original article

Expression of testis-specific genes, *TEX101* and *ODF4*, in chronic myeloid leukemia and evaluation of TEX101 immunogenicity

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BACKGROUND AND OBJECTIVES: Cancer-testis (CT) antigens are a group of antigens with a restricted expression in normal tissues, except testis, and they have aberrant expression in different tumors. This pattern of expression has made them promising targets for immunotherapy and cancer detection. Our aim was to find new members of this group that might be useful as markers in the detection of cancer and immunotherapy.

DESIGN AND SETTING: A descriptive study conducted in referral centers of Tehran University of Medical Science from january 2008 to January 2009.

PATIENTS AND METHODS: We analyzed the expression of two testis-specific genes named *ODF4* (outer dense fiber of sperm tails 4) and *TEX101* (testis expressed 101) in 20 chronic myeloid leukemia (CML) and 20 normal samples by reverse transcription-polymerase chain reaction and sequencing. Immunogenicity of *TEX101* was evaluated by means of enzyme-linked immunosorbent assay.

RESULTS: These two genes were expressed in 30% of CML patients but not in any of the healthy donors. Humoral response against *TEX101* was not detected in any samples.

CONCLUSIONS: *TEX101* and *ODF4* are CT genes useful for detection of CML. Unlike many CT genes, overexpression of *TEX101* was not shown to induce immunologic responses in these samples. According to the previous studies, overexpression of *TEX101* leads to suppression of cancer invasion and metastasis; thus, the induction of the expression of *TEX101* in cancer by epigenetic mechanisms may be a treatment strategy.

Chronic myeloid leukemia (CML) is a malignant clonal disorder of the hematopoietic system that leads to an increased number of myelocytes, erythrocytes, and thrombocytes in peripheral blood. The molecular hallmark of CML is the Philadelphia chromosome, a reciprocal t(9,22) translocation. This abnormal chromosome is found in cells from the myeloid, erythroid, megakaryocytic, and B lymphoid lineages, indicating the presence of a "cancer stem cell" that is capable of producing several types of differentiated cancer cells.[1]

The development of leukemia-specific immunotherapies, such as the application of cancer vaccines for leukemia patients, depends on the molecular definition of immunogenic leukemia-associated antigens that are specifically recognized by the immune system. One important group of tumor-associated antigens is cancer-testis (CT) antigens. The limited expression of these genes in normal tissues and their expression in various types of tumors make them appropriate candidates for immunotherapy. As these genes are expressed almost only in male germ cells and trophoblast among normal cells. Since these cells do not express major histocompatibility complex class I molecules, their expression cannot result in presentation of the antigenic peptides. In addition, tight junctions between Sertoli cells along the basolateral aspect and between capillary endothelial cells have a role in maintaining the mechanical barrier between blood and testis, and testis is considered an immune-privileged site.[2-4] If testis-specific genes are expressed in other tissues, they can elicit immune responses. For effective specific immunotherapies, extended studies on the expression and function of these genes, and their immunogenicity in leukemia are re-

quired. In the last decade, increasingly more CT antigens have been identified in hematological malignancies. The stem cell model of cancer suggests that certain cancer cells acquire stem cell–like characteristics via genetic mutations and epigenetic defects or fusion of circulating stem cells with cancer cells. Some CT antigens such as NRAGE (MAGED1, melanoma antigen family D, 1), NY-ESO (CTAG1B, cancer/testis antigen 1B), MAGE-1(melanoma antigen family A, 1), and SSX (synovial sarcoma, X) are expressed in human mesenchymal stem cells of the bone marrow, suggesting that the expression of CT antigens may be a hallmark not only of gametogenesis, but also of a stem cell marker. It has been suggested that the CT antigen expression in tumor tissues is restricted to cells that retain stem cell properties, favoring tumor maintenance, proliferation, and metastasis.[5]

CT antigens not only are expressed in hematological malignancies, but also induce B cell immunity in these patients. High-titer immunoglobulin G antibodies have been observed for many of them in different hematological malignancies.[6] We have analyzed expression of two testis-specific genes, *TEX101* (testis expressed 101) and *ODF4* (outer dense fiber of sperm tails 4), in CML samples at the mRNA and protein levels to find an appropriate candidate for detection of tumor cells as well as targets for new therapeutic approaches.

ODF4 is a testis-specific gene, expressed in the outer dense fibers of the tails of mature sperm. It was first isolated from a subtracted complementary DNA (cDNA) library of mouse testis.[7] Later, the human ortholog of ODF4 was cloned and its mRNA was expressed exclusively in the testis; the 30 kDa protein encoded by the mRNA was detected in the flagellae of ejaculated sperm, and its gene was mapped to chromosome 17.[8] Then the full-length cDNA of *ODF4* gene was isolated using 5' RACE and 3' RACE.[9] The *ODF4* expression was assessed in basal cell carcinoma and prostate adenocarcinoma, but it was not expressed in either of them.[10,11]

TEX101 gene encodes a glycophosphatidylinositol-anchored cell surface protein known as testis-expressed protein 101 or cell surface receptor NYD-SP8. Coimmunoprecipitation experiments showed that NYD-SP8 binds to urokinase plasminogen activator receptor/urokinase plasminogen activator complexes and may be involved in extracellular matrix proteins degradation.[12] Overexpression of *TEX101* reduces activities of the following three major classes of proteases known to be involved in extracellular matrix degradation: uPA, matrix metalloproteinases, and cathepsin B, which lead to suppression of both in vitro and in vivo cancer cell invasion and metastasis.[12] TEX101 mRNA has 4 alternative splicings, (http://www.ncbi.nlm.nih.gov/gene) and this mRNA has been shown to be expressed in basal cell carcinoma.[10]

We evaluated the expression of these two testis-specific genes in 20 normal healthy donors and 20 CML samples, and we screened the sera from CML patients for antibodies against TEX101 to estimate its capacity to induce humoral immune responses in vivo, which might be used as diagnostic or prognostic tools in CML.

PATIENTS AND METHODS

Blood samples were collected from 20 patients with confirmed CML diagnosis by molecular and cytogenetics methods and from 20 healthy people. All participants had given informed consent. The approval was obtained through the Investigation Review Board at Tehran University of Medical Sciences. This study was also approved by the Ethics and Clinical Studies Committee of Tehran University of Medical Sciences.

RNA Extraction, cDNA synthesis, and RT-PCR

Total RNA was isolated from leukocytes of patients using Tripure isolation reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions with minor modifications. The mRNA expression of these CT antigens was evaluated using reverse transcription (RT) and polymerase chain reaction (PCR). RT was carried out in each sample using random hexamer primers (Pharmacia, Sweden) and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) on 1 mg of total RNA. PCR reactions were performed using specific primers for *ODF4*, *TEX101*, and *HPRT1* for checking cDNA quality. The primers were:

ODF4-F: 5'-GCTTATCCTATACTTCACCT-GCG-3'
ODF4-R: 5'-GCCAGGAGTTCAGAAAA-GATTACAC-3'
TEX101-F: 5'-GGGAGTTCAGTGAGACCA-CAG-3'
TEX101-R: 5'-TGCCACCTCCAGTGATCT-CAAG-3'
HPRT-F: 5'-GCATTGTTTTGCCAGTGT-CAA-3'
HPRT-R: 5'-ATTGTAATGACCAGTCAA-CAGGG-3'

All primers were positioned in different exons of each gene to avoid false positive results caused by genomic DNA contamination of the RNA preparation. Primers for *TEX101* amplification were designed in a way that they could amplify all possible transcripts of this gene. PCR was performed using 30 amplification

cycles in a thermal cycler at annealing temperature of 60°C for *TEX101* and *HPRT1;* and 55°C for ODF4. Each set of PCR reactions contained a positive control of cDNA derived from normal testis and a negative control containing all of the PCR reaction mixture except for cDNA that was substituted with water. The integrity of RNA was checked in each PCR reaction by multiplex amplification for the *HPRT1* gene segment. PCR products were subjected to electrophoresis and run in a 1.8% agarose gel containing ethidium bromide, and then photographed under ultraviolet light. The specificity of primers was also confirmed by sequencing of PCR products using ABI Prism3130 Genetic Analyzer (Applied Biosystems, Foster City, CA).

TEX101 *cloning*

Open reading frame of *TEX101* gene was amplified using specific primers having BamH1 restriction site (F: CGGGATCCATGGGAACCCCTCGTATCCA, R: CGGGATCCAAGTGCCTTCTTAGGAAAAGTG). PCR was performed on cDNA of a normal testis sample. The PCR product was then inserted in BamH1 restriction sites of the Pmal-C2X vector. Competent cells from XL1-Blue *Escherichia coli* strain were transformed by a recombinant vector. Positive clones were selected by PCR using a specific primer for *TEX101* gene and a specific primer for the vector (M13_PUC_F: CCCAGTCACGACGTTGTAAAACG). The recombinant vector sequence was then confirmed by sequencing.

TEX101 *protein expression*

A stock culture of *E coli* expressing a TEX101 protein was streaked on selective agar plates containing antibiotics and incubated for 16 hours at 37°C. One colony was picked using a sterile wooden stick and inoculated in a test tube with 2 mL of Luria broth (LB) that contained antibiotics. The tube was placed in a 37°C shaking incubator and incubated for 8 hours. The culture was then transferred to a 2-mL microcentrifuge tube and centrifuged for 1 minute at 14 000g. A 2-mL volume of fresh LB with antibiotics was used to resuspend the cell pellet. This was then added to 50 mL of LB with antibiotics and placed in a 37°C shaking incubator until the optical density at 600 nm reached approximately 0.5.

The recombinant protein expression in bacterial cells was then induced by isopropyl-beta-thio galactopyranoside, and bacterial cells were cultured for 3 hours. A 12.5% polyacrylamide gel was prepared for SDS-PAGE analysis. A 5-µL aliquot of each elution was transferred to a fresh microcentrifuge tube, to which 3 µL of 3× loading dye was added. The samples were mixed, boiled for 2 minutes, and then loaded onto the polyacrylamide gel. Western blot analysis was done on the recombinant TEX101 protein as well as the testis tissue lysate, using mouse polyclonal antibody to TEX101 (ab69522) and rabbit polyclonal secondary antibody to mouse IgG-H&L (HRP) (ab6728).

Enzyme-linked immunosorbent assay

100 µL of 20 µg/mL elution of the recombinant TEX101 protein was coated on enzyme-linked immunosorbent assay (ELISA) wells. The ELISA experiment was done using 100 µL of patients' plasma samples. After 1 hour incubation in 37°C and 3 times washing, 1/2000 elution of rabbit polyclonal secondary antibody to human IgG-HandL (HRP), preadsorbed (ab7160) was added to wells. After 1 hour incubation in 37°C and 3 times washing, the reaction was visualized using 100 µL 3,3',5,5'-tetramethyl benzidine (US Biological, Marblehead, MA, USA).

RESULTS

Gene expression analysis

RT-PCR amplifications of cDNAs obtained from samples were performed using specific primers for mentioned genes. None of normal samples expressed *ODF4* or *TEX101*. Each specific band for *TEX101* and *ODF4* was seen in 6 of 20 (30%) patients' samples (**Figure 1**). Sequencing of PCR products confirmed specificity of primers (**Figure 2**). The size of the amplicon in CML patients was the same as in the case of testis (**Figure 1**), and the DNA sequence analysis confirmed no mutations. Available demographic data of patients showed no significant association with the gene expression pattern in sex, age, and other parameters. No overlap for *TEX101* and *ODF4* expression was observed in 20 CML samples.

TEX101 *cloning and recombinant protein production*

Colonies containing the favorable fragment were selected by PCR using a specific primer for *TEX101* gene and a specific primer for the vector, and the DNA sequence of the insert was assessed. PCR and sequencing results of selected colonies are shown in **Figures 3 and 4**, respectively. Colonies having the true inserted *TEX101* fragment were selected for further steps of recombinant protein induction. In addition, DNA sequencing of the inserted fragment using an upstream primer from the vector confirmed that the inserted fragment has no mutation. The result of induction of

the recombinant TEX101 protein production is shown in **Figure 5**. As an internal control and to compare the protein size of vector containing the insert with the intact vector, the Pmal-C2X vector was induced to produce protein (**Figure 5,** lane 2). Uninduced vectors also were run as negative controls (**Figure 5,** lanes 1 and 3). The size comparison of the induced recombinant protein with the protein size marker showed the predicted protein size for the TEX101 recombinant protein.

We tested the presence of circulating anti-TEX101 antibody using the purified recombinant TEX101 protein in the ELISA system in sera of CML patients. The optical density assessment of samples at 450 nm showed no significant difference between patients and normal samples, so we concluded that the humoral response against TEX101 was not detected by ELISA in our CML patients. As ELISA results for patient samples were not positive, further Western blot analysis was not done on samples.

DISCUSSION

CML is caused by a t(9;22)(q34;q11) chromosomal translocation and expression of the Bcr-Abl fusion protein. Enhanced, unregulated tyrosine kinase (TK) activity of the Bcr-Abl is causative for CML.[13] Bcr-Abl fusion protein is one of the most studied CML antigens as a potential target for immunotherapy, but attempts to develop immunotherapy approaches against this protein have failed. Therefore, identifying novel tumor antigens that can be targeted to develop immunotherapy against CML is urgently needed.[14]

A highly restricted expression of CT antigens in normal tissues and a broad expression in a wide range of different tumor types have made CT antigens attractive targets for immunotherapy. Earlier we have demonstrated the diagnostic and therapeutic significance of TEX101 in prostate cancer and basal cell carcinoma.[10,11] As CT antigens are known for their ability to cause spontaneous humoral and cellular immune responses in vivo, they are considered targets for active immunotherapy. The ELISA methodology can be used for screening of patients' sera for the presence of antibodies against CT antigens and for the identification of patients having high-titer serological responses to these antigens. In immunotherapeutic approaches, first it is necessary to identify patients who will benefit from a specific treatment. The differential expression of molecules involved in the sensitivity of the tumor cells to a specific therapy might explain the varying responses of patients to different therapeutic approaches such as immunotherapy, and might provide important tools for investigating this aspect in the clinic. In this study,

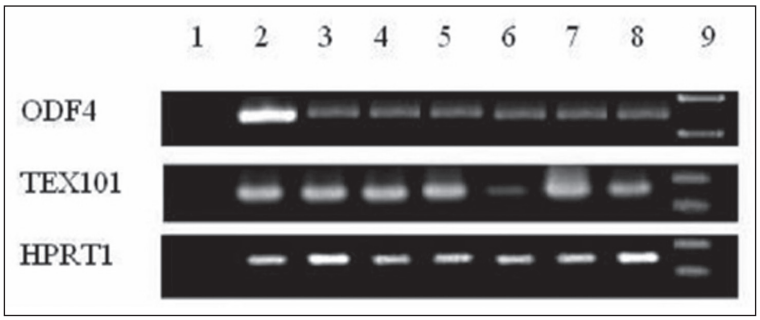

Figure 1. RT-PCR results for *ODF4, TEX101,* and *HPRT1.* Lane 1: negative control; lane 2: positive control; lanes 3-8: different positive samples; lane 9: 100 bp DNA marker.

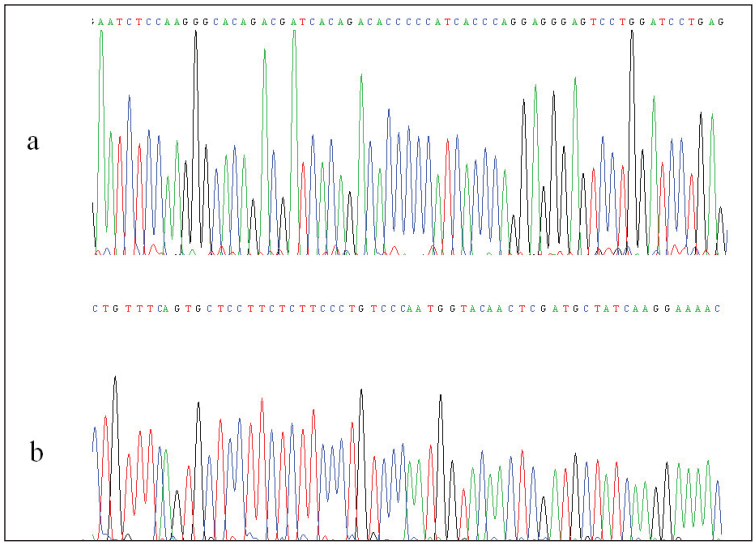

Figure 2. Sequencing results. a: *ODF4*, b: *TEX101*

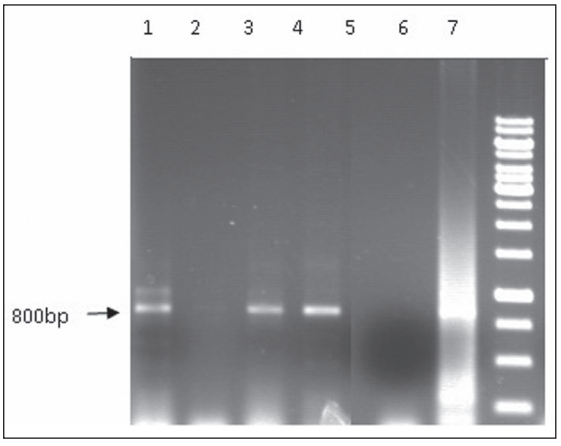

Figure 3. Colony PCR results: colonies 1, 3, and 4 show specific bands of *TEX101.* Lane 7: positive control; lane 8: 100 bp DNA marker.

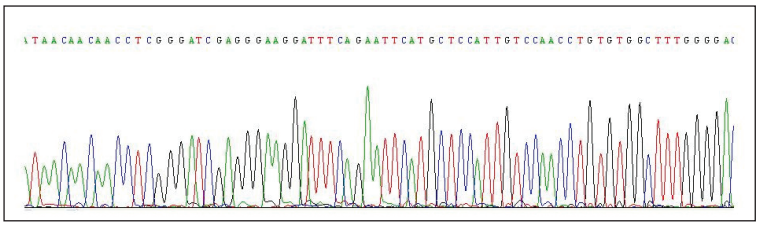

Figure 4. Sequencing result of a selected recombinant colony.

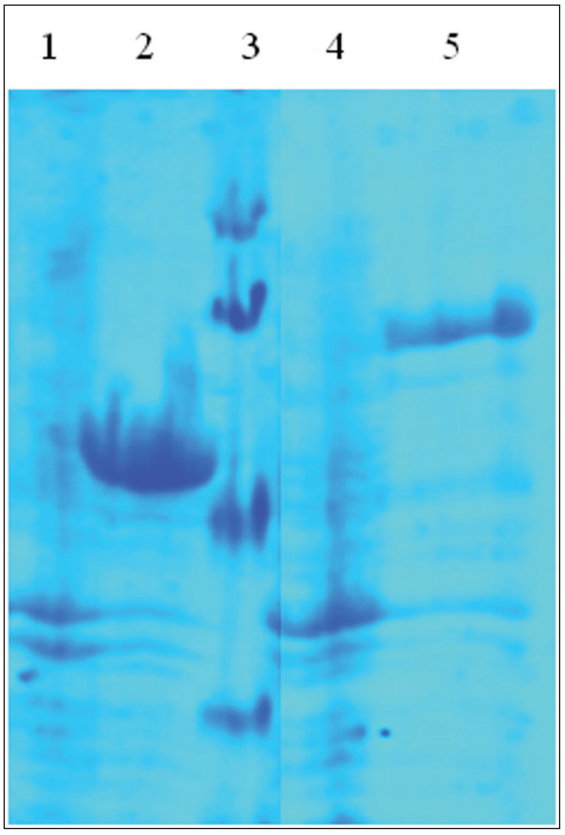

Figure 5. Recombinant protein induction. Lanes 1 and 2: uninduced and induced bacteria having intact Pmal-C2x vector, respectively; lane 3: protein size marker; lanes 4 and 5: uninduced and induced bacteria having recombinant fragment, respectively.

we reported the expression of *TEX101* and another testis-specific gene named *ODF4* in CML patients and searched for the presence of specific antibodies against TEX101 in patients' sera. Although TEX101 mRNA was found in 30% of patients, none of them had antibodies against it. The absence of humoral immune responses against the majority of CT antigens might be explained by their low expression, but the cause of inability of TEX101 to induce immune responses in CML patients is unclear. However, in our study, the lack of detection of anti-TEX101 humoral response in patients may be because of insufficient sample size, low level of protein expression, and the fact that most of studied patients were in the initial phases of their disease.

In a study, it has been shown that 90% of CML patients have a humoral response against SPAG9, a known CT antigen.[14] In contrast, in some other studies, the humoral response against some CT antigens was not detected in spite of high mRNA expression.[15] So the lack of antibody response observation in this study was not an unpredictable event. However, further studies with greater sample size are recommended.

A relationship between some CT antigen expression and higher tumor grades has been shown in transitional cell carcinomas of the urinary bladder,[16] in certain types of brain tumors,[17] and in endometrial cancer.[18] It has been suggested that overexpression of *TEX101* suppresses cancer cell invasion and metastasis;[12] therefore, in contrast with most CT antigens, an aberrant expression of *TEX101* in CML patients should be correlated with a better prognosis and reduced metastasis and needs to be assessed in further studies. Although in most patients, the immunologic response against CT antigens is considered a good prognostic factor, in the case of *TEX101*, the function of overexpressed antigen may make this issue different. The lack of immunologic response in these patients despite the expression of TEX101 at the mRNA level, may be a good prognostic factor and needs to be investigated in future studies.

The TEX101 protein binds to the urokinase plasminogen activator receptor (UPAR) and reduces the activity of uPA. In addition, interactions of UPAR in cells with a variety of signal transduction molecules including TKs have been described.[19] So if the TEX101 protein is expressed in CML, it may indirectly have an interaction with the Bcr-Abl fusion protein.

The primary regulator of CT gene expression in normal and cancer cells is an epigenetic mechanism, particularly DNA methylation. It was shown that demethylation agent 5-aza-2'-deoxycytidine can induce MAGE-A1 transcription in cell cultures, and the site-specific hypomethylation of MAGE-A1 in tumor cells depends on demethylation and then a persistent local inhibition of remethylation.[20] It is quite likely that overexpression of *TEX101* and *ODF4* in CML patients may be because of hypomethylation of these genes and warrants further investigation. A combinatorial therapeutic approach involving epigenetic modulatory drugs and CT antigen immunotherapy is suggested for many types of cancers.[21] In the case of *TEX101*, it seems that therapeutic approaches should

focus on its overexpression to reduce invasion and metastasis in cancers. *TEX101* is one of few CT genes reported to be expressed at the surface of cells; therefore, it is a suitable potential target of molecular manipulations to eradicate disease. Furthermore, the expression of *TEX101* in different types of tumors may imply a potential role for it in the molecular mechanism of carcinogenesis or it may be simply a by-product of hypomethylation in cancer. In future studies, the TEX101 expression should be assessed in different tumor types regarding its correlation with tumor grade and stage. Briefly, the therapeutic strategy regarding *TEX101* can be to enhance its expression by epigenetic tools. This strategy can both be protective against metastasis and trigger an immune response because of its restricted expression in testis. As discussed before, because of existence of blood-testis barrier, if testis-specific genes are expressed in other tissues at a detectable level for the immune system, they can elicit immune responses. If future studies reveal the expression of *TEX101* at the protein level and the presence of humoral responses against this gene in cancers, it can be more suitable target for immunotherapy. However, the expression pattern of *ODF4* in testis and CML makes this gene appropriate for immunotherapeutic approaches as well as detection of cancer.

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