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Original Article

Genotypic frequency of Caveolin-1 (CAV1) T29107A polymorphism in the Iranian patients with breast cancer

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Abstract

Introduction: Caveolin-1 (Cav-1) is a scaffolding protein found in special structures of plasma membrane, known as Caveolae. Cav-1 can regulate many intracellular processes, including signal transmission and cholesterol metabolism. This protein plays an important role in the growth and differentiation of breast tissue and acts as a tumor suppressor gene as well. The aim of this study was to determine the genotypic frequency of Cav-1 T29107A (rs7804372) polymorphism and its association with susceptibility to breast cancer among the female population in Kermanshah, Iran.

Methods: A total of 120 patients with breast cancer and an equal number of non-cancer individuals (control group), matched for age and gender with the patients, were selected in this study. The paraffin tissues of the patients from 2006 to 2013 were collected from Imam Reza hospital, Kermanshah, and 2.5 cc blood sample was taken from non-cancer individuals. The genomic DNA was extracted from paraffin tissues and blood by salting out method. The genotype of samples was determined by RFLP-PCR method, and Sau3A1 enzyme was used for RFLP analysis.

Results: The distribution of Cav-1 T29107A genotype was found to be significantly different between breast cancer patients and control group (p=0.004). Among the patients, 84 (70%) samples had genotype TT, 29 (24.78%) genotype AT and 7 (5.83%) genotype AA. As for the control group, however, 59 (49.17%) samples had genotype TT, 49 (40.83%) genotype AT and 12 (10%) genotype AA.

Conclusion: The results of this study showed that genotype TT is associated with an increased risk of susceptibility to breast cancer.

Introduction

Breast cancer is the most common type of cancer among women in various communities, including Iran with the diagnostic rate of 32% (1). According to a study analyzing the common cancers of central province, Iran during 2001-2006, breast cancer was reported to be the most prevalent type of cancer among the female population. Based on a report by Almasi Nourkiani et al. in Kermanshah, Iran during 1990-2003, the average age of breast cancer was found to be lower than the western countries and incidence of breast cancer was higher among the women aged <40 years (2-3).

Breast cancer is originated from the breast tissues, often from the cells lining the milk ducts and the lobules around the ducts (4). Cav-1 was identified in 1989 as a tumor suppressor gene (5). Its protein is a major structural component of Caveolae in membrane which is found in many cells, including fat cells, endothelial cells, fibroblast cells and epithelial cells of breast (6).

The human Cav-1 gene contains three exons located at 7q31.1. This gene encodes the 22 kDa protein Caveolin-1 with 178 amino acids, the main structural and functional proteins of caveolae, and plays a pivotal role in many signaling pathways, molecular transmissions, and cell proliferation and differentiation (5-6). Caveolae are scaffolding structures in plasma membrane in which there exist high levels of cholesterol, sphingolipid and caveolin proteins (8). Cav-1 plays a major role in the growth and differentiation of breast tissue and functions as regulator of cholesterol level. It is also involved in inactivation of cellular pathways such as Her/NEO, EGFR, PDGFR tyrosine kinase, RAS/P42/44MAP cascade pathways, a number of guanine-linked heterodimer proteins, G protein Sre-like Kinase, protein kinase C alpha, GTPase-dependent Ras and NO (9-10).

Studies have shown that overexpression of Cav-1 can cause cell cycle arrest in G0/G1 stage. Recently, it has been found that Cav-1 acts as tumor and metastasis inhibitor in breast cells (11-12). This study was conducted to determine the genotypic frequency of Cav-1 T29107A (rs7804372) single nucleotide polymorphism and its association with susceptibility to breast cancer among female population in Kermanshah, Iran.

Materials and methods

Sampling

The statistical society of this study comprised of 120 tumor samples of women with breast cancer undergoing surgery during 2006-2013 (4 out of the 10 samples of 2006 were alive at the time of completing the questionnaire) at Imam Reza hospital, Kermanshah. Also, 120 blood samples (2.5 cc in CBC tubes containing EDTA) were taken from the non-cancerous volunteers at the same hospital as control group. The control group and patient group samples were homogeneous in terms of age and gender. To collect the tumor samples from the patients, first their pathology files were analyzed and 120 female samples with the mean age of 49.2 years were selected. Based on the data of the files as well as the questionnaire (interview with patients), the patients were found to have no relatives with breast cancer and no history of cancers or genetic diseases. A specialist confirmed that the tissues were cancerous. It should be noted that the selected statistical population in this study was determined by Charles Cochran formula.

DNA extraction from tumor tissue by sodium chloride saturation

To analyze T10927A single nucleoid polymorphism in Cav-1 gene, DNA was extracted from the tissues of 120 samples. First, 5 µm sections were prepared from 20-25 mg segments of paraffin blocks by microtome and kept in 1.5 ml microtubes. These segments were then deparaffinized by xylene and ethanol in two stages, i.e. 25-60 mg equal to 1-3 sections (5 µm) were placed in separate microtubes. In the first stage, only xylene was used, and in the following stages, absolute ethanol and 70% ethanol were used, respectively and the supernatant was discarded in each phase. The samples were then washed with PBS, and 300 µl lysis buffer, 30 µl proteinase K and 25 µl 10% SDS were added afterwards and placed in bain-marie for 24 hours at 50 °C. Next, the samples were exposed to 95 °C temperature for 10 minutes in order to inactivate proteinase K. Then, 6 M NaCl was added to one third of the sample size and the mixture was centrifuged for 30 minutes at 3500 rpm. The separated supernatant was transferred to a new microtube and an equal amount of cold isopropanol was added. After a partial vortex, the samples were centrifuged for 15 minute at 1200 rpm. The extracted DNA was dissolved in 50 µl TE buffer following washing with 70% ethanol twice. To ensure the presence of DNA in the samples obtained from extraction, 5 µl of it was electrophoresed in 1% agarose and after staining with ethidium bromide, the extracted DNA was quantified by a gel doc device (Figure 1a).

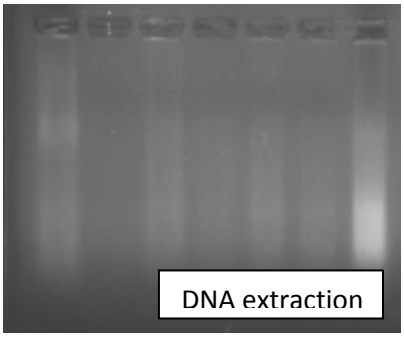

Figure 1a. DNA extraction from paraffin tissue

DNA extraction from blood by sodium chloride saturation

To evaluate T29107A (rs7804372) single nucleoid polymorphism in Cav-1 gene, DNA was extracted from 120 blood samples of control group. A total of 300 µl blood was poured in 1.5 ml microtubes and red blood cells were separated by 85% ammonium chloride during several stages. Then, 200 µl lysis buffer and 30 µl 10% SDS were added and placed in bain-marie for 60 minutes at 65 °C. Next, 120 µl 5 M NaCl was added and centrifuged at 13200 rpm for 3 minutes, and supernatant was separated and placed in a new microtube. After that, 1 cc cold absolute ethanol was added by a tube shaker and exposed to -20 °C temperature for 20 minutes. It was then dissolved in 70 µl deionized water (65 °C) after washing with 70% ethanol. To dissolve the DNAs, the samples were kept at room temperature, and after a short microfuge, they were kept at 4 °C for one day and transferred to -20 °C conditions for long-term storage. To ensure the presence of DNA in the extracted samples, 5 µl of it was electrophoresed in 1% agarose, and after staining with ethidium bromide, the extracted DNA was quantified by gel doc device (Figure 1b).

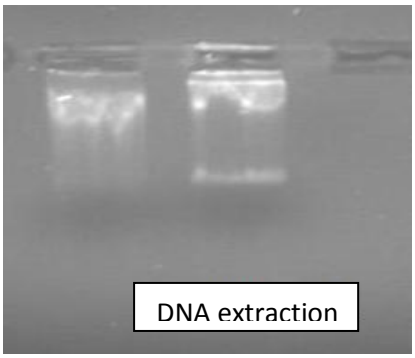

Figure 1b. DNA extraction from blood

PCR method

Using PCR method and two specific primers with the following sequences, proposed by Chih-Hsueh Lin et al. (2014), 336 kb segment was proliferated from Cav-1 intron.

Forward	**3'-GCCTGAATTGCAATCCTGTG-5'**
Reverse	**3'-ACGGTGTGAACACGGACATT-5'**

Sequences of forward and reverse primers for Cav-1 proliferation

PCR reaction was carried out with final volume of 25 µl containing 10 µl Taq premix (Master Mix), 1 µl forward primer and 1 µl reverse primer along with 5 µl model DNA and 7 µl deionized water. The temperature schedule of PCR was as follows: the single-stranded stage at 94 °C for 5 minutes in one cycle, connection of primers and proliferation in 35 cycles (30 sec at 94 °C, 30 sec at 55 °C and 30 sec at 72 °C) and final proliferation at 72 °C for 10 minutes in one cycle were performed in a thermocycler (Eppendorf Co, Germany). For greater certainty, the proliferated products were electrophoresed on 1.5% agarose gel, were stained with ethidium bromide and were imaged by gel doc device (Figure 2).

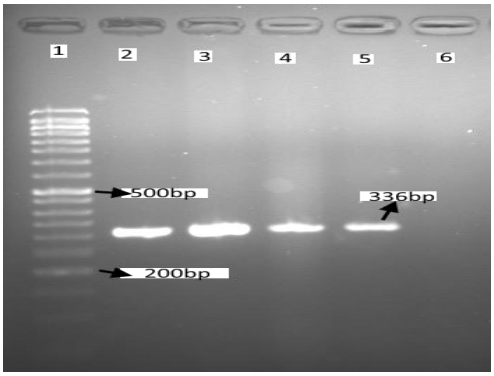

Figure 2. rs7804372 was proliferated by specific primers. After loading on 1.5% agarose, the given bonds with the length of 336 bp are indicated in columns 1-5, and column 6 is negative control. Also, 50 bp ladder is used.

Enzymatic digestion

The restriction enzyme used for this position was Sau3A1 (Fermentaz Co), that was able to detect the restriction site. To digest the PCR products, 5 µl 10X Buffer B and 2 µl (1u/µl) Sau3A1 enzyme as well as 10 µl PCR DNA and 34.8 µl deionized water were mixed. Optimal enzymatic digestion was carried out at 37 °C for 3 hours.

The enzymatic digestion products included a 336kb segment for genotype AA, 164+172 kb segments as well as 336 kb segment for genotype AT and 164+172 kb segments for genotype TT. The genotypes were observed by electrophoresis on 2.5% agarose gel and staining was performed with ethidium bromide (Figure 3). The obtained data were analyzed by SPSS (version 22) software. The frequency of genotypes and differences between control and patient groups were analyzed by chi-square test. The confidence level was set at 95% and $p<0.05$ was considered significant.

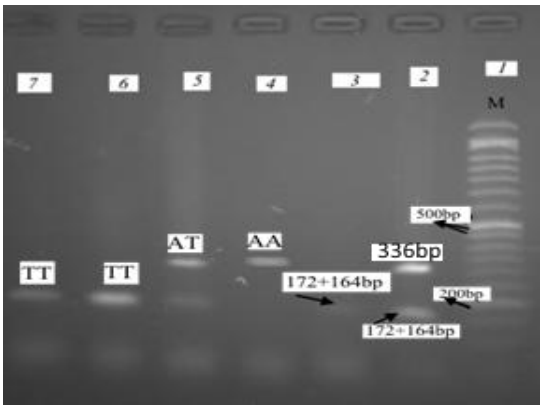

Figure 3. PCR product was cut by Sau3A1 enzyme at 37 °C for 3 hours. After loading on 2.5% agarose gel, the bonds of the given segment are shown in columns 2-7. Columns 3, 6 and 7 are cut and contain genotype TT. Columns 2 and 5 include two 336 bp and 164+172 bp bonds and contain genotype AT. Column 4 is not cut and has genotype AA.

Results

The tumor and disease phase were graded according to the pathological data of the patients. The findings showed that 10% of the patients were at stage 1 at the time of surgery, 69.17% at stage 2, 13.33% at stage 3 and 7.5% at stage 4. The obtained results indicated an increased risk for genotype AT (OR(Exp)=2.406, CI=95%, 1.364-4.424), but genotype AA showed a reduced risk (OR(Exp)=2.441, CI=95%, .907-6.567). On the other hand, as shown above, since the frequency of genotype AT in control group is higher, allele A has probably a protective role against this disease. Also, because genotype TT in the patient group is significantly increased, this genotype is possibly accompanied by an increased risk of breast cancer (Table 1).

All extracted DNAs had a good quality for PCR analysis. Figure 1 shows the results of a PCR sample. Figure 2 indicates an RFLP-PCR sample in which a 336kb segment for genotype AA, 164+172 kb segments along with 336 kb segment for genotype AT and 164+172 kb segments for genotype TT are shown.

The 50 bp DNA marker was used to determine the size of segments. The distribution of different genotypes and alleles of rs7804372 in control and patient groups is shown in Tables 2 and 3. The results indicated a significant difference between study groups in terms of genotypic and allele frequency. The homozygote genotypes AA and TT with frequency rates of 5.83% and 70% in the patient group compared with frequencies 10% and 49.17% in the control group, respectively, and heterozygote genotype AT with frequency of 40.83% in the control group compared with frequency of 29.87% in the patient group revealed a significant difference between these genotypes (P=0.001). Moreover, the frequency of allele T in control group (69.58%) in comparison with that of the patient group (82.1%) and frequency of allele A in the patient group (17.9%) compared with that of the control group (30.42) showed a significant difference between study groups. The presence of allele T increased the risk of breast cancer (Tables 2 and 3). On the other hand, 50% of the patients were reported to have polymorphism TT in stage 1, whereas about 5.7% of patients had polymorphism TT in stage 4 (Table 1).

Table 1. Frequency of TT, AT and AA genotypes in different stages of disease in patients with breast cancer

genotype* stage		stage				Total
		I	II	III	IV	
Genotype	TT	6	53	16	9	84
	AT	0	29	0	0	29
	AA	6	1	0	0	7
Total		12	83	16	9	120
P value[a]		0.0001				

a. P based on Chi-square test.

Table 2. Genotypic frequency, chi-square and OR in Cav-1 T29107A (rs7804372) polymorphism in the patient and control groups

Genotype* patient		Patient(number-frequency)		Total
		Patient group	Control group	
Genotype	TT	84(70%)	59(49.2%)	143
	AT	29(24.2%)	49(40.8%)	78
	AA	7(5.8%)	12(10%)	19
Total		120	120	240
P value[a]		0.004		

a. P based on Chi-square test.

Table 3. Allele frequency of Cav-1 T29107A (rs7804372) polymorphism in the patient and control groups

Allele*patient				
		Patient group	Control group	
Allele	T	197(82.1%)	167(69.58%)	364
	A	43(17.83%)	73(30.43%)	116
Total		240	240	480
P value [a]		0.001		

a. P based on Chi-square test.

Discussion

The studies carried out on different modes of a gene location suggest that factors such as genetic polymorphisms may express individual differences in susceptibility to cancer, early incidence of cancer and development of cancer. Breast cancer is the second most common type of cancer after skin cancer among the women worldwide (15). Breast cancer is the leading cause of death among women in industrialized countries as well as developing countries, including Iran (12-13).

Caveolin-1 (Cav-1) is a scaffolding protein in specific structures of plasma membrane called caveolae (12). Cav-1 can regulate many intracellular processes, including signal transmission and cholesterol metabolism. This protein plays a key role in the growth and differentiation of breast tissue. It also acts as a tumor suppressor gene. Cav-1 has been reported to mutate in 132 codon (P132L) in more than 16% of the patients with breast cancer (7-13). The American and Japanese groups have shown that single nucleoid variation of Cav-1 gene plays a major role in the risk of cancer (7).

The T29107 (rs7804372) polymorphism is located at intronic region of Cav-1 gene, and its polymorphic variation in individuals does not directly lead to encoding different amino acids. The variation of T29107A intronic polymorphism from Cav-1 may alter the normal expression or protein function of Cav-1 gene by alternative splicing and regulating mRNA sustainability (22). Further studies would help find whether this SNP can influence the downstream processing of protein and mRNA. In support of this idea, Hsu et al. studied the correlation of genotype and phenotype in hepatocellular carcinoma and showed that the liver tissue of the people with genotype AA and AG in Cav-1 G14713A single nucleoid polymorphism has higher levels of mRNA and protein than the people with genotype GG (21).

The present study evaluated Cav-1 T29107A (rs7804372) single nucleoid polymorphism in the patients with breast cancer and analyzed the correlation of the obtained genotypes with the data such as age and tumor stage recorded in the pathology files of the patients.

The data analysis showed that 70% of the patients and 49.17% of the control group had genotype TT, indicating a significant difference between study groups (p=0.004). Moreover, the frequency of the mutated allele was almost similar to that of the global population, as the frequency rates of mutated allele A in the patient and control groups were calculated to be 17.9% sand 30.42%, respectively, which is close to the global frequency reported for allele A (25.72%).

However, the association of the genotype and phenotype is unknown for T29107A (rs7804372). This study was the first study conducted in Iran to show that genotypic variation in T29107A(rs7804372) single nucleoid polymorphism is associated with susceptibility to breast cancer, and allele T may be a code to increase mRNA levels and protein in the patients with breast cancer.

A review of literature shows that only Liang-Chih Liu et al. conducted a study in Taiwan on the patients with breast cancer and examined 6SNP in Cav-1 gene. They reported an increase in the percentage of TT genotypes for T29107A (rs7804372), so genotype TT was found to be associated with the risk of cancer (7). Our study finding confirms this result.

Further, Satoru Sugi et al. investigated the correlation between T29107A polymorphism and prostate cancer and reported that the risk of prostate cancer is associated with genotype AA (17). The results of the present study indicated that T29107A polymorphism in breast cancer, in contrast to prostate cancer, is accompanied by an increase in genotype TT (17).

In addition, Ye Zhang et al. analyzed the relationship of rs3807987/rs7804372 genotypes with H-pylori and gastric cancer in China. They concluded that the risk of gastric cancer in the patients with H-pylori is correlated with G14713A genotype but has no correlation with T29107A. Therefore, allele A in G14713A interacts with H-pylori (15). Moreover, Chih-Hsueh Lin et al. found no significant association between T29107A and gastric cancer (17).

Conclusion

Given the significance of this gene in the development of breast tissue, this polymorphism is suggested to be studied in a larger population of women with breast cancer or other prevalent cancers in Iran. The correlation of this SNP and other SNPs and mutations of this gene with breast cancer and other genes involved in breast cancer is also recommended to be analyzed among Iranian population.

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