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PIWIL2 and *PL2L60* (Cancer/Testis genes) Expression in Breast Cancer

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ABSTRACT

Background: Cancer/testis antigens (CTAs) are members of a group of proteins which are normally expressed in testis germ cells and to a lesser extent in the ovaries. Because of recent reports about their aberrant and specific expression in some tumoral tissues, they may play a role as new candidates for targeted therapy. Therefore, the study of the expression pattern of these biomarkers and its relationship with clinical features of the patients is a subject of great interest.

Methods: In this study, expression of *PIWIL2* and genes was studied by multiplex RT-PCR in 65 breast tissue samples including 30 invasive ductal carcinomas (IDC), 30 normal adjacent tissue samples and five normal breast tissue samples and 2 normal testicular tissue samples as positive controls. beta actin was considered as internal control.

Results: Results of gel electrophoresis analysis demonstrated no significant expression of target genes in any sample except testis. Simultaneously, beta actin was expressed in all the samples.

Conclusions: The present study indicates lack of *PIWIL2* and *PL2L60* expression at mRNA level in breast cancer. Although cell lines can be used in cancer research, they are not representative of tumor tissues. More studies investigating the expression of these genes at protein level will help us decide whether to apply these candidate genes as tumor markers or not.

Introduction

Researchers are looking for new biomarkers to use them in diagnosis, prognosis or as a target for therapy. The Cancer/Testis (CT) antigens are one of these new promising biomarkers.[1] They are a number of proteins which are normally expressed in testis as an immune privileged organ and to a lesser extent in the ovarian germ cells and trophoblasts.[2] So, the ectopic expression of these antigens in other tissues like tumoral tissues could establish a novel role as specific biomarkers for them and can also pave the way for them to being used as stimulators of the human immune system.

PIWIL2 – a type of CT antigen – is one of the eight members of the Argonaute family proteins which its over-expression may cause tumorigenesis[1,3] Recent reports have demonstrated *PIWIL2* expression in some cancers in which

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it was associated with older age and more aggressive histologic types of the tumor.[4] Alternative splicing of the *PIWIL2* gene produces several distinct mRNAs among which *PL2L60* was specifically reported in breast cancer cell lines.[5]

Most of the studies about these genes of interest were performed in cell lines.[6] Although cell lines can be used in cancer research, they are not representative of primary tumors. In order to make concrete clinical decisions about biomarkers, it is necessary to evaluate them in the setting of tissues as well. Therefore, the expression pattern of *PIWIL2* and *PL2L60* genes was investigated by multiplex reverse transcription-polymerase chain reaction (RT-PCR) in the tumor, tumor-adjacent normal tissue and normal tissue of the breast and its relationship with clinico-pathological features of the patients was assessed.

Methods

Patients and tissue specimens

A total of 65 breast tissue specimens including 30 invasive ductal carcinomas (IDCs), 30 tumor-adjacent normal tissue specimens and 5 normal tissue specimens were obtained from the Breast Cancer Research Center BioBank (BCRC-BB).[7] After excision, tissue samples were frozen by using liquid nitrogen and then stored at -70°C. BCRC-BB is obliged to follow ethical guidelines and recommendations for biobanks on the storage and use of human biological samples. Patients' clinico-pathological features are summarized in table 1 as follows: age, grade of tumor, stage of tumor, human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR) and treatment regimen.

Two normal testicular tissue samples were taken from the Avicenna infertility clinic (AIC) and used as positive controls.

RNA extraction and cDNA synthesis

Tissues (8-20 mg) were excised on dry ice and homogenized in 1 ml of RNX-Plus (Cinnagen, Iran) to isolate total RNA according to the manufacturer's instructions. Quantity and purity of extracted RNAs were assessed by a spectrophotometer (Hitachi, U-0080D, Japan). The integrity of RNA was confirmed by electrophoresis on a 0.8% agarose gel. An amount of 1 µg of total RNA was reversely transcribed to cDNA by using a QuantiTect Reverse Transcription Kit (Qiagen, Germany).

Primer Design

All primers were designed using Gene Runner v.3.05 and confirmed with Primer Express v3.0. The sequence of the primers is listed in Table 2. The *ACTB* primer was designed as an internal control.

Multiplex RT-PCR

PCR was performed with a final volume of 25 µl. Each reaction contained buffer 1X; 0.2mM deoxynucleoside triphosphate; 1.5mM MgCl2; 0.5 µM primers *PIWIL2, PL2L60;* 0.5 µM primer *ACTB*; and 0.25 U of *Taq* DNA polymerase (Pars Tous, Iran). Template cDNA was added to the mixture and then overlaid with mineral oil. The PCR program consisted of an initial denaturation step at 95°C for 5 minutes (min), followed by 30 amplification cycles consisting of denaturation at 95°C for 30 seconds (s), annealing at 57°C for 40 s and a final extension phase at 72°C for 5 min. PCR products were analyzed by 2% agarose gel. To confirm the results, RT-PCR was performed for each primer separately.

Results

Table 1 summarizes clinico-pathological characteristics of study subjects. The mean age of patients was 48 (range: 31-74 years). ER, PR and HER2 expression was detected in 57%, 47% and 30% of patients, respectively. A total of 68% and 91% of the participants received hormone therapy and chemotherapy regimens, respectively. The gel electrophoresis illustrated that mRNAs of the target genes (*PIWIL2* and *PL2L60*) were not detected in any sample except testis, while *ACTB* was shown to be expressed in all the samples by using multiplex RT-PCR (Figure 1). For greater certainty of the obtained results, RT-PCR was performed for each primer separately on all samples and the result was the same (Figure2).

Table 1. Clinical and histopathological features of enrolled patients (N= 31)

	N (%)
ER status	
Negative	13 (43%)
Positive	17 (57%)
PR	
Negative	16 (53%)
Positive	14 (47%)
HER2	
Negative	21 (70%)
Positive	9 (30%)
Grade	
G1	4 (15%)
G2	16 (59%)
G3	7 (26%)
Stage	
I	2 (7%)
II	13 (47%)
III	11 (39%)
IV	2 (7%)
Hormone therapy	
No	6 (32%)
Yes	13 (68%)
Chemotherapy regimen	
Negative	2 (9%)
Positive	19 (91%)

Table 2. The list of primer sequences

Name	Nomenclature number	Primer sequence	Length of amplicon
PIWIL2	NM_001135721.1	F: ATCGCCCTCTGGTCCTGAC R: ACATCCAGCACACAGTCATTCC	583bp
PL2L60	AK027497	F:ATAGCTTCACGATGTCTGATGG R:CCTTCAATCTTATGTACATCCTTT	376bp
ACTB	NM_001101.3	F: CCT GGC ACC CAG CAC AAT R: GGG CCG GAC TCG TCATCAT	144bp

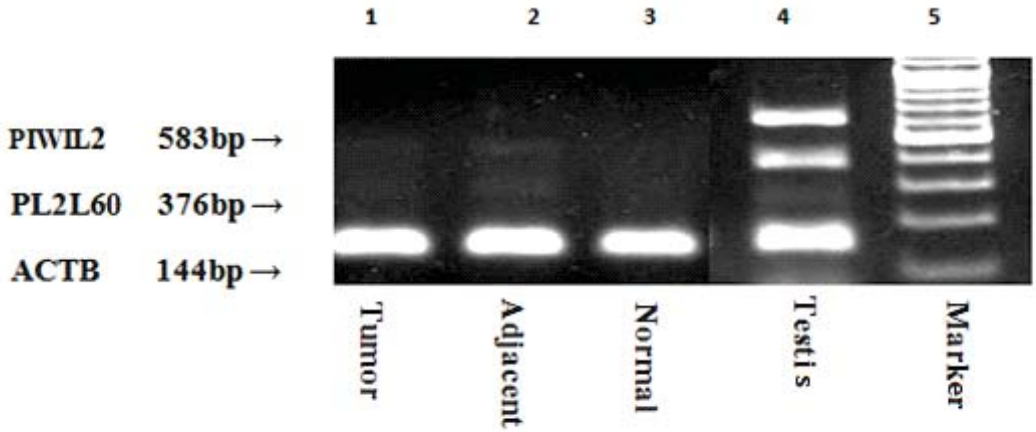

Figure 1: The gel electrophoresis of multiplex RT-PCR products, with *PIWIL2* and *PL2L60* as candidate genes and *ACTB* as internal control. Only expression of *ACTB* was detected in all samples (Lanes 1-3). *PIWIL2* and *PL2L60* could not be detected in any sample except testis (Lane 4). A 100 bp DNA marker was used (Lane 5).

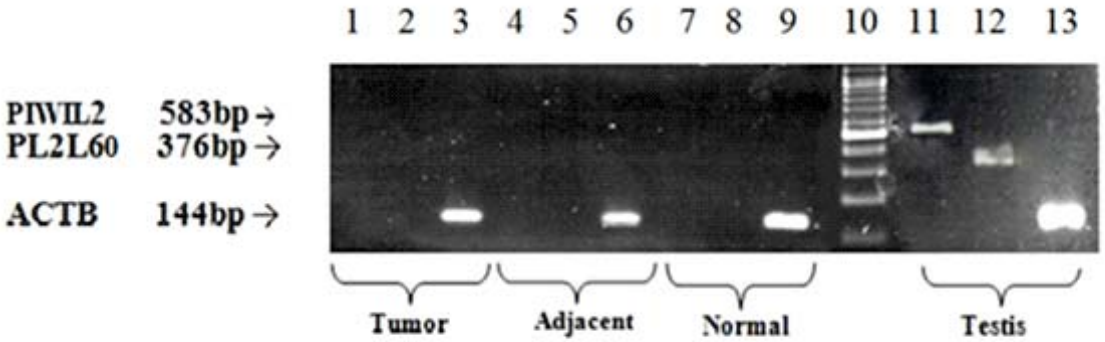

Figure 2: The gel electrophoresis of RT-PCR products with *PIWIL2*, *PL2L60* and *ACTB* primers separately. *PIWIL2* and *PL2L60* transcripts could not be found in all three samples (Lanes 1, 2, 4, 5, 7 and 8). *ACTB* was detected in all samples (Lane 3, 6 and 9). A 100 bp DNA marker was used (Lane 10). Testicular RNA was used as positive control and all three genes were detected in testicular tissue (Lanes 10-13).

Discussion

In this study 30 tumor and 30 tumor-adjacent normal tissue samples from breast cancer patients, five normal breast tissue samples from healthy cases and two normal human testis samples were evaluated for expression of *PIWIL2* and *PL2L60* by using multiplex RT-PCR. *PIWIL2* and *PL2L60* expression could not be detected at the mRNA level in all 65 cases, but *ACTB* was detected in all samples which verified the procedure. All the target genes were expressed only in testis samples, as positive controls.

Some studies have reported the expression of *PIWIL2* and its variant (*PL2L60*) at different stages of breast cancer.[5,6] It is showed that *PIWIL2* has an important role in development and growth of cancer cells. Also, the presence of *PIWIL2* at different stages of breast cancer and its correlation with ER and Ki-67 was detected previously.[8] Moreover, the expression of *PL2L60*, as a *PIWIL2* variant, was assessed in breast cancer and other cancer cell lines and in pre-cancerous breast cancer stem cells.[5,9] The discrepancy witnessed between our observations and the previous reports on *PIWIL2* expression can be due to three reasons; first, most of the studies about these genes were performed on cell lines.[9,10] Although cell lines are used as model systems for tumors, several studies show that their gene expression profile can be different from the primary tumor.[4,10] Second, despite overall correlation between mRNA and protein level, this correlation can sometimes be low in some genes as a result of regulation of transcription.[11] Therefore, protein level studies can resolve the conundrum. Third, ethnic and genetic diversity can be another reason. Distinct clinical characteristics and molecular subtypes of breast cancer have been reported among different populations.[12,13] Although a previous study on bladder cancer in Iranian patients also failed to show the expression of *PIWIL2*, there is a need for further experiments to confirm that *PIWIL2* has various expression profiles among different populations.[14]

According to the results of other studies in cell lines, *PL2L60* and *PIWIL2* may be used as cancer biomarkers for determining the prognosis and treatment outcome or as targets for therapy in cancer[5,8], but our study on primary tumors at mRNA level showed opposing results in Iranian samples.

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