

A potential association between the number of CA repeats in the promoter region of the ADAMTS9 gene with lymphatic metastasis of breast cancer

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Aim: We investigated the effect of the number of cytosine-adenine (CA) repeats in the ADAMTS9 promoter region on breast cancer lymphatic metastasis.

Materials and methods: Thirty-one postoperative breast cancer patients were selected and examined retrospectively. The patients were classified into 2 groups: metastatic or nonmetastatic. Thirty healthy women were selected as the control group, and their peripheral blood was obtained. Following DNA isolation from the cancer tissue specimens and peripheral blood, the promoter region of the ADAMTS9 gene was directly sequenced and the number of CA repeats was determined.

Results: The number of CA repeats ranged between 19 and 21 in the control and metastatic groups. However, in the nonmetastatic group, the number of CA repeats ranged between 17 and 18. This difference in the median number of CA repeats between the control group and the nonmetastatic group was statistically significant.

Conclusion: A potential relationship may exist between lymphatic metastasis in breast cancer and the number of CA repeats in the promoter region of the ADAMTS9 gene. Our study indicates a potential association between the number of CA microsatellite repeats in the promoter region of the ADAMTS9 gene and breast cancer lymphatic metastasis.

Key words: Breast cancer, lymphatic metastasis, ADAMTS9 gene, promoter region, dinucleotide repeats

1. Introduction

Breast cancer is one of the most common malignant tumors among women, as it constitutes more than 30% of all cancers in women (1). Environmental factors are known to play a role in the pathogenesis of breast cancer, and the genetic factors that contribute to the progression of breast cancer have also been identified in recent studies (2).

Cancer is a disease initiated by cells that accumulate inappropriately following the loss of normal cell cycle regulation and their escape from cellular control systems. Genetic control mechanisms are involved in every step of these events (3,4). Most tumors start to spread microscopically when they reach a volume of 1 mm³

(5). However, tumor cells are recognizable by means of mammography or palpation when they reach larger volumes.

Metastasis is directed by 2 main principles. The first is that tumor cells are heterogeneous populations whose genotypes and phenotypes change over time, and the second is that the process of metastasis is based on the metastatic properties of the tumor cells and an array of events relating to their interactions with the microenvironment during the course of metastasis. For example, the amplified synthesis of growth factor receptors, angiogenic factors, matrix metalloproteinases, and integrin receptors are key regulators of metastasis. Cancer

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cells that can induce angiogenesis at the metastatic site are able to invade surrounding blood vessels and enter the bloodstream to seed additional metastases (2,3). Cancer cells can also hijack normal cells and use their enzymes to invade the lymphatic system and blood vessels to initiate metastasis. In addition, cancer cells promote angiogenesis and increase the synthesis of invasion precursor enzymes, such as matrix metalloproteinase-9 (MMP-9), cysteine and cathepsin proteases, heparinase, and other matrix metalloproteinases. Thus, cancer cells enhance their own proliferation, spreading, and tissue invasion properties. However, changes in the behavior of tumor cells, due to their environment and their biological characteristics, should be considered (6).

ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) gene products were found to be involved directly in several steps that control gene expression, mRNA binding, and protein production in both normal and disease processes (7). ADAMTS proteinases were also shown to have roles in cell proliferation, apoptosis, and the direct interaction of cancer cells with the extracellular matrix.

ADAMTS9 is a member of the ADAMTS gene family located at 3p14.2 (8), and it is expressed in all embryogenic tissues and a subset of adult tissues (9). While ADAMTS9 has catalytic activity on the cell surface and performs its proteolytic activities on other cells through its thrombospondin repeats, ADAMTS9 also possesses versicanase and aggrecanase activities in its extracellular region (7).

With the maximum number of thrombospondin repeats among the members of the ADAMTS gene family, ADAMTS9 drew attention due to the long cytosine-adenine (CA) microsatellite repeats in its promoter. The purpose of this study is to determine the effect of the CA repeat frequency on cancer and metastasis formation. Microsatellite sequences have been shown to be implicated in specific gene expression (10). Variations in the length of CA microsatellite repeats within a promoter region can change gene expression (11). Thus, CA microsatellite repeats may be considered as a candidate modulator of gene expression. Collectively, these repeats might be a molecular marker in certain conditions. Thus, we aimed to demonstrate whether the number of CA repeats could be a useful biomarker in predicting the risk of lymphatic metastasis of breast cancer. Similar studies were previously performed with the MMP-9 (12), insulin-like growth factor 1 (IGF-1) (13), and poly(ADP-ribose) polymerase-1 (PARP-1) (14) genes, which are in the same gene family or have similar functions as ADAMTS9. To our best knowledge, no studies have been performed to date to investigate the potential role of ADAMTS9 gene CA repeats in breast cancer.

2. Materials and methods

2.1. Patients

This retrospective study was approved by the Institutional Review Board of the Fatih University School of Medicine. Informed consent for the genetic study was obtained from cancer patients and healthy volunteers. Thirty-one postoperative breast cancer patients were included in our retrospective study. After obtaining informed consent, the medical records of all patients were reviewed. Phase 4 male patients who had palliative resection surgery were excluded. The patients were grouped as having either metastatic or nonmetastatic breast cancer. Histological tissue sections of the tumors from each patient were annotated according to the pathology records, and the regions including cancerous tissue from each patient were labeled under a light microscope. We then conducted our experiments on tissue samples obtained from paraffin blocks and corresponding to the regions labeled under the light microscope. The control group consisted of 30 volunteers who received a diagnosis of any chronic disease at Fatih University hospital, and they permitted the use of their peripheral blood samples for DNA isolation.

2.2. Determining the number of CA repeats in the ADAMTS9 promoter region

Paraffin-embedded tissues were purified using established methods, and genomic DNA from tissue samples and peripheral blood leukocytes were isolated using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The sequences for the forward and reverse primers used for polymerase chain reaction (PCR) amplification of the ADAMTS9 promoter region were 5' CTTCTGAGGGCTGGTTAAA 3' and 5' CTCCCATCTCTAAACCCCTG 3', respectively, and the expected product length was 180 bp.

PCR amplification was carried out in a 25- μ L PCR mix (QIAGEN, Hilden, Germany) containing 12.5 μ L of Taq PCR master mix, 9.5 μ L of dH₂O, 2 μ L of DNA, and 0.5 μ L of each primer. The samples were denatured at 95 °C for 5 min and then subjected to 35 cycles of amplification at 95 °C for 30 s, annealing at 62 °C for 30 s, and elongation at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The amplicons were analyzed in ethidium bromide-stained 1.5% agarose gels, which showed single bands of the expected size in both the patient and control groups. Next, the amplicons were sequenced in an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each individual in our study was classified into 1 of 3 groups: lymphatic metastatic breast cancer, lymphatic nonmetastatic breast cancer, or control. The numbers of CA microsatellite repeats were determined for each individual, and the results were interpreted collectively for each group.

2.3. Statistical analysis

An analysis of data normality (including age, survival time, number of lymph nodes removed, and number of CA repeats) was performed graphically and by using a Shapiro–Wilk test. All variables, except for survival time, showed a normal distribution. To represent the descriptive statistics, numbers and percentages were used for the categorical variables. The medians and standard deviations were used as measurement variables depending on the normal distribution. A Kruskal–Wallis test was used to compare CA repeat frequencies among the control, metastatic, and nonmetastatic groups. A Bonferroni-corrected Mann–Whitney test was referenced in the post hoc pairwise comparisons. A chi-square test was used to investigate the differences between the categorical variables (pathological grade, histological group, receptors, etc.) in the metastatic group. Student's t-test was used to examine the differences due to the presence of metastases on survival time. All statistical analyses and calculations were performed using MS Excel 2003 and SPSS 15.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

The distributions of the number of CA repeats in the control, metastatic, and nonmetastatic groups are shown in Figure 1. Differences in the number of CA repeats among the groups were determined using Bonferroni-corrected post hoc pairwise comparisons ($\chi^2 = 31.162$; $P < 0.001$).

We observed that the median number of CA repeats in the control group was significantly greater than that in the nonmetastatic group ($Z = 4.757$, $P < 0.001$; $Z = 5.180$, $P < 0.001$), but this was not significantly different with respect to the metastatic group ($Z = 0.601$; $P = 0.548$). Additionally, the median number of CA repeats in metastatic patients was significantly higher than in nonmetastatic patients ($Z = 5.180$; $P < 0.001$). When sorted by the median value for CA repeats, the smallest CA repeat number belonged to the nonmetastatic group, and the highest median was observed in the metastatic group. The medians of the control and metastatic groups were similar.

The tumor grade and phase were considered as they correlated with the median number of CA repeats, and the results are shown in Tables 1 and 2. The median number of CA repeats was statistically similar according to their grade ($Z = 1.205$; $P = 0.246$; Table 1). However, the median of at least one phase was different from those of the other phases ($c^2 = 17.264$; $P < 0.001$; Table 2). To determine which phase caused this difference, the Bonferroni-corrected Mann–Whitney test was employed. Accordingly, there was no statistically significant difference between the medians of Phase 1 and Phase 2 (2A + 2B) patients ($Z = 1.625$; $P = 0.126$). However, there was a statistically significant difference between the median values of Phase 1 and Phase 3 (3A + 3B) patients ($Z = 3.757$; $P < 0.001$). Moreover, the difference between the median values of Phase 2 (2A + 2B) and Phase 3 (3A + 3B) was statistically significant ($Z = 2.994$; $P = 0.003$). When the patients were ranked according to the values of their (CA)_n medians, the smallest median belonged to Phase 1 patients, and the highest median belonged to Phase 3 (3A + 3B) patients.

Next, we evaluated the number of CA repeats in the estrogen receptor (ER), progesterone receptor (PR), c-erbB2 receptor (human epidermal growth factor receptor 2, HER2, also known as Neu), and ER + PR groups individually. However, we noted no significant differences with respect to these groups (respectively, $Z = 1.944$, $P = 0.061$; $Z = 1.394$, $P = 0.193$; $Z = 0.549$, $P = 0.549$; $Z = 1.944$, $P = 0.061$; data not shown).

4. Discussion

We investigated the CA repeat length in the promoter region of the ADAMTS9 gene to uncover a potential association with breast cancer lymphatic metastasis. With regard to the median numbers of CA repeats, we observed no significant difference between the metastatic group and the control group ($Z = 0.601$; $P = 0.548$). However, the median number of CA repeats was significantly higher in the control group when compared to the nonmetastatic group ($Z = 4.757$; $P < 0.001$). These results suggest that expansion in the number of CA repeats from normal repeats

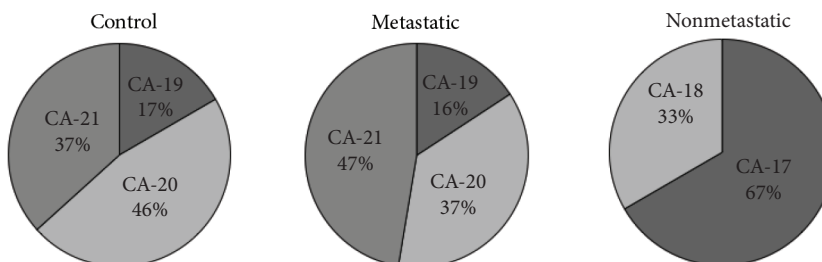


Figure 1. Circle chart representations of the number of CA repeats in each group. Distribution of the number of CA repeats in control, in metastatic, and in nonmetastatic groups. CA: cytosine-adenine dinucleotide. Number following dash indicates the number of CA repeats in the ADAMTS9 promoter region.

Table 1. (CA)n median values after analysis with respect to grade.

Grade	n	%	Median	CAG	Z / χ^2	P
Grade 1	14	45.2	18.5	2.3	Z = 1.205	0.246
Grades 2 + 3	17	54.8	20.0	4.0		

Table 2. (CA)n median values after analysis with respect to phase.

Phase	n	%	Median	CAG	Z / χ^2	P
Phase 1	7	22.6	17.0	1.0	$\chi^2 = 17.264$	<0.001
Phase 2 (2A + 2B)	11	35.5	19.0	3.0		
Phase 3 (3A + 3B)	13	41.9	21.0	1.0		

may not affect tumor formation but may potentially reduce the risk of metastasis in the tumor microenvironment. We hypothesize that CA repeat contraction in the microsatellites located in the ADAMTS9 promoter may block RNA polymerase binding to the ADAMTS9 promoter. If this scenario is correct, cancer-related gene expression, such as that of ADAMTS9, in tumors would decrease (15,16), and lower matrix metalloproteinase activity would translate into a reduced ability to invade lymphatic vessels and metastasize. Porter et al. reported that the expression of ADAMTS9 in breast cancer tissues was down-regulated with respect to noncancerous breast tissues and uncovered a potential association between ADAMTS9 and breast cancer (17). Since ADAMTS9 maps to regions known to be frequently deleted in breast cancer and other malignancies (8,9,13,16), we await with interest further observations regarding the possible implication of CA repeat polymorphisms in tumorigenesis and metastasis.

Similar studies were previously performed with the MMP-9 (12), IGF-1 (13), and PARP-1 (14) genes, which are in the same gene family as or have similar functions to ADAMTS9. Zaremba et al. studied the effects of PARP-1 activity in 118 cancer patients and 56 healthy control patients. Variations in PARP-1 activity caused by polymorphisms in the composition of the PARP-1 gene or protein were thought to affect the patients' response to cancer therapy. Although both PARP-1 activity and protein expression were similar in the control and cancer patient groups, the number of CA repeats in the PARP-1 promoter was found to be shorter in the patient group compared to the control group (14). In the study by Javadi et al., the number of CA dinucleotide repeats in the IGF-1 promoter was determined for 215 female patients diagnosed with breast cancer. Although the alleles and genotypes were similar in both the 215 cancer patients and the 224 matched controls with or without a family history of breast cancer (first- and second-degree relatives), and the number of CA repeats was approximately 19, it was

observed that the risk of breast cancer increased with the expansion of 2 CA repeats and decreased with the contraction of 2 CA repeats (13). Shimajiri et al. studied CA repeat length in the promoter region of the MMP-9 gene in esophageal cancer cell lines (12). In their study, the CA repeat length was thought to regulate MMP-9 gene transcription and enzymatic function. MMP-9 plays an important role in tumor growth, invasion, and metastasis. It is thought that a decrease in the number of CA repeats induces down-regulation of the MMP-9 transcription (12). In all of these aforementioned studies, the investigated genes were similar to the ADAMTS9 gene with regard to their association with cancer and metastasis. Moreover, they resemble ADAMTS9 in their cysteine-rich structures, matrix metalloproteinase properties, extracellular thrombospondin motifs, and aggrecanase and antiangiogenic activities. Our finding that a lower median value for the number of CA repeats, which was associated with low metastasis risk, was detected in the ADAMTS9 promoter of the nonmetastatic group compared to the other groups is consistent with previous findings.

The axillary lymph nodes are the major drainage area for the breast, and a negative correlation between axillary metastasis and prognosis has been shown by Nemoto et al. (18), Haffty et al. (19), and Fisher et al. (20). In our study, metastatic axillary lymph nodes were present in 61.29% of patients (n = 19). The median number of CA repeats in the control and metastatic lymph node groups was significantly greater than in the nonmetastatic lymph node group (Z = 4.757; P < 0.001). This result suggests a potential relationship between the number of CA repeats and lymph node metastasis.

The findings by Hanahan and Weinberg are in accordance with the findings of the present study. In their study, the metastatic tumor cell itself had evolved over time and reacquired the ability to metastasize, which was considered a result of extracellular matrix degrading protease (e.g., MMP-9) (21). Similarly, we interpret the similar number of CA repeats in both the metastatic and

control groups as an adaptation of the tumor cells to the microenvironment.

The primary factor affecting the prognosis of breast cancer is the status of metastasis (22). The most critical step for both regional and distant metastases is to traverse the extracellular matrix surrounding the tumor. Several matrix metalloproteinases are known to contribute to the invasion and traversal of the extracellular matrix in metastatic spreading. Together with ADAMS, the ADAMTS proteinases have been shown to play a role in cancer development and progression (16). Because ADAMTS9 is a matrix metalloproteinase, ADAMTS9 gene expression may be related to tumor progression and metastatic spreading.

Studies in the field of prognostic biomarkers that attempt to predict the course of disease have gained considerable importance due to their indisputable value in cancer surveillance. Genetic factors are involved in carcinogenesis and are known to influence prognosis (5). For this purpose, the MMP, ADAM, and ADAMTS gene families are of particular interest for further study. These gene families have been found to promote tumor formation, propagation, metastasis, and angiogenesis in various cancer types (23,24).

The median value for the number of CA repeats in the control group was similar to that of the metastatic group, but the nonmetastatic group had a significantly lower median value. Hence, a potential relationship between the number of CA repeats in the ADAMTS9 promoter and lymphatic metastasis of breast cancer may exist. We hypothesized that a CA repeat contraction may inhibit the tumor invasion metastasis cascade due to ineffective

binding of RNA polymerase to the target region on the promoter and the subsequent decrease in ADAMTS9 expression. As a result, ADAMTS9 cannot carry out its matrix metalloproteinase activity and fails to perform lymphatic vessel invasion, and thus prevents metastasis by this mechanism. One report suggested that shortened microsatellite CA sequences down-regulate promoter activity of the MMP-9 gene (12). Genetic mutations in CA microsatellites that are located in the gene's promoter, or increasing cytosine methylation of the promoter, may cause promoter activity augmentation. It is considered that a scarcity in the number of CA repeats induces down-regulation of the promoter region of MMP and MMP-related genes, such as ADAMTS9, and this down-regulation stimulates a couple of oncogenic mechanisms that cause an increased risk of cancer incidence. Another explanation is cytosine expansion (21). Cytosine expansion may affect the transcription factors binding to the promoter. The metastatic tumor cell reacquiring the ability to metastasize was considered a result of CA repeat expansion. Vilar and Gruber investigated the effect of microsatellite instability in colorectal cancers (25). Microsatellites in the human genome are formed by 2 or more nucleotide repeats and are used as molecular markers for mismatch repair system defects. By coming together, in particular (CA)_n and (CAG)_n sequences accumulate mutations and inhibit the binding function of DNA polymerase to DNA effectively in the synthesis (25). A limitation of our study was the lack of expression analysis in tumor samples. An increasing number of ADAMTS studies (26–28) and further experiments will elucidate the ADAMTS9 gene (Figure 2)

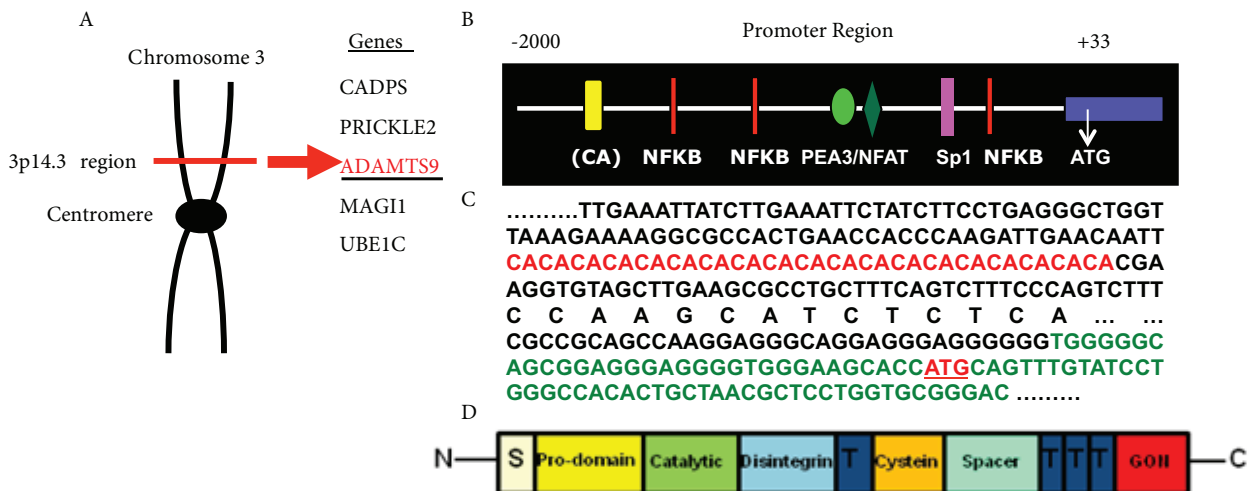


Figure 2. Chromosomal location and promoter of the ADAMTS9 gene. A) Illustration of the ADAMTS9 gene on 3p14.3. B) Binding sites of putative transcription factors (NF-κB, NFAT, Sp1) on the ADAMTS9 gene. Potential binding site, e.g. RunX, is also involved in the ADAMTS9 promoter. C) Partial sequence of the ADAMTS9 gene; promoter region has 19 CA repeats located around -1489 (red); green capital letters show exon 1. Transcription start site (ATG) is in red capital letters and underlined. D) ADAMTS9 protein domains. T: thrombospondin (TSP); S: signal peptide. ADAMTS9 has 15 TSP domains.

architecture, function, and promoter anatomy in cancer progression. Recently, Li et al. demonstrated that CA repeats within the endothelin-converting enzyme-1 (ECE-1c) gene promoter are highly polymorphic and harbor transcriptional start sites and CA repeat length, indicating shifted allelic frequency distributions. These data indicate that genomic repeat composition constitutes a novel core promoter element and affects transcriptional start site determination (29).

In conclusion, we found that both the control and metastatic groups had similar median numbers of CA repeats, but the higher median value of the number of CA repeats was significant compared to that of the nonmetastatic group. Therefore, the ADAMTS9 gene may renew itself by adapting to the host cell and imparting the tumor cell with the ability to metastasize through CA repeat composition. These data should also encourage the study of ADAMTS' role in breast cancer pathogenesis

(30–34) as a potential novel target for rational therapeutic strategies.

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