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

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Received July 1, 2016; Accepted February 13, 2017

DOI: 10.3892/mmr.2017.6489

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 the corresponding normal homozygote (OR, 4.093; P=0.001). Furthermore, the estimated risk of breast cancer in individuals with a homozygote [odds ratio (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.

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; Takapouzi 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, Frederikssberg, 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 χ² 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'-GGCCACCGACGCA-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
in patterns observed on the agarose gel. In the present study, allele 0 is defined as having a single copy of the sequence (5'-GGCCACGACGCAA-3'), whilst allele 1 has 2 tandem copies of this sequence. Notably, the sequence directly adjacent (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 | Allele 0 | Allele 1 | P-value | χ² |
|-------------|---------|---------|---------|----|
| Control (%) | 190 (64.4) | 85 (28.8) | 20 (6.8) | 0.001 | 29.6 |
| All breast cancer cases | 393 (46.6) | 215 (53.4) | 0.001 | 55.21 |
| Control | 465 (78.8) | 125 (21.2) | 0.001 | 2.286 |
| Familial breast cancer cases | 167 (54.9) | 137 (45.1) | 0.131 | 2.86 |
| Control | 465 (78.8) | 125 (21.2) | 0.001 | 2.286 |
| Non-familial breast cancer cases | 226 (74.3) | 78 (25.7) | 0.131 | 2.86 |
| Control | 465 (78.8) | 125 (21.2) | 0.001 | 2.286 |

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 | χ² |
|-------------|--------------|--------------|---------|----|
| All breast cancer cases | 393 (46.6) | 215 (53.4) | 0.001 | 29.6 |
| Control | 465 (78.8) | 125 (21.2) | 0.001 | 55.21 |
| Familial breast cancer cases | 167 (54.9) | 137 (45.1) | 0.131 | 2.286 |
| Control | 465 (78.8) | 125 (21.2) | 0.001 | 2.286 |
| Non-familial breast cancer cases | 226 (74.3) | 78 (25.7) | 0.131 | 2.286 |
| Control | 465 (78.8) | 125 (21.2) | 0.001 | 2.286 |

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 FANC F by aberrant promoter methylation (25,26) and BACH1/FANCF 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.
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. Nevertheless, larger studies are required to compare the frequency of FANCA gene variations 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.

Acknowledgements

The current study was supported by Tehran University of Medical Sciences and Health Services (grant no. 91-01-31-14816).

References

1. WHO: Global Burden of Disease. Geneva, Switzerland. 2004.
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun MJ: Cancer statistics, 2008. CA Cancer J Clin 58: 71-96, 2008.
3. Akbari ME, Abachizade K, Tabatabaei M, Ghanbari Motlagh A, Magid Jabari Z and Khayamzadeh M: Cancer in Iran. Darolfeiki Publications, Ghom, 2008.
4. Kamangar F, Dores GM and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: Defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 24: 2137-2150, 2006.
5. Stratton MR and Rahman N: The emerging landscape of breast cancer susceptibility. Nat Genet 40: 17-22, 2008.
6. Hoeij H and Patel KJ: The emerging genetic and molecular basis of Fanconi anemia. Nat Rev Genet 2: 446-457, 2001.
7. Tischkowitz MD and Hodgson SV: Fanconi anaemia. J Med Genet 40: 1-10, 2003.
8. Pronk JC, Gibson RA, Savoia A, Wijker M, Morgan NV, Melchionda S, Ford D, Temtamy S, Ortega JJ, Jansen S, et al: Localisation of the Fanconi anaemia complementation group A gene to chromosome 16q24.3. Nat Genet 11: 338-340, 1995.
9. Han SS, Tompkins VS, Son DJ, Han S, Yun H, Kamberos NL, Dehoedt CL, Gu C, Holman C, Tricot G, et al: CDKN1A and FANCD2 are potential oncotargets in Burkitt lymphoma and multiple myeloma. Exp Hematol Oncol 4: 9, 2015.

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

| Category                        | Genotype | All | 01 | 11 | 00 | P-value |
|---------------------------------|----------|-----|----|----|----|---------|
| Age at menarche                 |          |     |    |    |    | 0.001   |
| ≤12 years (%)                   |          | 220 | 100 (76.3) | 16 (38.1) | 26 (61.9) | 104 (80.6) | 0.775 (0.428-1.405) | 0.148 (0.069-0.316) | 1 |
| >12 years (%)                   |          | 82  | 31 (23.7)  | 26 (61.9)  | 25 (19.4)  | 1       |
| Age at breast cancer onset      |          |     |    |    |    | 0.335   |
| ≤40 years (%)                   |          | 118 | 57 (44.2)  | 14 (34.1)  | 47 (36.4)  | 1       |
| >40 years (%)                   |          | 181 | 72 (55.8)  | 27 (65.9)  | 82 (63.6)  | 1       |
| Age at menopause                |          |     |    |    |    | 0.693   |
| ≤50 years (%)                   |          | 39  | 20 (27.8)  | 5 (19.2)   | 14 (25.5)  | 1       |
| >50 years (%)                   |          | 114 | 52 (72.2)  | 21 (80.8)  | 41 (74.5)  | 1       |
| Metastasis status               |          |     |    |    |    | 0.348   |
| Yes (%)                         |          | 37  | 14 (10.8)  | 8 (19)     | 15 (11.6)  | 1       |
| No (%)                          |          | 264 | 116 (89.2) | 34 (81)    | 114 (88.4) | 1       |
| Cancer status                   |          |     |    |    |    | 0.001   |
| Familial (%)                    |          | 152 | 81 (61.8)  | 28 (66.7)  | 43 (32.8)  | 1       |
| Non-familial (%)                |          | 152 | 50 (38.2)  | 14 (33.3)  | 88 (67.2)  | 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.
Biallelic mutations in BRCA1 cause a new Fanconi anemia subtype

Canada Consortium, Majewski J, Dyment DA, Innes AM. University of Washington Centre for Mendelian Genomics; FORGE Sawyer SL, Tian L, BRCA2: Implications for Fanconi anemia and familial breast cancer Waisfisz Q.

Bakker JL, Thirthagiri E, van Mil SE. Oncotarget 6: 20396-20403, 2015. Shen Y, Lee YH, Panneerselvam J, Zhang J, Loo LW and Fei P: A novel nucleotide polymorphism in the matrix metalloproteinase-1 promoter enhances lung cancer susceptibility. Cancer Res 61: 7825-7829, 2001.

Bond GL, Wu W, Bond EE, Robins H, Lutzker SG, Arna VC, Bargonneti J, Bartel F, Taubert H, Wuerl P, et al: A single nucleotide polymorphism in the MDM2 promoter enhances lung cancer susceptibility. Hum Mol Genet 16: 1051-1057, 2007.

Rodriguez-Lopez R, Osorio A, Sanchez-Pulido L, De La Hoya M, Baroso A, Caldes T and Benitez J: No mutations in the XRCC2 gene in BRCA1/2-negative high-risk breast cancer families. Int J Cancer 103: 136-137, 2003.

Smith TR, Miller MS, Lohman K, Lange EM, Case LD, Mohrenweiser HW and Hu JJ: Polymorphisms of XRCC1 and XRCC3 genes and susceptibility to breast cancer. Cancer Lett 190: 183-190, 2003.

Kiiski JI, Pelltari LM, Khan S, Freysteinsdottir ES, Reynisdottir I, Hart SN, Shimeles H, Vilks E, Kallioniemi A, Schleuter J, et al: Exome sequencing identifies FANCM as a susceptibility gene for triple-negative breast cancer. Proc Natl Acad Sci USA 111: 15172-15177, 2014.

Fearnhead NS, Wilding JL, Winney B, Tonks S, Bartlett S, Bicknell DC, Tomlinson IP, Mortensen NJ and Bodmer WF: Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. Proc Natl Acad Sci USA 101: 15992-15997, 2004.

Virt EL, Jankowska A, Mackay C, Glaas MF, Wiek C, Keliach SL, Lottmann N, Kennedy FM, Marchal C, Lehnert E, et al: AluY-mediated germline deletion, duplication and somatic stem cell reversion in UB2ET defines a new subtype of Fanconi anemia. Hum Mol Genet 24: 5093-5108, 2015.

Blanco A, Gutiérrez-Enríquez S, Santamariña M, Montalban G, Bonache S, Balmáñez J, Carracedo A, Diez O and Vega A: RAD51C germline mutations found in Spanish site-specific breast cancer and breast-ovarian cancer families. Breast Cancer Res Treat 147: 133-143, 2014.

Cleton-Jansen AM, Moerland EW, Pronk JC, van Berkel CG, Apostolosu S, Crawford J, Savoia A, Auerbach AD, Mathew CG, Callen DF and Cornelisse CJ: Mutation analysis of the Fanconi anemia A gene in breast tumours with loss of heterozygosity at 16q24.3. Br J Cancer 79: 1049-1052, 1999.