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ORIGINAL ARTICLE

Cancer Testis Antigen Expression in Breast cancer Tissues and Cell Lines

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ABSTRACT

Cancer/Testis genes (CT-genes) are a gene family which only express in testis tissue but not in somatic tissues. Although some of them are randomly expressed in some types of cancers. The aim of this study was to analyze the expression of several cancer-testis genes in breast cancer and cancer cell lines. 32 breast cancer tumor samples and 14 cancer cell lines were prepared. Expression of NY-ESO-1 1a, NY-ESO-1 1b, SSX-2 and MAGE-3genes as well as GAPDH (internal control) were studied by multiplex RT-PCR method. In a way that the cancer-testis gene and GAPDH were studied simultaneously. Three (9%) of 32 tumor samples expressed mRNA of NY-ESO-1 1a while six (19%) of 32 tumor samples expressed mRNA of NY-ESO-1 1b. Seven (22%) of 32 tumor samples expressed mRNA ofSCP1. Two (6%) of 32 tumor samples expressed mRNA of MAGE3. Thirteen (41%) of 32 tumor samples expressed one of them. two genes (NY-ESO-1 and SCP1) of this gene family might be considered as appropriate biomarker for breast cancer. It is suggested that more number of breast cancer tumor samples should be examined to evaluate expression of these two genes. SCP1 and NY-ESO-1 proteins may promote future of breast cancer immunotherapy.

Keywords: *breast cancer, biomarker, immunotherapy, cancer-testis genes.*

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INTRODUCTION

Breast cancer is currently the most common malignancies in women and affects about 1 of 8 women.[1].According to statistics of U.S.A Cancer Association; breast cancer incidence was 207090 cases in the U.S.A in 2010 and 40000 people die with this type of cancer annually. Besides high rate of breast cancer prevalence in iran, Iranian women compared to developed countries, suffer from that one decade earlier which may be due to population statistics and dermatographic issues in Iran.[3]. Despite therapeutically treatments given after primary surgery, a great deal of patients show metastatic steps of cancer.Of course the average survival rate of patients with metastatic breast cancer increases through hormonal and chemical therapeutically approaches like Trastuzumab Antibody but a metastatic disease will be fatal Eventually [4]. So after all, development of the science of immunotherapy can be a great help to cure breast cancer.

The prerequisite for the development of tumor-specific immunotherapeutic strategies is the identification early biomarkers and development of molecular-targeted therapeutic drugs [2]. Cancer testis (CT) antigens can be defined by predominant expression in various type of cancer but not in normal tissues, except for testis. Their restrictive expression makes them attractive for immunotherapy [6, 7]. Due to testis blood barrier and the immune privileged status of germinal cells, expression of CT

genes in tissues other than testis can trigger immune response. They can be considered as tumor specific markers and represent ideal target for vaccine [8, 9]. Restricted pattern of these proteins' expression has made the immune system of cancerous patients who express these proteins in their tumor cells, able to identify them repeatedly. Humoral immune response to Cancer/Testis antigens has been observed in many tumors, for example, antibodies against *SCP-1* in pancreas cancer [10], against *SCP-1* and *NY-ESO-1* in breast cancer [1, 11], against *CTSP-1* in prostate, thyroid and breast cancer and against *NY-ESO-1*, *SSX2* and *MAGEA3* in multiple myeloma have been identified [12]. However their tissue-specificity and immunogenicity has made the possibility of using them as a candidate for anticancer vaccination so that the immune response is stimulated and fight against tumor growth [9, 11].

According to data available in C/T genes Database (www.cta.lncc.br), more than 100members of C/T gene family has been identified but some of breast cancer types express some of these antigens [1, 7, 8]. In this study probable expression of 5 transcripts of these antigens (*NY-ESO-1 1a*, *NY-ESO-1 1b*, *SCP1*, *MAGEA3* and *SSX2*) in 32 tumor samples from breast cancer patients and 14 cancer cell lines was analyzed by Multiplex RT-PCR. This research can be considered as the first research in this field, to analyze the frequency of these Cancer/Testis genes in breast cancer samples in Iran.

MATERIALS AND METHODS

Preparing tumor tissue samples of breast cancer

Tumor tissue samples were collected from patients suffering from breast cancer who referred to Imam Khomeini Hospital and also from Iranian Center for Breast Cancer of the Academic Center for Education, Culture and Research of the University of Tehran,between May and December of 2011. After selecting women suffering from sporadic breast cancer and filling out the testimonial, tumor tissue samples were collected for this study by the corresponding doctor during the operation. In this research 32 tumor samples were collected with the observance of all ethical and medical issues.Regarding the importance of minimizing the time between sample collection and RNA extraction, the samples were transferred immediately to the nitrogen tank and taken to Iran Pasteur Institute for molecular studies and kept in -70˚C freezer till RNA extraction.

Preparing cancer cell lines

Firstly, 14 cell lines (Table 1) were prepared from Cell Bank of Iran Pasteur Institute. Shortly after cell lines delivery, they were cultured in their optimized conditions in the presence of antibiotic, and after 48 hours incubation in 37˚C and 5% Carbon Dioxide the cells were analyzed and counted using invert microscope and were passaged if necessary to reach approximately to ten million and get ready for RNA extraction.

RNA extraction and cDNA synthesis

For RNA extraction from tumor tissue Guanidinium Thiocyanate-Phenol-Chloroform method was used [13]. RNA extraction from cell lines was performed using Rneasy Mini Kit (Qiagen, Germany). The amount of each RNAextracted was determined by ultraviolet spectrophotometry at 260 nm.also cDNA synthesis from extracted RNAs was performed using Quantitect Reverse Transcription (Qiagen, Germany).

Multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) assay and electrophoresis

In this research expression of 6 transcripts of mRNA was analyzed by ordered primers (Table 2) by thermal cycler (BioRad, California) with these conditions: a primary denaturation step of 5 minutes at 94˚C, 32 cycles of 94˚C for one minute, 60˚C for one minute and 72˚C for one and a half minute, and one step of ten minutes at 72˚C.GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) transcript was used as internal control. Each reaction tube contained 22 microliters (including 1X PCR Buffer, 1.5mM $MgCl_2$, 0.2M dNTP mix and 1M spermidine) and 10picomole of forward and reverse primers, 200ng of cDNA and 0.5unit of Taq DNA Polymerase (Qiagen, Germany) was used.

Since the aim was to analyze the internal control and the gene of interest simultaneously in order to minimize the rate of false negatives in the results, the optimization of conditions for simultaneous analysis of both transcripts was done which included the optimization of temperature gradient, change in the amount of enzyme, primers, PCR steps' duration and the amount of cDNA added. The mRNA transcript of study and GADPH were analyzed in multiplex.

And also each reaction was analyzed along with a positive control (one of cell lines that expresses the gene of interest beside GADPH), a negative control (one of cell lines which only expresses GADPH) and a blank control (without cDNA to minimize the probability of false positives). In order to confirm the authenticity of the tests, after performing PCR, the acquired product was run on 1.5% agarose gel electrophoresis with 100bps or 1Kb markers, at the voltage of 100V in one hour.

RESULTS

Expression frequency of transcripts in cell lines

*NY-ESO-1 1a*transcript was expressed in A-375 cell line.*NY-ESO-1 1b* transcript was expressed in A-375 and 1321N1cell line.*MAGEA3* transcript was expressed in A-375 and U-373MG cell line.*SCP*-1 transcript was expressed in GC-1spg cell line. *SSX-2* transcript was expressed in K562 cell line.These cell lines was used as positive control in the analysis of tumor samples and MDA-MB-231 cell line was used as negative control for *NY-ESO-1 1a*, *MAGEA3* and *SSX2* transcripts.SW742 cell line was used as negative control for *NY-ESO-1 1b* gene. The results about expression of genes of interest in cell lines are revealed in table 3.

Expression frequency of transcripts in breast cancer tumor tissue samples

All 32 breast cancer tumor samples were analyzed through Multiplex PCR method. Some results of their electrophoresis are shown in figures 1 and 2. 3 (9%) of 32 tumor samples expressed *NY-ESO-1 1a* transcript. 6 (19%)of32 tumor samples expressed *NY-ESO-1 1b* transcript and totally 9 samples (28%) expressed *NY-ESO-1* gene. 7 (22%) of 32 tumor samples expressed *SCP-1* transcript and 2 (6%) samples out of 32 expressed *MAGEA3* transcript. None of the samples expressed *SSX2* gene transcript. Of course all the samples expressed *GAPDH* gene (graph 1).

The study of simultaneous expression of C/T genes in breast cancer samples

In this study one sample (3%) out of 32 tumor samples expressed all three transcripts, *NY-ESO-1 1a*, *NY-ESO-1 1b* and *MAGEA3*. 2 samples (6%) expressed 2 transcripts, *NY-ESO-1 1b* and *SCP1*. 11 samples (34%) expressed one of *NY-ESO-1* or *SCP1* gene.

13 samples (41%) expressed at least one of studied C/T genes.

Comparison between expression of C/T genes in breast cancer tumor tissue samples and breast cancer cell lines:

While 41% of breast cancer tumor samples expressed one of studied genes, none of the three breast cancer cell lines (MCF-7, BT-20 and MDA-MB-231) expressed none of the studied C/T genes.

DISCUSSION

An ideal tumor marker mRNA, is one which would express in cancer cells but would not express in non cancer cells. In this research Multiplex RT-PCR technique was selected to evaluate the expression rates and this was due to high precision and accuracy of this technique. Of course the advantage of this method over RT-PCR (to analyze GAPDH gene first and then C/Tgene separately) is that it reduces the possibility of false negatives. On the other hand, by taking negative controls into account, a comparison can be made between negative tumor samples and negative controls. In each test one sample without any cDNA (blank) was also considered to reduce the possibility of false positives.

Results of studying *NY-ESO-1* gene in tumor samples of breast cancer patients in different studies were 13 out of 129 samples (10.1%) [14], 37 out of 88 (42%) [15], 9 out of 50 (18%) [16], 80 out of 403 (20%) [17] and 11 out of 49 (22%) [18], while this study has evaluated the expression frequency of these genes in studied samples in Iran as 28%.

The results of studying *SCP1* gene in breast cancer were 44 samples out of 129 (34.1%) [14] and 64 out of 98 (65%) [1] while the expression of this gene was evaluated in this study 22%.

The results of studying *MAGEA3* gene expression in breast cancer were 25 samples out of 67 (37%) [19], 3 out of 28 (11%) [20] and 11out of 98 (11%) [1] in other studies and in this study the expression rate of this gene was 6%.

The results of *SSX2* gene expression in breast cancer were 8 samples out of 98 (8%) [1] and 5 out of 129 (3.9%) [14] in other study and in this study this gene expressed in none of tested samples.

Of course the cause of this difference in reports on these genes' expressionare not clear but it may be due to difference between genetic pools of different populations studied or divergences caused by technical differences or difference in sample volumes. And of course it has to be taken into account that tumor samples had been collected in different steps of the illness and this may affect the results too. Also, genetic heterogeneity in cancer can be effective and in this case maybe variances that exist in the definition and diagnosis of breast cancer stages can be effective on this issue.

Furthermore, results of this study about breast cancer cell lines in comparison with breast cancer tumor samples are so different and this difference can be due tolow number of studied cell lines or that their expression profile. Anyhow, by studying more breast cancer cell lines for the expression of these genes more accurate and trustable results can be obtained.

The approach to reach for cancer immunotherapy is based on C/T antigens, the study of C/T genes transcripts expression in cancers and also the study of translation into proteins for these genes [7, 8]. Although proteins coded by C/T genes are called C/T antigens, but not all of them are able to raise the response of immune system. By the way, antibodies against *NY-ESO-1* and *SCP1* are defined in breast

cancer [1, 11]. All these instances make Cancer/Testis antigens promising goals for breast cancer immunotherapy and raise the hope for producing vaccines from them to provoke the immune system and inhibit the tumor growth [8, 9, 21 and 22]. Clinical assessment of cancer-specific immunotherapy on patients suffering from Gastroenteric Carcinoma by administration of MAGE peptide is being carried out and led to good results in some cases, but there is a vital problem in this route and it is the lack of a proper candidate for immunotherapy, as the necessary prerequisite for immunotherapy is the expression of C/T genes in cancer cells [14].

So it can be concluded like that *NY-ESO-1* and *SCP1* can be thought of as appropriate biomarkers for breast cancer. And it can be suggested to study the expression rate of *NY-ESO-1* and *SCP1* in more breast cancer tumor samples to gain more perfect data. Also, the study of mentioned gene transcripts expression can be carried out along with pursuit of clinical status of the patients to gather information on possible correlations between the expression of these genes and breast cancer prognosis. And of course, the expression of *NY-ESO-1* and *SCP1* genes can be studied in the protein level in breast cancer tumor cells and in the case of their expression in protein level and their possible immunogenicity, there will be a hope to be able to use these C/T antigens to develop breast cancer immunotherapy.

Table 1: list of cell lines and their properties

Cell lines	Tissue	Morphology	Culture medium
Hela	Cervix	Epithelial-like	EMEM + FBS 10%
A-375	Skin	Epithelial-like	DMEM + FBS 15%
U-373MG	Brain	Epithelial-like	RPMI 1640 + FBS 10%
1321N1	Brain	Glial-like	DMEM + FBS 10%
RPMI 8226	Hematopoietic	Lymphoblast	RPMI 1640 + FBS 10%
MCF-7	Breast	Epithelial-like	EMEM + FBS 10%
MDA-MB-231	Breast	Epithelial	L-15 + FBS 10%
PC-3	Prostate	Epithelial	F12K+AA1%+FBS 10%
BT-20	Breast	Epithelial-like	RPMI 1640 + FBS 10%
HNCF-PI52	Cervix	Fibroblast-like	RPMI 1640 + FBS 10%
Calu-6	Lung	Epithelial-like	RPMI 1640 + FBS 10%
SW742	Colon	Epithelial-like	L-15 + BCS 10%
K562	Pleural efusion	Lymphoblast- like	RPMI 1640 + FBS 10%
GC-1spg	Testis	Epithelial-like	DMEM + FBS 10%

Table 2: list of primers used in this study. F=Forward and R= Reverse.

transcript	Accession Number	primer sequences and corresponded region in mRNA		PCR PRUDACT SIZE
GAPDH	NM_002046	F	5′-GTC AAC GGA TTT GGT CGT ATT-3 (124-144)	540 base pair
		R	5′-AGT CTT CTG GGT GGC AGT GAT-3′ (643-663)	
NY-ESO-1 1a	NM_001327.2	F	5′-AGT TCT ACC TCG CCA TGC CT-3′ (319-338)	386 base pair
		R	5′-TCC TCC TCC AGC GAC AAA CAA-3′ (684-704)	
NY-ESO-1 1b	NM_0011327.2	F	5′- ATG GAT GCT GCA GAT GCG G-3′ (271-289)	328 base pair
		R	5′-GCT TAG CGC CTC TGC CCT G-3′ (580-598)	
SCP1	NM_003176.2	F	5′-GTA CAG CAG AAA GCA AGC AAC TGA ATG-3′ (1383-1409)	565 base pair
		R	5′-GAA GGA ACT GCT TTA GAA TCC AAT TTC C-3′ (845-872)	
MAGE3	NM_005362.3	F	5′-GAA GCC GGC CCA GGC TCG-3′ (48-65)	423 base pair
		R	5′-GGA GTC CTC ATA GGA TTG GCT-3′ (450-470)	
SSX2	NM_175698.1	F	5′-GTG CTC AAA TAC CAG AGA AGA TC-3′ (149-171)	435 base pair
		R	5′-TTT TGG GTC CAG ATC TCT CGT G-3′ (562-583)	

Table 3: The results about expression of transcripts in cell lines. (ND = Not Determined).

Cell Line	NY-ESO-1 1a	NY-ESO-1 1b	MAGE-3	SCP-1	SSX-2	GAPDH
Hela	ND	Neg	ND	Neg	ND	Pos
A-375	Pos	Pos	Pos	Neg	Neg	Pos
U-373MG	Neg	Neg	Pos	Neg	Neg	Pos
1321N1	ND	Pos	ND	Neg	Neg	Pos
RPMI 8226	ND	Neg	ND	Neg	Neg	Pos
MCF-7	Neg	Neg	Neg	Neg	Neg	Pos
MDA-MB-231	Neg	Neg	Neg	Neg	Neg	Pos
PC-3	ND	Neg	ND	Neg	Neg	Pos
BT-20	ND	Neg	ND	Neg	Neg	Pos
HNCF-PI52	ND	ND	ND	Neg	Neg	Pos
Calu-6	ND	Neg	ND	Neg	Neg	Pos
SW742	ND	ND	ND	Neg	Neg	Pos
K562	ND	ND	ND	Neg	Pos	Pos
GC-1spg	ND	ND	ND	Pos	Neg	Pos

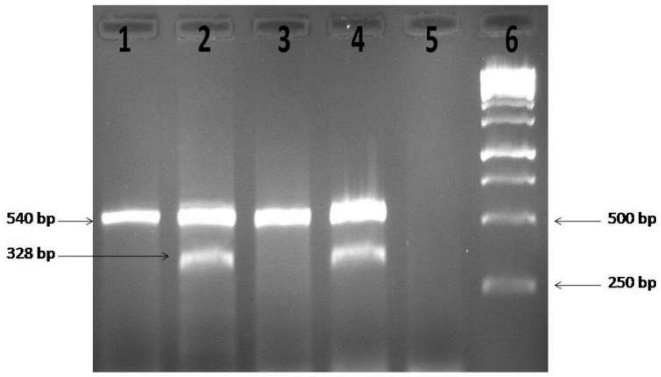


Figure 1: Electrophoresis result for Multiplex RT-PCR products with *NY-ESO-1 1b* and *GAPDH* primers in some tumor samples: all the samples expressed *GAPDH* gene. Well no.1: tumor sample without expression of *NY-ESO-1 1b* gene. Well no.2 is tumor sample expressing the mentioned gene. Well no.3 is SW742 cell line as negative control. Well no.4 is A-375 as positive control. Well no.5 is the sample without cDNA. Well no.6 is 1Kb size marker

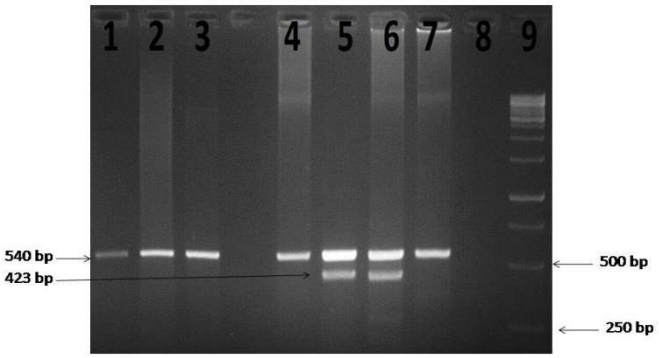
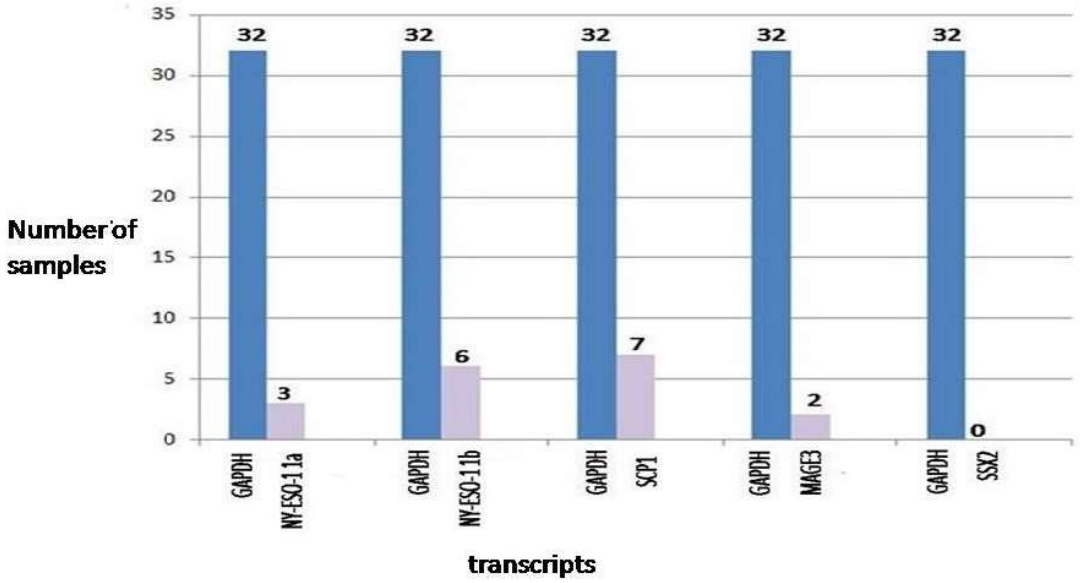


Figure 2: Electrophoresis result for Multiplex RT-PCR products with *NY-ESO-1 1b* and *GAPDH* primers in some tumor samples: all the samples expressed *GAPDH* gene. Wells no.1, 2, 3 and 4 are tumor samples which only expressed *GAPDH* gene. Well no.5 is a tumor sample which has expressed *MAGEA3* gene. Well no.6, 7, 8 and 9 are A-375 cell line as positive control, MDA-MB-231 as negative control, the sample without cDNA and 1Kb size marker respectively



Graph 1: comparison between the expression of studied C/T genes and *GAPDH* in 32 breast cancer tumor samples: *NY-ESO-1* gene (*NY-ESO-1 1a* and *NY-ESO-1 1b* transcripts) had the most expression rate (28%) and the 2nd rate was of *SCP1* gene of 22%. *MAGEA3* and *SSX2* has the least expression rate of 6% and 0% respectively.

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