a frequency of 35.4 and 21.2% in patients with breast cancer and normal controls, respectively. There was a significant increase in the frequency of the duplication allele in patients with familial breast cancer compared with controls (45.1%, P=0.001). Furthermore, the estimated risk of breast cancer in individuals with a homozygote [odds ratio (OR), 4.093; 95% confidence intervals (CI), 1.957-8.561] or heterozygote duplicated genotype (OR, 3.315; 95% CI, 1.996-5.506) was higher compared with the corresponding normal homozygote genotype. In conclusion, the present study indicated that the higher the frequency of the duplicated allele, the higher the risk of breast cancer. To the best of our knowledge, the present study is the first to report *FANCA* gene duplication in patients with breast cancer.

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Introduction

Breast cancer is the most common type of invasive cancer in women and accounts for 16% of all female cancers, with the second highest mortality rate among women worldwide (1), while in USA and Iran it comprises 26 and 23% of all female cancers (2-4), respectively. In addition, breast cancer is an important public health issue; with ~8,000 women being diagnosed annually in Iran, of which 97.2% are women. However, due to the relatively early diagnosis, it is the fifth most common cause of cancer death in Iran (3). The two major susceptibility genes, *BRCA1* and *BRCA2*, account for a maximum of 20% of familial breast cancer cases worldwide. The remaining cases may be explained by mutations in other cancer susceptibility genes together with environmental factors (5).

The Fanconi anemia complementation group A *(FANCA)* gene is located at 16q24.3, and is primarily recognized as a gene involved in Fanconi anemia (FA), which is a rare autosomal recessive disorder with a prevalence of ~1-5 per million in the western world (6). FA genes are assigned into the following 8 distinct complementation FA groups: *FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF* and *FANCG*. *FANCA* is the most common FA subtype in the majority of populations and is defective in >65% of FA cases; *FANCC*, *FANCG* and *FANCD2* each account for ~5-15% of cases, with the remaining subtypes being rare (6-8). Furthermore, *FANCA* is a potential tumor suppressor gene due to its role in the repair of DNA damage, and it remains an attractive candidate as either a cancer predisposition gene or a target of genetic sporadic cancer (9). The proteins encoded by FA genes are closely associated to each other in molecular pathways involved in DNA repair, and interact directly to form a multi-subunit nuclear complex (10) that is required to respond to DNA damage (11,12). Notably, the 16q24.3 genomic region, where *FANCA* resides, is a common target for loss-of-heterozygosity in breast tumors (13), and in addition, an intronic *FANCA* single nucleotide polymorphism has been previously associated with an 8% increase in breast cancer risk (14). Only 1 potential *FANCA* missense mutation has been identified in UK families with breast cancer (15), and 4 large *FANCA* deletions have been previously reported in sporadic acute myeloid leukemia (16). As polymorphisms in promoters alter the transcription or regulation of a gene, it was hypothesized that

the *FANCA* gene duplication in the promoter region may alter the risk of developing breast cancer in an Iranian population, similar to previous reports in other populations (17), and may be associated with an altered risk of developing breast cancer. The present study therefore performed a case control study to determine whether duplication allele may be responsible for an increase in the risk of breast cancer among a population of Iranian women.

Materials and methods

Subjects. A total of 304 breast cancer cases were systematically ascertained through Imam Khomeini Hospital Complex (Tehran University of Medical Sciences, Tehran, Iran). Of these cases, 50% (152) were selected on the basis of a family history of breast cancer (defined as ≥2 cases of breast cancer in a first- or second-degree female relative). Blood was taken from all recruits who consented to molecular analysis for breast cancer predisposition genes at the Central Laboratory of Imam Khomeini Hospital Complex hospital. The age range of the all breast cancer participants was 28-74 with a mean age of 49.41±10.44 years (familial and non-familial breast cancer). The controls were selected from the same population from which the cases arose and consisted of 295 healthy female volunteers who were attending the Cancer Institute of Imam Khomeini Hospital Complex for a checkup. The age range of the controls was 28-74 with a mean age of 49.28±10.48 years. None of the control individuals had any history of breast cancer or any other neoplastic diseases, and had no family history of breast cancer diagnosed at the same clinics. Women with hysterectomy and artificial menopause, or who had been exposed to any kind of radiation, including X-rays, or chemotherapy in their life time were excluded from the study. Control and cancer groups were drawn from the same geographical area. Demographical and epidemiological risk factor data was collected from a short, structured questionnaire, which included information on age at menarche, age at menopause, marriage status, race, age at breast cancer onset, number of pregnancies and children, age at first child birth and average lactation term. An ongoing protocol to collect and store blood samples for future genomic tests was approved by the institutional review board and appropriate ethics committee. Peripheral blood was collected and genotyping analysis was performed for selected regions in the *FANCA* gene.

Ethical approval and consent to participate. The study was approved by the Research Ethics Committee for Tehran University of Medical Sciences (*IR.TUMS.REC.1395.2500*). Informed consent for testing and publication was obtained from all participants prior to participation in the present study (or their parents/legal guardians).

Molecular genetic analysis. DNA for genotyping was prepared from the peripheral blood sample of patients and controls. The DNA promoter region containing the duplication (164 bp) was amplified using primers designed by Primer3 (version 0.4.0) software and positive control primers were used to amplify estrogen receptor 1 gene exon 4 (329 bp), as published elsewhere (18). Primer sequences are presented in Table I.

Phenol-chloroform DNA extraction. DNA was isolated using AccuPrep® (high pure phenol-chloroform) Genomic DNA extraction kit (Bioneer Corporation; Takapouzist Co., Tehran, Iran). Lysis buffer (200 μl) was added to 200 μl whole blood and incubated at 60˚C for 10 min, 500 μl phenol was then added and centrifuged at 12,000 x *g* at 4˚C for 5 min. Chloroform (500 μl) was added to the upper phase and centrifuged for 5 min at 12,000 x *g* at 4˚C. Sodium Acetate (1:10) plus cold 100% ethanol (2:1) was added to the upper phase, frozen for 20 min at -20˚C and centrifuged for 10 min at 12,000 x *g* and 4˚C. To precipitate DNA, 70% ethanol was added and centrifugation was performed for 15 min at 12,000 x *g* and 4˚C. The DNA was measured using a spectrophotometer; the amount of DNA was calculated in μg/ml (85 μg/ml) by absorbance at 260 nm and the purity was tested by determining the 260/280 nm ratio (a ratio of 1.7 was detected).

Polymerase chain reaction (PCR) conditions. The following was added to each 50 μl PCR reaction tube: 43.5 μl master mix (5X HOT FIREPol® Blend; TAG Copenhagen A/S, Frederiksberg, Denmark); 2 μl (200 nM) primers synthesized by TAG Copenhagen A/S; 2 μl (50 ng) DNA template (extracted genomic DNA); and 2.52 μl Taq DNA polymerase (0.5 U Super Taq enzymes; Cambridge Bioscience, Ltd., Cambridge, UK) and PCR was performed in an Eppendorf thermo-cycler following the protocol in Table II. The first set of PCR primers amplified a 151-base pair product for allele 0 (normal) and a 164 base pair product for allele 1 (duplication). The DNA ladder (Biotium) was also purchased from Cambridge Bioscience, Ltd. The products were separated by electrophoresis on 3% agarose gel and stained by 1% ethidium bromide (Cambridge Bioscience, Ltd.). PCR products from the three genotypes were sequenced on the 96-capillary ABI 3730xl (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) by the Sanger sequencing technique.

Statistical analysis. The Hardy-Weinberg equilibrium was assessed by the standard methods (19). The data were considered using normal (00; women without a copy of the duplication allele), heterozygotes (01; women with 1 copy of the duplication allele) and homozygotes (11; women with 2 duplication alleles). Data was analyzed using SPSS, version 17.0 (SPSS, Inc., Chicago, IL, USA). Stratification with respect to demographics and risk factors was performed and post-stratification Pearson's χ^2 analysis was used to calculate the significance and odds ratio (OR) with a 95% confidence interval. All tests were two-sided and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of the FANCA promoter duplication. Variation in the *FANCA* gene promoter region (NCBI reference sequence, NM_000135.2) was screened using PCR analysis. Variant bands were purified and sequenced directly with forward and reverse primers. The comparison of band sequences and the sequence of the *FANCA* promoter demonstrated that a 13-base pair sequence (5'-GGCCACGACGCAA-3') located from -98 to -110 bases upstream of the transcription start point, which was present either as a single or double copy, causes variation

Table I. Polymerase chain reaction primers.

Primer	Sequence 5'→3'	Base pairs
Duplicated region *FANCA* primers	F: CCAAACGCAAAAACTACCTCACCG R: CGCTGCCTTCCTATTGGCTGC	164
ESR1 exon 4 primers (positive control)	F: ACCTGTGTTTTCAGGGATACGA R: GCTGCGCTTCGCATTCTTAC	329

FANCA, Fanconi anemia complementation group A; *ESR1*, estrogen receptor 1 gene exon 4; F, forward; R, reverse.

Table II. Polymerase chain reaction cycling conditions.

Step number	Reaction	Temperature (˚C)	Duration (min)	Number of cycles
1	Primary denaturation	94	4	1
2	Denaturation	94	1	35
3	Annealing	67	1	35
4	Extension	72	1	35
5	Final extension	72	5	2

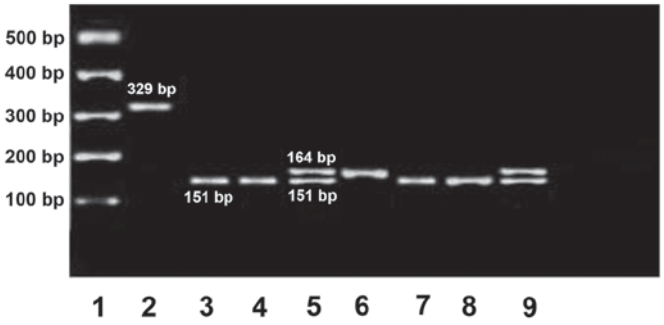

Figure 1. Genotyping the Fanconi anemia complementation group A promoter polymorphism by polymerase chain reaction. Allele 0 amplifies as a band of 151 bp and allele 1 as a band of 164 bp. All 3 genotypes are readily distinguishable on a 3% agarose gel run at 100 V for 1 h. Lane 1, ladder; lane 2, estrogen receptor 1 exon 4 gene, positive control; lanes 3,4,7 and 8, 00 homozygote; lane 5, 01 heterozygote; and lane 6, 11 homozygote. bp, base pairs.

in patterns observed on the agarose gel. In the present study, allele 0 is defined as having has a single copy of the sequence (5'-GGCCACGACGCAA-3'), whilst allele 1 has 2 tandem copies of this sequence. Notably, the sequence directly adjacent to (in the downstream direction) the 13-base pair sequence shares homology with the duplicate sequence.

Case-control study of FANCA variations. A single set of primers was used to amplify both alleles by PCR in the promoter region (Fig. 1). The frequency of the polymorphism was determined in patients with breast cancer and controls to assess if the presence of either allele was associated with a predisposition to breast cancer. Table III presents the genotypic frequency distribution in patients with breast cancer and controls. The distribution of the genotypes within overall breast cancer and familial breast cancer groups deviated significantly from those expected under Hardy-Weinberg equilibrium with a P-value of 0.001. In patients with familial breast cancer, the estimated risk was 1.3 fold higher for individuals who were 01 heterozygote duplication (OR, 1.27; 95% CI, 0.825-1.955) or 1.5 fold higher for those who were 11 homozygote duplication (OR, 1.511; 95% CI, 0.73-3.131) compared with 00 homozygotes. Therefore, the results indicated that a higher frequency of allele 1 may be associated with an increased risk of developing familial breast cancer. However, the genotypic frequency did not show significant (P=0.365) elevation among the-non family history group. Furthermore, the frequency of the duplication allele (allele 1) in all breast cancer patients and familial breast cancer patients only was significantly higher compared with controls (P=0.001), with exception of non-familiar breast cancer patients (P=0.131; Table IV). Promoter duplication genotypes were compared with selected clinical breast cancer features, including age at menarche, age at breast cancer onset, age at menopause and lymph node metastasis in patients with breast cancer. The only significant association was for age at menarche, as indicated by the ORs presented in Table V. Genotype frequencies exhibited significantly different distributions in age at menarche (<12 years old vs. ≥12; P=0.001). The estimated risk was higher for individuals who were 11 homozygotes for the duplication (OR 0.148, 95% CI: 0.069-0.316) compared with the corresponding 01 heterozygotes (OR, 0.775; 95% CI, 0.428-1.405). Furthermore, the estimated risk of developing breast cancer (familiar breast cancer vs. non-familiar breast cancer) was 4-fold higher for those who were 11 homozygotes (OR 4.093, 95%; CI, 1.957-8.561) or 3-fold higher for those who were 01 heterozygotes (OR, 3.315; 95% CI, 1.996-5.506) compared with the corresponding 00 homozygotes. The results suggest that the higher the frequency of allele 1, the greater the risk of developing breast cancer.

Discussion

To the best of our knowledge, the present study was the first to evaluate the association between variations in the *FANCA* gene, in 304 breast cancer cases, and breast cancer risk in Iranian women. As this duplication was relatively common in healthy individuals, it may be regarded as a polymorphism. It is already established that alterations in the FANC group

Table III. The distribution of Fanconi anemia complementation group A gene promoter polymorphism genotypes and estimated risk in breast cancer cases and controls.

	Study group			
Group	00	01	11	P-value
Control (%)	190 (64.4)	85 (28.8)	20 (6.8)	
All breast cancer cases				0.001
Number (%)	131 (43.1)	131 (43.1)	42 (13.8)	
OR (95% CI)	1.0	2.235 (1.57-3.17)	3.046 (1.71-5.424)	
Familial breast cancer cases				0.001
Number (%)	43 (28.3)	81 (53.3)	28 (18.4)	
OR (95% CI)	1.0	1.27 (0.825-1.955)	1.511 (0.73-3.131)	
Non-familial breast cancer cases				0.365
Number (%)	88 (57.9)	50 (32.9)	14 (9.2)	
OR (95% CI)	1.0	4.211 (2.686-6.601)	6.186 (3.189-11.998)	

OR and CI for 01 and 11 groups are presented relative to that of 00. 00, normal genotype; 01, duplication heterozygote; 11, duplication homozygote; OR, odds ratio; CI, confidence interval.

Table IV. The distribution of Fanconi anemia complementation group A promoter polymorphism allelic frequencies in familial and non-familial breast cases compared with controls.

Study group	Allele 0 (%)	Allele 1 (%)	P-value	χ^2
All breast cancer cases	393 (46.6)	215 (53.4)	0.001	29.6
Control	465 (78.8)	125 (21.2)		
Familial breast cancer	167 (54.9)	137 (45.1)	0.001	55.21
Control	465 (78.8)	125 (21.2)		
Non-familial breast cancer	226 (74.3)	78 (25.7)	0.131	2.286
Control	465 (78.8)	125 (21.2)		

P-values indicate comparisons between the number of individuals with allele 0 and allele 1. 0, normal single copy allele; 1, duplication allele.

of genes are associated with several types of cancer (20-23). Following the identification of *BRCA2* as a member of the FANC family (*FANCD1*), researchers hypothesize that variations in other FANC genes, such as *FANCA*, may increase breast cancer susceptibility (24). For example, the absence of *FANCF* by aberrant promoter methylation (25,26) and *BACH1*/*FANCJ* mutations (27) have been previously identified in breast cancer cases. Furthermore, deletions (28) and duplications (17) in the *FANCA* gene are pathogenic and have been previously detected in patients with breast cancer.

As even single base changes in gene sequences may change gene expression regulation and lead to tumorigenesis, particularly if this affects a transcription factor binding site (17,29,30), the 13-base pair duplication alleles in the promoter region identified in the present study are important. Considering the common occurrence of both *FANCA* polymorphism alleles, neither allele is expected to be a high penetrance predisposition allele. The current study evaluated whether duplication in the promoter region of the *FANCA* gene may increase breast cancer susceptibility. The results of the present study were consistent with previous epidemiological studies, which indicated the role of *FANCA* gene variations in increasing the risk of breast cancer (31). According to the results of the present study, *FANCA* gene mutations generally increase the risk of breast cancer and contribute to the development of familial breast cancer. Previous research has highlighted the role of various genes involved in DNA repair, other than *BRCA1* or *BRCA2*, in increasing breast cancer susceptibility. For example, a study on the Finnish population reported that *FANCM* mutations caused a strong predisposition to breast cancer (31-35). Johnson *et al* (31) reported missense variants in the *BRCA1*, *BRCA2*, *ATM*, *CHEK2* and *ATM* genes to be significantly associated with breast cancer risk among cases with bilateral disease (P=0.005), particularly for less common alleles (P=0.00004). However, despite the evidence to support the potential role of the *FANCA* family in increasing susceptibility to breast cancer and other types of cancer (15), the investigation of the role of these genes in cancer susceptibility in a monoallelic context has been limited. However, heterozygous *FANCA* deletions have been reported as potential low penetrance alleles for acute myeloid leukemia (16). Recently, Virts *et al* (36) in the US demonstrated the *FANCT* gene to be a rare cancer susceptibility gene. In a study on a Spanish population, Blanco *et al* (37) introduced RAD51 paralog C, a novel FA gene necessary for homologous recombination repair, as a rare susceptibility gene for hereditary breast and ovarian cancer. By contrast, Cleton-Jansen *et al* (38) reported the absence of mutations in 19 breast cancer patients with 16q24.3.

Table V. Estimated risk of breast cancer for major risk factors in different genotypes.

Category	All	Genotype			P-value
		01	11	00	
Age at menarche					0.001
≤12 years (%)	220	100 (76.3)	16 (38.1) 26 (61.9)	104 (80.6)	
>12 years (%)	82	31 (23.7)	26 (61.9)	25 (19.4)	
OR (95% CI)		0.775 (0.428-1.405)	0.148 (0.069-0.316)	1	
Age at breast cancer onset					0.335
≤40 years (%)	118	57 (44.2)	14 (34.1)	47 (36.4)	
>40 years (%)	181	72 (55.8)	27 (65.9)	82 (63.6)	
OR (95% CI)		1.381 (0.838- 2.276)	0.905 (0.432-1.893)	1	
Age at menopause					0.693
≤50 years (%)	39	20 (27.8)	5 (19.2)	14 (25.5)	
>50 years (%)	114	52 (72.2)	21 (80.8)	41 (74.5)	
OR (95% CI)		1.126 (0.508-2.497)	0.697 (0.221-2.199)	1	
Metastasis status					0.348
Yes (%)	37	14 (10.8)	8 (19)	15 (11.6)	
No (%)	264	116 (89.2)	34 (81)	114 (88.4)	
OR (95% CI)		0.917 (0.423-1.987)	1.788 (0.699-4.576)	1	
Cancer status					0.001
Familial (%)	152	81 (61.8)	28 (66.7)	43 (32.8)	
Non-familial (%)	152	50 (38.2)	14 (33.3)	88 (67.2)	
OR (95% CI)		3.315 (1.996-5.506)	4.093 (1.957-8.561)	1	

OR and CI for 01 and 11 groups are presented relative to that of 00. 00, normal genotype; 01, duplication heterozygote; 11, duplication homozygote; OR, odds ratio; CI, confidence interval.

Genotyping of the promoter polymorphism indicated a significant difference in the allele or genotype distribution between patients with breast cancer and normal controls. The present study had 80% power to detect an OR ≥1.27 for heterozygous carriers of the duplication and an OR ≥1.511 for homozygous carriers of the duplication in hereditary breast cancer. Furthermore, the frequency of the allele 1 was significantly higher in patients with a family history of breast cancer compared with the control group (45 and 21%, respectively; P=0.001), indicating that allele 1 may increase the risk of breast cancer development. Nevertheless, larger studies are required to compare the frequency of *FANCA* gene sequence variants between breast cancer patients and healthy controls with different ethnicities. In conclusion, the current study was the first to report a promoter region variation in the *FANCA* gene among women with breast cancer in Iran. The results of the present study confirmed the allelic variants in the *FANCA* promoter region as a tumor suppressor gene. This gene affects cell activities, such as the basal rate of transcription or the regulation of transcription, which increase the risk of breast cancer. However, a more extensive evaluation of the role of other FA pathway genes in hereditary susceptibility to cancer is required. Further studies are required to clarify the full implication of *FANCA* gene variation in breast cancer susceptibility and predisposition to other types of cancer. Such genetic markers may be useful as an early breast cancer diagnosis tool in developing countries such as Iran.

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