Interactive Effects of Common Haplotypes of Two Leukocyte Diapedesis-Related Genes, LFA-1 and JAM-A on Breast Cancer Risk

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ABSTRACT

Leukocyte diapedesis is an important process in breast cancer etiopathogenesis. Therefore, Junctional adhesion molecule-A (JAM-A) and lymphocyte function-associated antigen-1 (LFA-1) genes are among potential candidate genes involved in breast cancer development. In the present study, JAM-A rs790056 (T>C), LFA-1 rs8058823 (A>G) and LFA-1 rs2230433 (C>G) gene variations and their associations with breast cancer risk were investigated in breast cancer patients and healthy subjects. The JAM-A and LFA-1 genotypes were determined in 108 breast cancer patients and 63 healthy controls with Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) assay. LFA-1 rs8058823 common AA genotype (χ^2= 6.062, p= 0.014) and A allele frequency (p= 0.001) and LFA-1 rs2230433 rare GG genotype frequency (p= 0.048) was higher in the patient group compared with controls. The TA haplotype (JAM-A rs790056-T, LFA-1 rs8058823-A alleles) frequency was significantly increased in the patient group compared with controls (p= 0.0173), while the TG haplotype (JAM-A rs790056-T, LFA-1 rs8058823-G alleles) and CG haplotype (LFA-1 rs2230433-C, LFA-1 rs8058823-G alleles) frequencies were significantly lower in the patient group compared with controls (p= 0.0051 and p= 0.002, respectively). In addition, the TCG haplotype (JAM-A rs790056-T, LFA-1 rs2230433-C, LFA-1 rs8058823-G alleles) frequency was significantly lower in the patient group compared with controls (p= 0.0096). Haplotype analysis confirmed that the LFA-1 rs8058823 is more effective in breast cancer risk than LFA-1 rs2230433 and JAM-A rs790056. LFA-1 rs8058823 A allele may be related to breast cancer risk, influencing leukocyte diapedesis. Our findings indicate that functional gene variations associated with leukocyte diapedesis may affect breast cancer risk.

Keywords. Breast cancer, Gene, Junctional adhesion molecule-A, Lymphocyte function-associated antigen-1, Haplotype

ÖZET

Lökosit Diapeziyle İlişkili LFA-1 ve JAM-A Genlerindeki Yaygın Haplotiplerin Meme Kanseri Riskinde İnteraktif Etkileri

Lökosit diapedezi meme kanseri etyopatojenezinde önemli bir süreçtir. Bu nedenle, Bağlantı Adezyon Molekülü-A (JAM-A) ve lenfosit fonksiyon ilişkili antijen-1 (LFA-1) genleri meme kanseri gelişiminde rol oynayan potansiyel aday genler arasındadır. Bu çalışmada meme kanseri hastaları ve sağlıklı bireylerde JAM-A rs790056 (T>C), LFA-1 rs8058823 (A>G) ve LFA-1 rs2230433 (C>G) gen varyasyonlarının meme kanseri riski ile ilişkileri araştırıldı. JAM-A ve LFA-1 genotipleri 108 meme kanseri hastası ve 63 sağlıklı kontrolde polimeraz zincir reaksiyonu- Restriksiyon Fragmanı Uzunluk polimorfizmi (PZR-RFUP) teknikleri kullanılarak belirlenmiştir. Hasta grubunda kontrol grubuyla karşılaştırıldığında LFA-1 rs8058823 yaygın AA genotipi (χ^2= 6.062, p= 0.014), A allel frekansı (p= 0.001) ve LFA-1 rs2230433 nadir GG genotip frekansı (p= 0.048) yüksektir. Hasta grubunda kontrole kıyasla TA haplotip (JAM-A rs790056-T, LFA-1 rs8058823-A allelleri) frekansı anlamlı olarak yüksek gözlenirken (p= 0.0173), TG haplotip (JAM-A rs790056-T, LFA-1 rs8058823-G allelleri) ve CG haplotip (LFA-1 rs2230433-C, LFA-1 rs8058823-G allelleri) frekansları anlamlı olarak düşüktür (sırasıyla, p= 0.0051 ve p= 0.002). Ayrıca TCG haplotip (JAM-A rs790056-T, LFA-1 rs2230433-C, LFA-1 rs8058823-G allelleri) frekansı hasta grubunda kontrole göre anlamlı olarak düşüktür (p= 0.0096). Haplotip analizi LFA-1 rs8058823'in meme kanseri riskinin belirlenmesinde LFA-1 rs2230433 ve JAM-A rs790056'den daha etkili olduğunu doğrulamaktadır. LFA-1 rs8058823 A alleli lökosit diapedezini etkileyerek meme kanser riski ile ilişkili olabilir. Bulgularımız lökosit diapedezi ile ilişkili fonksiyonel gen varyasyonlarının meme kanseri riskini etkileyebileceğine işaret etmektedir.

Anahtar Kelimeler: Meme kanseri, Lökosit Bağlantı Adezyon Molekülü-A, lenfosit fonksiyon ilişkili antijen-1, Haplotip

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer related deaths among females worldwide.[1] Extravasation of cancer cells involves rolling, adhesion and transmigration (diapedesis) that are similar to extravasation of leukocytes (transendothelial migration, leukocyte diapedesis).[2] Junctional adhesion molecule-A (JAM-A), also known as F11R, JAM-1, or CD321, is a type I transmembrane immunoglobulin G glycoprotein, which is expressed at epithelial cells tight junctions and intercellular borders of endothelial cells, and also on the surface of platelets and some leukocytes.[3] JAM-A has important functions including intercellular junction assembly and cell polarity[4], cell morphology[5], angiogenesis[6], and leukocyte migration.[7]

The effect of JAM-A expression on risk of breast cancer remains controversial. Many studies have suggested a positive correlation between JAM-A and progression, migration, and invasion of breast cancer.[8-10] However, some studies yielded conflicting results.[11,12]

JAM-A is expressed at lower levels and limited mainly to areas of epithelial cell-cell contact in normal breast tissue[13] and has been shown to be overexpressed in breast cancer.[8] Moreover, JAM-A promotes cell migration in breast cancer via downstream effects on β1-integrin activation. Adhesion of natural killer cells to the target cells/tumor cells following the stimulation by activating receptors is mediated by leukocyte integrin LFA-1, resulting in apoptosis of the target cells/tumor cells. A higher percentage of LFA-1+ breast tumor cells was observed in patients with better outcome /without recurrence and expression of LFA-1 increased in patients with good prognosis.[14] Additionally, heterophilic interaction of JAM-A and LFA-1 causes transendothelial migration of leukocytes which is also a process involved in tumor cell extravasation.[7]

To date, there have been a limited number of studies investigating associations of variations of LFA-1 gene with development of breast cancer. It was suggested that the LFA-1 gene variations may predict the sporadic infiltrative duct breast carcinoma risk and prognosis factors in Chinese Han women.[15] Because both of JAM-A and LFA-1 interfere with leukocyte diapedesis, they may be potential candidate genes involved in the development of breast cancer. However, there was no study investigating JAM-A gene variation alone and together with LFA-1 gene variations in breast cancer patients. Therefore, we aimed to investigate not only the distribution of the JAM-A and LFA-1 genes variants among patients with breast cancer and controls, but also the possible amplified effects of these genetic variations together on the development risk of breast cancer.

PATIENTS and METHODS

Study Population

The patient group consisted of 108 breast cancer patients with median age 50.84±12.69 years, who were admitted to Breast Services at the Department of Surgery, Cerrahpasa Medical School of Istanbul University. In addition, 63 healthy women, presenting a mean age of 51.83±5.37 years, were used as a control group. The control group consisted of age- and sex-matched healthy subjects, subjects with a personal or family history of any cancer and chronic diseases such as cardiovascular or cerebrovascular disease, diabetes mellitus, hypertension, or renal disease were excluded from the study. Control subjects were not taking any regular medication during the study. Informed consent was obtained from all participants. All of our breast cancer patients had previously undergone appropriate surgery. Questionnaires, medical records, and pathological reports were used to confirm the diagnosis and cancer status.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study protocol was approved by the Local Ethical Committee at Istanbul University.

Immunohistochemical Analysis of Breast Cancer Samples

The estrogen receptor (ER) and progesteron receptor (PR) status of the patients was defined by immunohistochemistry on formalin-fixed, paraffin-

embedded sections of clinical specimens as part of routine pathological interpretation. Immunohistochemistry was performed using a rabbit monoclonal antihuman ER antibody (clone SP1; Thermo-Scientific, MA, USA) and a polyclonal rabbit antihuman PR antibody (clone 16, Novocastra, Leica Microsystem, Wetzlar, Germany). Nuclear staining of > 10% of cells were accepted as positive for ER or PR status.

Genotyping

Peripheral blood samples were collected in tubes containing EDTA, and genomic DNA samples were extracted from whole blood with salting out procedure. Polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) protocols slightly modified from Fu et al.[15] were performed to detect rs8058823 (in 3′-untranslated region (3'-UTR A>G) and rs2230433 variations (in exon 21 (2120 G>C) (Arg707Thr)) of the LFA-1 gene, while rs790056 variation (in intron 6 T>C) of the JAM-A gene was detected according to an PCR-RFLP method developed in our laboratory and primers used to amplify this gene region were designed according to Ensembl genome database using Primer3 program (forward 5′-GCAGTACAAAGGAGAGCCTCT-3′ and reverse 5′-TGGGACACCCATGACTTCA-3′).

Statistical Analysis

Statistical analysis was performed using SPSS software for Windows, version 21.0 (SPSS Inc. Chicago, USA). Allele frequencies were calculated by using gene counting method. The difference in the occurrence of the genotypes in the case and control groups was evaluated with chi-square test. In order to determine the relative risks, odds ratios and 95% confidence intervals were used. Haploview program was used for haplotype analysis[16] and p value less than 0.05 was considered to be statistically significant.

RESULTS

Clinical Investigation

Almost half of the patient group consisted of premenopausal women and the other half was postmenopausal women. The positive expression rates of ER and PR among patients were 69.7% and 51.3% and histological grade proportions of clinical stages 1-2 and 3 among patients were 56.5% and 43.5%, respectively. Lymphatic vascular and perineural invasion were present in 45.3% and 31.4% of the patients, respectively and 55.4% of tumors were positive for c-erb expression. Moreover, 87.3% had invasive ductal carcinoma, while the rest had other subtypes of breast carcinoma. There was no significant difference in mean age between patients and control subjects (50.84±12.69 vs. 51.83±5.37, p= 0.495).

Distributions of the JAM-A and LFA-1 Genotypes

JAM-A rs790056 genotypes and allele frequencies were similar between the study groups (p> 0.05). LFA-1 rs8058823 normal AA genotype (χ^2= 6.062, p= 0.014) and A allele frequency (p= 0.001) and LFA-1 rs2230433 rare GG genotype frequency (p= 0.048) were found to be higher in the patient group compared with controls. In the patient group 21 subjects were heterozygous for the LFA-1 rs8058823 rare G allele (AG), but none were homozygous for this allele (GG). In the control group, 47 subjects were heterozygous for the LFA-1 rs2230433 rare G allele (GC), but none were homozygous for this allele (GG) (Table 1). As such, statistical evaluation of data was performed on LFA-1 rs8058823 AG and LFA-1 rs2230433 GC heterozygous genotypes for subsequent analysis.

Haplotype Analysis

It was also analyzed JAM-A rs790056, LFA-1 rs2230433 and LFA-1 rs8058823 haplotypes for association with breast cancer as sets of two and three alleles together. The haplotype analysis with two alleles confirmed the association of JAM-A and LFA-1 gene variations with breast cancer and revealed that the TA haplotype (JAM-A rs790056 T and LFA-1 rs8058823 A alleles) frequency was significantly increased in the patient group as compared with controls (p= 0.0173), while the TG haplotype (JAM-A rs790056 T and LFA-1 rs8058823 G alleles) and CG haplotype (LFA-1 rs2230433 C and LFA-1 rs8058823 G alleles) frequencies were significantly lower in the patient group as

Table 1. Distributions of JAM-A and LFA-1 genotypes in the study groups

	Groups	
	Control (n= 63)	**Patient (n= 108)**
JAM-A rs790056 Genotypes		
TT	39 (61.9%)	69 (63.9%)
CC	8 (12.7%)	9 (8.3%)
TC	16 (25.4%)	30 (27.8%)
JAM-A rs790056 Alleles		
T	94 (74.60%)	168 (77.78%)
C	32 (25.40%)	48 (22.22%)
LFA-1 rs8058823 Genotypes		
AA	40 (63.5%)	87 (80.6%) *
GG	7 (11.1%)	-
AG	16 (25.4%)	21 (19.4%)
LFA-1 rs8058823 Alleles		
A	96 (76.19%)	195 (90.28%) **
G	30 (23.81%)	21 (9.72%)
LFA-1 rs2230433 Genotypes		
CC	16 (25.4%)	21 (19.4%)
GG	-	7 (6.5%) ***
GC	47 (74.6%)	80 (74.1%)
LFA-1 rs2230433 Alleles		
C	79 (62.70%)	122 (56.48%)
G	47 (37.30%)	94 (43.52%)

*, p= 0.014; **, p= 0.001 (Fisher's exact test); ***, p= 0.048 (Fisher's exact test)

compared with controls (p= 0.0051 and p= 0.002, respectively). Haplotype analysis confirmed that the LFA-1 rs8058823 A allele is the most effective in breast cancer risk and its detrimental effect on breast cancer kept in presence of JAM-A rs790056 T allele (Table 2).

The haplotype analysis with triple alleles (JAM-A rs790056 - LFA-1 rs2230433 and LFA-1 rs8058823) revealed that the TCG haplotype (JAM-A rs790056 T- LFA-1 rs2230433 C and LFA-1 rs8058823 G alleles) frequency was significantly lower in the patient group compared with controls (p= 0.0096) (Table 3).

Table 2. The frequencies of two alleles haplotype sets of JAM-A rs790056 (T>C), LFA-1 rs8058823 (A>G) and LFA-1 rs2230433 (C>G) in the study groups

	Frequencies				
Haplotype	**Overall**	**Patient**	**Control**	**Chi square**	**p**
JAM-A rs790056- and LFA-1 rs8058823					
TA	0.666	0.713	0.587	5.667	0.0173
CA	0.182	0.185	0.175	0.058	0.8095
TG	0.100	0.065	0.159	7.847	0.0051
CG	0.052	0.037	0.079	2.852	0.0913
JAM-A rs790056 and LFA-1 rs2230433					
TC	0.441	0.437	0.448	0.04	0.8405
TG	0.325	0.341	0.298	0.669	0.4134
CC	0.147	0.128	0.179	4.652	0.1986
CG	0.087	0.094	0.075	0.369	0.5435
LFA-1 rs2230433 and LFA-1 rs8058823					
CA	0.462	0.482	0.429	0.896	0.3439
GA	0.386	0.417	0.333	2.332	0.1267
CG	0.126	0.083	0.198	9.59	0.002
GG	0.026	0.019	0.040	1.389	0.2386

Table 3. The frequencies of triple alleles haplotype sets of JAM-A rs790056, LFA-1 rs2230433 and LFA-1 rs8058823 in the study groups

Haplotype (JAM-A rs790056- LFA-1 rs2230433- LFA-1 rs8058823)	Frequencies				
	Overall	Patient	Control	Chi square	p
TCA	0.352	0.377	0.311	1.511	0.2189
TGA	0.313	0.336	0.274	1.391	0.2383
CCA	0.109	0.104	0.116	0.128	0.7207
TCG	0.091	0.060	0.144	6.711	0.0096
CGA	0.074	0.082	0.060	0.54	0.4622
CCG	0.036	0.024	0.056	2.365	0.1241
CGG	0.016	0.012	0.021	0.406	0.524

DISCUSSION

The present study is the first to investigate JAM-A gene variation alone and together with LFA-1 gene variations in breast cancer patients. Several studies demonstrated the roles of JAM-A in carcinogenesis, such as being involved in signaling events inducing breast cancer cell migration via activation of Rap1 GTPase and β1-integrin.[9] Transforming growth factor-β1 has been shown to induce breast cancer cell invasion through the downregulation of JAM-A expression.[12] Also, microRNA-145 reduces breast cancer cells migration and invasion by targeting JAM-A.[17] In another study, microRNA-495 induced breast cancer cell progression by repressing JAM-A .[11] It was observed that JAM-A was mainly overexpressed on breast, lung and kidney tumor tissues and injections of anti-JAM-A antibody in vivo resulted in a significant tumor growth inhibition of xenograft human tumors.[18] Overexpression of JAM-A in non-small cell lung cancer was correlated with tumor progression and knockdown of JAM-A resulted in G1 cell cycle arrest and growth inhibition of lung cancer cells.[19] Inactivation of JAM-A reduced pancreatic tumor development by increasing antitumor immune response via promoting dendritic cell and T lymphocyte infiltration in pancreatic carcinoma.[20] Loss of the tight junction plaque molecule zonula occludens-1 (zo-1), that associates with the cytoplasmic domain of JAM-A, has been found to be correlated with poor prognosis in breast cancer.[8] Gutwein et al. reported that JAM-A was downregulated in tumor samples of patients with renal cell carcinoma and the downregulation of JAM-A in renal cell carcinoma led to a disassembly of tight junction and to the loss of cell-cell contacts promoting the migration of tumor cells.[21] It was also reported that low expression of JAM-A is significantly associated with metastasis and poor clinical outcome in pancreatic cancer[22] and JAM-A expression was found to be negatively correlated with histologic grade, myometrial invasion, and stage in endometrial carcinoma.[23] Moreover, low JAM-A expression was significantly associated with tumor size, lymphatic vessel invasion, lymph node metastasis, TNM stage, and poor survival in gastric cancer patients and promoted cell migration and invasion.[24]

Recently, it was shown that the silencing of JAM-A protein expression in breast cancer cells significantly reduces breast cancer cell adhesion and migration[9] due to JAM-A functions in promoting epithelial cell spreading[5] and leukocyte migration.[7] It has been also shown that signaling events resulting with high JAM-A expression may promote the migration of breast tumor cells associated with tumor invasion and metastasis.[8] In addition, downregulation of JAM-A reduces breast cancer progression by increasing cell apoptosis.[10] However, it was found that JAM-A expression was decreased in breast cancer tissues and was inversely correlated with the mobility of breast cancer cells[11], it was also reported an inverse relationship between JAM-A expression in breast cancer cells and their invasive ability.[12]

Binding of lymphocyte function-associated antigen-1 (LFA-1) to the membrane-proximal domain 2 of JAM-A destabilizes the homophilic JAM-A interaction allowing leukocyte transendothelial migration.[25] LFA-1 also called αLβ2 integrin, CD11a/CD18 or ITGAL, is a αβ heterodimeric transmembrane glycoprotein expressed on the surface of leukocytes.[26] The expression of LFA-1 in breast cancer cell line can result in the transendothelial migration of breast cancer cells.[27] Likewise, it was observed that the LFA-1 participates in the immunological synapse between T cells and antigen presenting cells in T cell activation.[28] It was shown that the LFA-1 engagement at the immune synapse between cytotoxic T cells and tumor cells is required for cytotoxic immune synapse maturation, resulting in synaptic release of both cytokines and lytic granules and, by that, in tumor cell lysis. Moreover, LFA-1 is important for natural killer cell cytotoxicity as it mediates natural killer-cell binding to intercellular adhesion molecule-1 (ICAM-1) on target cells, producing activating signals that lead to polarization of the actin cytoskeleton and cytotoxic granules followed by perforin and granzymes exocytosis.[14]

Considering the roles of JAM-A and LFA-1 in cancer, it is remarkable to analyze JAM-A and LFA-1 genes variations alone and together in breast cancer. In a study including 537 Chinese Han female infiltrative duct breast carcinoma patients and 577 age-matched Chinese Han healthy women, it was found that the LFA-1 rs8058823 and LFA-1 rs2230433 single nucleotide polymorphisms (SNPs) were related to infiltrative duct breast carcinoma.[15]

Mechanistically, 3′-UTR gene region can regulate gene expression and mediate stability, degradation and subcellular localization of mRNA[29], the rs8058823 SNP located in the 3′ UTR was investigated and it was suggested that this variation may affect LFA-1 expression.[15] Similarly, the rs2230433 variant of LFA-1 gene resulting in the amino acid substitution from arginine to threonine, is located within the integrin-alpha-2 domain of the LFA-1 gene, which forms interactions with EGF2, FG-GAP, and Integrin-β-tail domains. Thus, the rs2230433 SNP is predicted to be involved in splicing regulation.[15] Also, the rs790056 SNP of the JAM-A gene is an intronic variation. Intronic variations can cause a blockage of translation and decrease in the expression of protein[30] since sequence variations occurring in exons or introns may affect the correct processivity of the mRNA by disrupting the splice site or altering the secondary structure of the mRNA.[31] Therefore, in this study, we investigated the potential effects of JAM-A and LFA-1 gene variations together on breast cancer development risk and their protective effects against breast cancer for the first time.

It was suggested that the LFA-1 rs8058823 AA genotype (p= 0.00000418) and A allele (p= 0.00000267) may be protective against infiltrative duct breast carcinoma and rs8058823 AG genotype may increase infiltrative duct breast carcinoma risk (p= 0.00000747).[15] Concurrently, they reported that the LFA-1 rs2230433 GG genotype (p= 0.0316) and G allele (p= 0.0480) may be protective against infiltrative duct breast carcinoma and rs2230433 CG genotype may increase infiltrative duct breast carcinoma risk (p= 0.0397). Contrary, in the present study, it was observed that both LFA-1 rs8058823 normal AA genotype (p= 0.014) and A allele frequency (p= 0.001) and LFA-1 rs2230433 rare GG genotype (p= 0.048) may be related to risk of breast cancer in a Turkish population.

In the present study, the frequency of LFA-1 rs8058823 rare G allele in control and patient groups were 23.81% and 9.72%, respectively. The frequency of the LFA-1 rs2230433 rare G allele was seen 37.3% in controls versus 43.52% in breast cancer patients. The frequency of rs8058823 rare G allele is substantially higher than that reported in Han Chinese (Controls: 0.09%, patients with sporadic infiltrative duct breast carcinoma: 2.14%)[15], South Asian and European populations, but is similar to the African populations (12.0%) (Ensembl genome database, population genetics). In contrast, the frequency of LFA-1 rs2230433 G allele is much lower than the reported frequency in European (70.0%), African (58.0%) (Ensembl genome database, population genetics), and Han Chinese population (Controls: 83.22%, patients with sporadic infiltrative duct breast carcinoma: 79.94%),[15] yet is similar to the frequency of 29% in South Asian (Ensembl genome database, population genetics). The differences in frequencies of

the LFA-1 rs8058823 and rs2230433 among study populations may reflect the importance of different ethnic origin, environmental background or other factors.

We also evaluated effects of JAM-A and LFA-1 genotypes on risk of breast cancer with haplotype analysis. The haplotype results suggested that JAM-A rs790056 T allele and LFA-1 rs8058823 A allele together increase breast cancer risk. However, JAM-A rs790056 T and LFA-1 rs8058823 G alleles together and LFA-1 rs2230433 C and LFA-1 rs8058823 G alleles together reduce breast cancer risk. In the absence of LFA-1 rs8058823 A allele, JAM-A rs790056 T allele when combined with other LFA-1 alleles (LFA-1 rs2230433 C and LFA-1 rs8058823 G alleles) exhibits protective effect against breast cancer. Haplotype analysis confirmed that the LFA-1 rs8058823 was observed more effective in breast cancer risk than LFA-1 rs2230433 and JAM-A rs790056. In conclusion, findings of this study suggest that the LFA-1 and JAM-A gene variations may be useful for predicting breast cancer risk.

The main limitation in the present report is relatively small study population. Moreover, because of lacking JAM-A and LFA-1 expressions in this study, our hypothesis needs to be clarified. Therefore, future studies are needed to be conducted with greater sample size and addition of large number of SNP markers in LFA-1 and JAM-A genes, which effect JAM-A and LFA-1 expressions and leukocyte migration for breast cancer progression.

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