Research Article

Expression Analysis of *ARMC3*, a Testis-Specific Gene, in Breast Cancer Patients

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Abstract

Background: Breast cancer is the most prevalent cancer among women. Biomarkers that are expressed in tumors play a pivotal role in diagnosis and treatment. Cancer-testis (CT) antigens are predominantly expressed in the testis and also have inappropriate expression in various tumor types. In the case of expression in tumors, they will be used as immunotherapy targets.

Objectives: Expression of *ARMC3*, a CT antigen, was analyzed to determine its potential as a tumor marker for breast cancer.

Materials and Methods: Eighty samples including 40 tumor samples and 40 normal adjacent tissue samples, were gathered from the ICBC biobank. RNA extraction was carried out on all samples. The extracted RNA was treated by DNaseI, after which cDNA was synthesized. Expression of *ARMC3* with ACTB (internal control) was studied using Real-Time PCR (polymerase chain reaction).

Results: Overall, 43.6% of tumors and 25.6% of normal adjacent tissues expressed *ARMC3*. *ARMC3* was overexpressed in 41% of tumor samples (P = 0.00) and showed decreased expression in 46.2% (P = 0.00). Also, the expression of this gene in 12.8% of tumors was unchanged, which was statistically significant. It should be noted that all samples expressed *ACTB* gene.

Conclusions: Expression of *ARMC3* in tumor samples and normal adjacent tissue is very important. The expression of this gene in tumor-adjacent tissue may be associated with the stage of cancer; it may be that these tissues are affected by epigenetic and oncogenic changes of breast cancer. Accordingly, aberrant expression of *ARMC3* in tumor samples may be an attractive candidate for use as a tumor marker.

Keywords: Breast Cancer, Cancer-Testis Genes, Biomarker

1. Background

Breast cancer (BC) is viewed as the most frequent type of malignancy, accounting for 22% of all cancers diagnosed in women. BC is a heterogeneous and complex disease with distinct pathologies and specific histological features. In Iran, BC ranks first among cancers diagnosed in women, making up 24.4% of all malignancies. BC is characterized by the proliferation and abnormal differentiation of malignant immature cells that often carry aberrations that deregulate hundreds or even thousands of genes (1-5).

Cancer-testis (CT) antigens are named for their typical pattern of expressions as they are present in a significant subset of malignant tumors, whereas their expression in other tissues is limited (6-9). CT antigens were discovered in 1991 by van der Bruggen et al. when the first tumorous antigen was determined to significantly alter the immunologic response of tumors (10). In various clinical trials, several CT antigens have been successfully employed as target antigens (11-13). Aberrant CT antigen expression in cancer has been reported in previous studies, including studies on breast cancer (14).

There are now more than 100 gene families (such as *ARMC3*) that have been determined to be up-regulated in cancer; however, their biological function remains unclear (15, 16). The armadillo (ARM) is a repeating protein related to the Drosophila melanogaster armadillo protein, a protein essential for Wingless signal transduction. ARMC3 (armadillo repeat-containing protein 3), also known as the β-catenin-like protein, belongs to the CT81 family and is located at 10p12.31. ARMC3 encodes an 872-amino-acid protein that contains 12 ARM domains. ARM proteins are involved in a variety of processes such as cell migration, cell proliferation, tissue maintenance, and tumorigenesis. They are intracellular proteins that function in signal transduction and cell structure. ARMC3 has been identified in many cancer tissues and is thought to play a role

in tumor initiation. Some data has been published regarding ARMC3 mRNA expression in neoplasias such as colon and lung carcinoma and endometrial cancer (17, 18). The expression of several CT antigens has been analyzed in a variety of malignant neoplasms on an mRNA level (18).

The function of ARMC3 in breast cancer remains unclear. Therefore, further investigations are necessary to determine the functional role of ARMC3 in the formation of malignant phenotype cancer cells and cancer development.

2. Objectives

In order to analyze these CT antigens, we launched an investigation on the expression of *ARMC3* a testis-specific gene, at the mRNA level, in a series of 80 breast tissue samples by quantitative real-time PCR (q-RT-PCR).

3. Materials and Methods

3.1. Study Population

Samples were obtained from the breast cancer research center Biobank (BCRC-BB) (19). The study protocol was approved by the ethics committee of Qazvin University of medical Science, in compliance with the Helsinki declaration. The BCRC–BB should observe ethical guidelines and recommendations for Biobanks on the storage and use of human biological samples. Two types of tissues, including invasive ductal carcinoma and normal adjacent tissue were obtained from BCRC-BB. Five human testis tissue samples were provided by the Royan institute as the positive control.

3.2. Sample Preparation

Tissues were cut into pieces on dry ice (8 - 20 mg) and homogenized in 1 mL of RNX-Plus (Cinnagen, Iran) to extract RNA, according to the manufacturer's protocol. Extracted RNA was checked qualitatively and quantitatively by gel electrophoresis and spectrophotometry, respectively. In order to verify the quality of extracted RNA samples, electrophoresis was carried out on 1% agarose. Then 28s and 18s ribosomal bands, as well as contamination of DNA, were assessed. The RNA was quantified by a spectrophotometer at 260 nm (Biochrom WPA Biowave II). Extracted RNA was treated with RNase-free DNase I (Takara) to prevent the proliferation of genomic DNA contamination. Next, cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen).

All primers and probes were designed using primer express V.3.0 software (applied biosystems). To check the sequence specificity and avoid any homology to other parts of the human genome, all sequences were verified at http://www.ncbi.nlm.nih.gov/BLAST/.

In order to achieve PCR efficiency, serial dilutions were performed using pooled cDNA from five testis cDNAs with equal proportions, then amplification efficiency for each primer was approximated by using four-fold cDNA serial dilutions and calculated using 7500 software system ver. 2.0. The sequences and final concentration of primers and probes are listed in Table 1.

Q-RT-PCR was carried out in triplicate using Precision $2 \times$ qPCR Mastermix (PrimerDesign Ltd, UK) in 15 μL reactions. The two-step real-time PCR protocol was used. Cycle profiles of real-time PCR were as follows: activation of polymerase at 95°C for 10 minutes, and 40 cycles of amplification (95°C for 15 seconds, 60°C for 30 seconds). The fluorescence was measured using applied bio-systems 7500 real-time PCR system. After reviewing the initial data, including the results of the amplified samples, threshold cycles (Ct) were collected. To determine the expression of target genes in tumor and normal adjacent tissue, normalization of each sample result was performed by subtracting the Ct for the target gene from the housekeeping gene Ct (β-actin) using the $2^{-\Delta\Delta CT}$ formula.

3.3. Statistical Analysis

Final relative gene expression data were analyzed using REST 2009 software. Descriptive and analytical analyses of data were carried out using SPSS software version 18.

4. Results

The mean age of patients with BC was 49.16 $\pm$ 1.713 years (range 29 - 74 years). Fifty percent of cases were estrogen-receptor (ER) positive and 58.7% were ER negative. Demographic data are summarized in Table 2.

The mean quantity of RNA measured by the spectrophotometer at 260 nm was 1.15 $\pm$ 0.30 μg μL^{-1}. According to the descriptive analysis, ARMC3 was expressed in 43.6% of breast tumor samples and 25.6% of normal adjacent tissue samples. ARMC3 was overexpressed in 41% of the tumor samples (P = 0.00) and showed decreased expression in 46.2% of the tumor samples (p = 0.00). The expression of this gene in 12.8% of tumors did not undergo change, which was statistically significant.

In normal testis samples, the highest levels of transcript were observed in comparison to breast tumor and adjacent samples (P = 0.00).

After normalization of the expression levels using β-actin (ACTB) as internal reference, no significant changes were observed in the expression of ARMC3 in breast cancer tumors in comparison to normal adjacent breast tissue.

Table 1. Information for the Studied Genes

Gene	Primer		PCR Product Length, bp	PCR Efficiency, %	Optimum Concentration, μM
ARMC3			138	97	0.3
	Forward	5'-GCAACGAGGCACAACAAGG-3'			
	Reverse	5'-GGTCAAACACATCCTTAGGAGG-3'			
	Probe	5'-CCTTCTTTATCTTTTTACCCATCCTGCCGG-3'			
ACTB			66	95	0.5
	Forward	5'-CAGCAGATGTGGATCAGCAAG-3'			
	Reverse	5'-GCATTTGCGGTGGACGAT-3'			
	Probe	5'-AGGAGTATGACGAGTCCGGCCCC-3'			

Table 2. Selected Characteristics of Breast Cancer Subjects[a]

Characteristics	Percent
Age[b]	49.16 (1.713)
Stage	
I	11.6
II	45.6
III	28.8
IV	13.8
Grade	
I	12.2
II	51.2
III	36.6
ER/PR	
ER/PR positive	50/41.3
ER/PR negative	50/58.7
HER-2	
Positive	41.3
Negative	58.7

[a] N = 80.
[b] Values are expressed as mean (SD).

The ARMC3 expression ratio was 0.770 (SD: 0.011 - 40.966; 95% CI: 0.000 - 11,275.530; P = 0.750).

5. Discussion

Although great strides have been taken in the treatment of cancer, breast cancer still accounts for severe cancer mortality among women. Considering this situation, finding biomarkers expressed in cancer would be a promising method for diagnosing and treating it. The first step for this purpose would be identifying biomarkers that are extensively expressed in various stages of cancer progression, especially the initial stages (2).

Cancer-testis genes express a number of immunologic proteins often exclusively expressed in normal testis tissues and some tumors. These antigens are proteins that are naturally expressed in male generative cells. There is evidence for the functional roles of CT antigens, including modulating gene expression and contributing to tumor signaling pathways, tumor cell division, and apoptosis (20). Due to the tissue-exclusivity and immunogenicity of the cancer-testis antigens, and their infrequent expression in normal tissues, one could construe their expression in tumorous tissues as a cancer biomarker. Unlike chemotherapy and radiotherapy, which kill healthy dividing cells in addition to tumor cells, CT antigens can be used for specific targeting of cancer cells using immunotherapy (21). Many CT antigens form the basis for peptide-based CT cancer vaccine trials (22), and phase 1 studies utilizing different types of these vaccines for a number of malignancies have identified these methods as harmless (23). The humoral immune response to CT antigens has been observed in numerous tumors, for instance antibodies against ARMC3 were identified in pancreatic, prostate, and endometrial cancers (18, 24).

There is a list of several known CT genes in breast cancer (25). Overexpression of some CT antigens in breast tumors (26) and a combination of different tumors was previously reported (27).

Armadillo/β-catenin (ARM) domains are imperfect 45-amino acid repeats that are involved in protein–protein interactions. ARM domain-containing proteins, such as ARMC3, are active in signal transduction, development, cell adhesion and mobility, and tumor initiation and metastasis (17).

In a study using the RT-PCR method in 2006, the ARMC3 expression at the mRNA level was reported at 33% and 38% for pancreatic and lung cancers, respectively (18). In the

present study, research on ARMC3 expression at the mRNA level showed that ARMC3 was expressed in 43.6% of tumors and 25.6% of normal adjacent tissues. ARMC3 expression increased in 41% and decreased in 46.2% of the tumor samples. The expression was not changed in 12.8% of the tumor samples, meaning tumorous and normal adjacent tissues had similar expressions.

The results of this study showed very low levels of ARMC3 transcripts in the breast tissue samples, whereas the highest levels of transcript were observed in normal testis with highly significant differences in the gene expression ratio in comparison with breast tissue samples, confirming the restricted expression pattern of the genes in normal testis.

There have not been sufficient studies on ARMC3, and the clinical significance of ARMC3, especially in human breast cancer, remains to be the subject of future research.

The expression of these genes in normal tissues adjacent to tumors could be related to the concretization stage. The glandular epithelial cells in the breast undergo cyclical proliferation, which favors neoplastic transformation, and could be attributed to genomic instability in normal breast lobules adjacent to the cancer focus (28). The expression of these genes in the adjacent tissues could therefore be caused by epigenetic and oncogenic changes in the breast cancer and point to biomarkers of vital importance.

The reason behind conflicting reports about the expression of these genes in tumors has not yet been identified. It may be due to varying genetic reserves of different populations being studied, or it could result from technical or sample volume discrepancies. The majority of studies mentioned were conducted on limited numbers of samples. It must be noted that tumorous samples in different studies are provided from various stages of cancer. This heterogeneity can influence the obtained results. The effect of genetic heterogeneity in cancers also needs to be considered, as well as the discrepancies inherent in the definition and diagnosis of progression stages of breast cancer in this respect (29, 30).

It is proposed that ARMC3 expression in breast cancer cell lines should also be considered, and the relation between ARMC3 expression and the clinicopathological characteristics of the disease should be assessed and compared to obtain further information about the genetic correlation and prognosis of breast cancer. In the next stages of the research, it is recommended that the generation and expression of antibodies against ARMC3 in the serum be considered. It is hoped that these cancer-testis antigens such as ARMC3 can be employed to advance immunotherapy of breast cancer, in that ARMC3 is expressed in the protein level and is immunogenic.

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