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Review Article

Cancer-Testis Antigens: Potential Targets for Cancer Immunotherapy

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Cancer-testis antigens are tumor antigens that their expression is almost limited to male germ cells in the testis. Some of cancer-testis antigens are also expressed in the ovary and in trophoblasts. Recently their expression has been seen in different types of tumors.

Many pathophysiologic studies suggest that a blood-testis barrier exists in the testis. Because spermatogenesis begins at puberty, new cell-surface antigens are expressed when the immune system has refined the ability to distinguish self from nonself. So, sperms in the testis do not stimulate immune responses. In addition, although antigen-presenting cells are commonly seen in the interstitial spaces of the testis, these cells are scarcely seen within the seminiferous tubules. So, testis is considered as an immune-privileged site, and testis-specific genes, if expressed in cancers can be immunogenic. For this reason cancer-testis antigens are promising candidates for cancer immunotherapy and have become a major focus for the development of vaccine-based clinical trials in recent years. In addition, these antigens can also be used as biomarkers for early detection of cancers.

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Introduction

Cancer-testis antigens are a group of tumor antigens, expressed in normal testis and different types of tumors. As their name implies, their expression is seen in germ cells of the testis but sometimes they are expressed in female reproductive organs and trophoblasts.[1–3] Immature germ cells of fetal ovary (oogonia and primary oocytes) express cancer-testis antigens but their expression has not been seen in oocytes in the resting primordial follicles. Cytotrophoblast and syncytiotrophoblast of the placenta express some cancer-testis antigens.[4] Expression of these antigens in the placenta is different from other antigens; it means that some of them are not expressed in the placenta but some are highly expressed, and their expression is not completely paralleled with their presence in the fetal germ cells.[5]

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Because some characteristics of malignant tissues, such as invasiveness, destructiveness, and metastatic features, are shared with trophoblastic cells, gene expression profile in the placenta can be similar to cancer. Some cancer-testis antigens can be expressed in nongametogenic tissues such as the pancreas, liver, and spleen at levels much less than germ cells.[6] In addition, it was recently reported that some cancer-testis antigens such as N-RAGE, NY-ESO, MAGE, and SSX are expressed in both adult and fetal human mesenchymal stem cells of the bone marrow but after differentiation of osteocytes and adipocytes, their expression is down-regulated.[7] It has been suggested that expression of cancer-testis antigen in addition to be a special characteristic of gametogenesis can be a stem cell marker. This restricted expression of these antigens in undifferentiated somatic and germ cells is suggestive of their essential role in embryonic development.[8] Cancer-testis antigens are considered as promising target molecules for cancer vaccines because of their highly tissue restricted expression.[3,6,9,10]

Until now at least 70 families of cancer-testis gene with 140 members have been attributed to

this group and their expression has been studied in different types of tumors.[11] Some of them are proved to be immunogenic.[6] Attributing genes to this gene group is based on some characteristics: 1) mRNA expression in normal tissues is almost limited to testis, fetal ovary, and placenta, 2) mRNA expression in different cancers. We have assessed expression of cancer-testis antigens in normal tissues by the means of digital differential display.[12] Those with more limited expression in normal tissues are mentioned in Tables 1 and 2.

Classification of cancer-testis antigens

About 50% of cancer-testis genes, including those which have been used in cancer immunotherapy, are located on X chromosome.[11] These cancer-testis-X genes usually form gene families connected to inverted DNA repeats. Study of the sequence of the human X chromosome has shown that about 10% of all genes of X chromosome are attributed to cancer-testis gene family.[37] In normal testis the cancer-testis-X genes are generally expressed in the spermatogonia, which are proliferating germ cells.[3]

Expression of cancer-testis-X antigens is different in different types of tumors. The highest expression frequency of them has been seen in bladder cancer, lung cancer, ovarian cancer, hepatocellular carcinoma, and melanoma. Cancer-testis-X genes are usually expressed in parallel, and tumors that express them tend to express several cancer-testis-X antigens. For example, in a study, it was revealed that 40% of breast tumors and 65% of melanomas expressed three or more cancer-testis-X antigens.[38]

On the other hand, the genes for non-X cancer-testis genes are distributed throughout the genome and do not generally form gene families and are not located within genomic repeats. In the testis, they are expressed more dominantly in later stages of germ cell differentiation, such as in spermatogonia.[3] Because these two groups of cancer-testis antigens are expressed during different stages of spermatogenesis, their function seems to be different.

Identification of cancer-testis antigens

Several strategies have been used to identify cancer-testis antigens.

T cell epitope cloning

Many antigens recognized by $CD8^+$ T cells have been discovered by transducing cDNA libraries constructed from tumor cells into target cells, which express the suitable HLA molecule, and then using antitumor T cells isolated from tumor infiltrates to discover the antigen epitopes presented by HLA on the surface target cells.[39] This approach was first used by Bruggen et al.,[40] and the first cloned antigen by this technique was the melanoma antigen MAGE-1. Other new tumor antigens including B melanoma antigen (BAGE) and G antigen (GAGE) gene family were identified by this strategy.[41,42]

Serological analysis of cDNA expression libraries (SEREX)

Both cellular and humoral immune systems participate in recognition of tumor antigens. So, existence of tumor-associated antibodies in blood indicates a significant host-tumor interaction. A new method was developed by Sahin and his colleagues who used antibody repertoire of patients with cancer for identification of antigens.[43] Using this method, antibody response can be detected. In this approach, a cDNA expression library is constructed from a fresh tumor specimen and cloned into phage expression vectors. Then, *E.coli* cells are transduced by these recombinant phages. Recombinant proteins expressed by bacteria are incubated with serum from the autologous patient. Clones reactive with high-titer antibodies are distinguished and nucleotide sequence of cDNA insert will be identified.[44] This technique was applied to identify cancer-testis antigens NY-ESO-1,[45] CT7/MAGE-C1,[46] SCP-1,[47] OY-TES-1,[48] HOM-TES-85,[49] CAGE,[50] cTAGE,[51] and NY-SAR-35.[52] But the clinical significance of these antitumor antibodies is unknown; so, the antigens recognized by these antibodies should be screened for T cells recognition by reverse T-cell immunology. In order to do this, antigen-presenting cells should be either loaded with selected major histocompatibility complex (MHC) class I binding peptides or transduced by cDNA of the antigen.[2]

Differential gene expression analysis

Differential display is a powerful tool for the comparison of gene expression between two or more mRNA populations. The first parts of this technique are PCR and denaturing polyacrylamide gel electrophoresis to provide DNA fingerprints of tissues. RNAs extracted from the sources to be compared are reverse transcribed with one of a possible set of four degenerate oligonucleotide

primers $(dT)_{12}VC$, $(dT)_{12}VA$, $(dT)_{12}VG$, or $(dT)_{12}VT$ where V is C, A, or G. First-strand cDNA is used as a template in the PCR with oligo(dT) primer mixture and a decamer sequence that has been randomly generated. The complex mixture of cDNAs are then separated by

Table 1. Cancer-testis antigens of X chromosome expressed in less than five normal tissues.

CT antigen	Chromosome location	Expression in normal tissues rather than testis	Expression in cancer according to digital differential display	Expression in cancer tissues	Function	References
HOM-TES-85(LUZP4)	Xq23	None	None	Hepatocellular carcinoma, lung cancer, ovarian cancer, melanoma, glioma, bladder cancer, breast cancer	Lucine zipper protein(possible transcriptional regulatory protein)	13 – 17
NY-ESO-1 (CTAG1B)	Xq28	Placenta, bone	Chondrosarcoma	Brain tumors, melanoma, ovarian cancer, nonsmall cell lung carcinoma, breast cancer, hepatocellular carcinoma, esophageal carcinoma	Unknown	18 – 24
FATE1	Xq28	Adrenal gland, placenta, brain	Adrenal tumor, germ cell tumor	Hepatocellular carcinoma	Unknown	25,26
MAGEB1	Xp21	Salivary gland, skin, brain, spinal cord	Skin tumor	Hepatocellular carcinoma, esophageal squamous cell carcinoma	Unknown	27,28
SAGE1	Xq26	Bone marrow, brain	Germ cell tumor, leukemia	Head and neck squamous cell carcinoma, sarcomas	Unknown	29
SPANXA1	Xq27	Bone marrow, liver	Germ cell tumor, leukemia, liver tumor	Melanoma	Association with nuclear envelope of human spermatids and spermatozoa	30
SPANXB2	Xq27	Connective tissue	Soft tissue/ muscle tissue tumor	Melanoma	Association with nuclear envelope of human spermatids and spermatozoa	30
SPANXC	Xq27	None	None	Melanoma	Association with nuclear envelope of human spermatids and spermatozoa	30
SPANXD	Xq27	Connective tissue, skin, liver	Soft tissue/ muscle tissue tumor, liver tumor, skin tumor	Melanoma	Association with nuclear envelope of human spermatids and spermatozoa	30
TAF7L	Xq22	Eye, lung, liver	Germ cell tumor, retinoblastoma	Head and neck squamous cell carcinoma	Transcription factor	31
TFDP3	Xq26	Skin	Skin tumor		Transcription factor	
NXF2	Xq22	Larynx, lung, placenta, skin	Germ cell tumor, skin tumor, head and neck tumor	Lung carcinoma, bladder carcinoma, sarcoma	Nuclear RNA export, association with nuclear envelop	32
PASD1	Xq28	Bone	Chondrosarcoma	Myeloma, B cell lymphoma	Sensors for light and oxygen in signal transduction	33,34
PAGE5 (GAGEE1)	Xp11	Liver, placenta, skin	Germ cell tumor, leukemia, skin tumor	None	Unknown	12

Table 2. Cancer-testis antigens of non-X chromosomes expressed in less than five normal tissues.

CT antigen	Chromosome location	Expression in normal tissues rather than testis	Expression in cancer according to digital differential display	Expression in cancer tissues	Function	References
PRAMEF2	1p36	Brain	Primitive neuroectodermal tumor	None	Unknown	12
BRDT	1p22	Brain, mouth, muscle, prostate	Germ cell tumor, head and neck tumor	Lung cancer	Possible transcriptional regulatory protein	35
SPO11	20q13	Brain, connective tissue	Soft tissue/ muscle tissue tumor		Formation of double-strand breaks in paired chromosome homologues	12
SYCP1	1p13	Connective tissue	Germ cell tumor, soft tissue/ muscle tissue tumor	Brain tumor	Major component of synaptonemal complexes	20
TPTE	21p11	None	Germ cell tumor, adrenal tumor	Hepatocellular carcinoma	Phosphatase and tensin homolog (PTEN)-related tyrosine phosphatase	26
ADAM2	8p11	Brain, connective tissue, prostate	Soft tissue/ muscle tissue tumor, prostate cancer	Multiple myeloma	Membrane-anchored protein structurally related to snake venom disintegrins, cell-cell and cell-matrix interactions	36

electrophoresis by a denaturing polyacrylamide gel.[53] Cancer-testis genes can be efficiently identified through comparison of testis and cancer tissue libraries.

Massively parallel signature sequencing (MPSS)

In this approach millions of short sequence tags associated to transcript from different RNA preparations are generated and MPSS data of normal testis and different cancer tissues are compared. Using this approach a new cancer-testis gene called CT45 was found which is frequently expressed in lung cancer.[54]

DNA microarrays

Microarrays are miniature devices having thousands of DNA sequences as gene-specific probes, immobilized on a solid support (nylon, glass, silicon). cDNA targets labeled with a radioactive, fluorescent, or chemiluminescent tag are hybridized with sequences on array, and the intensity of the signal generated by each bound probe indicates the relative abundance of that transcript in the sample.[55] Using this technology, it is possible to compare gene pool of tumor samples with DNA sequences derived from testis-specific genes. It has been applied to identify a new cancer-testis antigen named STK31 in colorectal cancer.[56]

Tissue microarray

Tissue microarray technology is a powerful tool for simultaneous analysis of hundreds of tissue specimens in a single experiment. A tissue microarray is constructed by taking core biopsies of paraffin-embedded tissues and re-embedding them on a single arrayed ‘‘master block’’. Tissue microarrays are dependent on a variety of techniques such as immunohistochemistry for protein expression and fluorescence in situ hybridization (FISH) to detect DNA alterations. Tissue microarrays have the advantage of examining a single gene product per experiment in a large number of samples.[55] So, it is possible to examine expression of a single testis gene in various tumor samples.

Serial analysis of gene expression (SAGE)

Serial analysis of gene expression (SAGE) is a method that has the ability to quantitate and compare large numbers of transcripts. Only a portion of the cDNA transcript, which is known as a SAGE tag, is needed to analyze the expression profile of each particular tissue. At first concatemers (DNA segments composed of repeated sequences linked end to end) of SAGE tags are made; then, up to 30 tags will be sequenced at once. The frequency of each tag in the concatenated sequence shows the abundance of the corresponding transcripts in that cell. So, expression levels of a sequence can be compared between two populations. SAGE libraries can be used to analyze the differences in gene expression between cells or tissues.[57]

Function of cancer-testis antigens

Although cancer-testis antigens are on the center of attention because of their probable usage

as tumor vaccines, their biologic function in both germ line and tumors is not well understood. Some of them, especially the members of MAGE family, may have a critical role in the process of tumorigenesis. Their putative function can be categorized in eight groups.

- Structural components of spermatozoa such as TSGA10.[58–62]
- Possible role in transcription regulation such as MAGE-A,[40] SSX,[63] HOM-TES-85,[50] E2F-like/HCA661,[64] TAF7L,[65] BRDT,[35] PLU-1,[66] BORIS,[67] NXF2.[68]
- Possible role in signal transduction such as LIP1,[69] SGY1,[70] MAGE.[71]
- Helicase-like features such as CAGE,[71] HAGE.[72]
- Cell to cell binding such as SPA17,[73] TPX1,[74] ADAM2.[69]
- Enzymatic actions such as ADAM2, [69] LIP1, [69] TSP50,[75] LDHC,[76] TPTE.[51]
- Probable role in inhibition of apoptosis such as CAGE.[77]
- Components of synaptonemal complex such as SCP1,[78] SPO11.[79]

The information about the function of cancer-testis antigens is incomplete, but it seems that most of these antigens may have a putative role in transcriptional regulation. Their products can also affect many different cellular processes, such as signaling, translation, and chromosomal recombination. Most of this information is derived from the study of adult male germ cells, but there are new coming data from their expression profiles in female gametes and embryonic tissues.

A critical question about cancer-testis antigens is whether their expression has a fundamental role in tumorigenesis or they are produced after cellular transformation without any relation to this process. There is strong evidence at least for some of them which show that they have a basic role in tumorigenesis. For instance, recent data indicate that expression of MAGE genes in cancer cells is related to the malignant phenotype and response to treatment. It was found that cell lines that expressing at least one of the three MAGE genes were more resistant to TNF-mediated cytotoxicity.[80] Transfection of cells with MAGEA2 or MAGEA6 genes also gives them a proliferative advantage, although the molecular mechanism is not clear. [81]

Another study shows that CAGE, a novel cancer- testis antigen, promotes motility of cancer cells through activation of focal adhesion kinase. Overexpression of it also promotes motility of several cell lines, whereas down- regulation of it by antisense CAGE cDNA, has a prominent effect in decreasing cell motility; so, it can have a role in metastasis. [82]

There is another report indicating that members of CAGE family when transfected into HeLa cells, make them resistant to apoptosis induced by either interferon-δ or by the death receptor FAS (TNF receptor superfamily, member 6).[77]

TSGA10 is a new cancer-testis gene whose function is somehow identified. Mouse homologue of TSGA10 mRNA, firstly detected in postmeiotic phase of spermatogenesis, is processed to a major fibrous sheath protein of sperm tail,[59] and has mitotic arrest deficient domain. Mitotic arrest deficient is a mitotic checkpoint protein. The mitotic spindle checkpoint monitors proper attachment of the bipolar spindle to the kinetochores of aligned sister chromatids. Recently, a protein–protein interaction between hypoxia inducible factor 1 (HIF-1), a transcriptional regulator of genes involved in oxygen homeostasis, and the TSGA10 was identified by yeast two- hybrid screening.[83] Recent models suggest that TSGA10 which is a fibrous sheath protein, after processing can also serve as scaffolds for protein complexes involved in regulating signal transduction and cell division processes.[59]

In another experiment it was suggested that SSX has a functional role in cell migration and a potentially similar function in cancer cell metastasis. It has been revealed that when SSX is down-regulated in a melanoma cell line expressing SSX, the migration of cells will decrease.[7]

Because many of the important features of cancer cells such as migration, invasion, immune subversion, apoptosis resistance, and induction of angiogenesis are also seen in gametogenesis or placentation processes, it is possible that cancer-testis antigen products controlling gametogenesis process give similar characteristics to cancer cells.[84]

Some authors believe that cancer-testis antigens play a part at earlier stages during embryonic development and in stem cell self-renewal. They suggest that expression of these antigens in tumor tissues is restricted to cells that maintain stem cell properties. Cancer-testis antigens may be true

hallmarks of cancer stem cells and can be considered as targets for interference in recurrence and metastatic processes. Cancer cells in which cancer-testis antigens are expressed may have lost their ability to differentiate. So, drugs developed to specifically target cancer-testis antigens, could be used to improve the treatment of cancer.[8]

Additional studies on expression of cancer testis-antigen and their molecular interactions in testis and tumors are needed to achieve a comprehensive knowledge about their function in tumorigenesis. Results of these studies may be useful in developing antitumor strategies such as immunotherapy.

Regulation of cancer-testis antigens expression

As mentioned before, expression of cancer-testis antigens is almost restricted to male germ cells in the testis and various malignancies. An important question regarding their expression is about the mechanism of their transcriptional silencing in normal tissues except testis and their derepression in malignancies. It is accepted that regulation of methylation has an important role in the control of their expression.[2,85] For example, several studies on cancer-testis antigens especially MAGE-A1 have shown that DNA methylation is the primary silencing mechanism for these genes and demethylation is necessary and sufficient to produce expression. It was also shown that heavy methylation represses gene expression in cells, despite the existence of transcription factors required for expression, and demethylation agent 5-aza-2'-deoxycytidine can induce MAGE-A1 transcription in cell cultures.[86] In another study, it was shown that the site specific hypomethylation of MAGE-A1 in tumor cells depends on demethylation and then persistent local inhibition of remethylation.[87]

Multiple sequence alignment results and comparison of the 5' flanking regions of the mouse and human TSGA10 genes indicate that the homologue of the first exon of the mouse gene is located at 8.3 kb upstream of human exon 1. This result may indicate TSGA10 genes use different exon 1 sequences and different promoters[59]; so, different mechanisms may act in different animals. The presence of an alternative promoter in human and pig TSGA10 genes compared with mouse and rat genes still needs to be investigated.

The mechanism of epigenetic regulation is somehow clear for some genes. For instance, recent data indicate that reciprocal binding of CCCTC-binding factor (zinc finger protein, CTCF) and CCCTC-binding factor like (BORIS) to the NY-ESO-1 promoter mediates epigenetic regulation of this cancer-testis antigen in lung cancer cells, and suggest that induction of BORIS may be a novel strategy to enhance immunogenicity of pulmonary carcinomas.[88]

It has also been shown that intratumoral heterogeneity of expression of cancer-testis antigens in melanoma is regulated by methylation and using the demethylation agent 5-aza-2'-deoxycytidine they could induce expression of several cancer-testis antigens.[89]

Immunogenicity of cancer-testis antigens

Many pathophysiologic research suggest that a blood-testis barrier exists in testis. Because spermatogenesis begins at puberty, new cell-surface antigens are expressed when the immune system has refined the ability to distinguish self from nonself. So, sperms in the testis do not stimulate immune responses. In addition, although antigen-presenting cells are commonly seen in the interstitial spaces of the testis, these cells are scarcely seen within the seminiferous tubules. So, testis is considered as an immune-privileged site.[90] The mechanical barrier is made by tight junctions between Sertoli cells along the basolateral aspect and between capillary endothelial cells.[91–93] The apparent lack of human leukocyte antigen (HLA) class I expression on the surface of germ cells is also important in making the testis as an immune privileged site.[94] For these reasons cancer-testis antigens are promising targets for immunotherapy.

Humoral responses to cancer-testis antigens have been seen in several tumors, for instance antibodies against SCP-1 in pancreatic cancer,[95] antibodies against NY-ESO-1, SCP-1, and SSX-2 in breast cancer,[96] antibodies against CTSP-1 in prostate, thyroid, and breast tumors,[9] antibodies against TSGA10 in hepatocellular carcinoma and malignant melanoma,[97] and antibodies against MAGEA3, SSX2, and NY-ESO-1 in multiple myeloma,[98] have been detected.

Cancer-testis antigens are also immunogenic to cytotoxic T lymphocytes. For instance, Sp17 specific HLA-A1 and B27 restricted cytotoxic T lymphocytes generated from peripheral blood of a healthy donor were able to kill HLA-matched myeloma cells.[99]

Ability of cancer-testis antigens to elicit cellular and humoral responses has led directly to the development of antigen-specific cancer vaccines.

Over 34 trials with different NY-ESO-1 vaccine formulations have been performed. NY-ESO-1 peptide, protein, and pox-NY-ESO-1 vaccines can all induce strong NY-ESO-1 humoral and cellular responses in patients with no pre-existing NY-ESO-1 immunity. The NY-ESO-1 Protein/ISCOMATRIX® trial conducted by Jonathan Cebon had some hopeful results and a Phase II randomized trial is now ongoing.[99] The salmonella/NY-ESO-1 vaccine, which has had considerable therapeutic effects in mice, is now being prepared for the clinic and NY-ESO-1 adenovirus constructs for vaccination will be developed in near future.[101] Although the field of antigen-specific cancer vaccine is still in its early steps, it is anticipated that cancer-testis antigens will be at the center of attention for immunotherapy in future.

Future aspect of cancer-testis antigens

The findings presented above indicate that expression of cancer-testis antigen often shows marked specificity for tumor cells. These markers can be used to target tumor cells for early detection and target specific gene-therapy or treatment of cancer. In addition, the immune-privilege of testis and concept of testis-specific genes, which are expressed in various cancers, can provide the lead for further development of tumor vaccines. Active immunotherapy is still in preclinical and clinical trial phase of development but it will become available in the clinics in near future. The growing knowledge in cancer-testis antigens and their ability to elicit cellular and humoral responses will provide new tools for active immunotherapy of patients.

Finally, as many changes in tumoral cells are caused by post-translational modifications which are not detected by DNA/RNA analyses and proteomics-based studies of many tumor types are now underway,[102] modifications of cancer testis antigens at protein level in tumoral cells can be detected and compared with normal cells to find new biomarkers for cancers.

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Cancer/Testis Antigens (CTAs) are a promising class of tumor antigens that have a limited expression in somatic tissues (testis, ovary, fetal, and placental cells). Aberrant expression of CTAs in cancer cells may lead to abnormal chromosome segregation and aneuploidy.Â Thus, all CTAs are in principle attractive targets for immunotherapy in cancer because the gonads are immune privileged organs and anti-CTA immune response can be tumor-specific. Vaccines using peptides derived from NY-ESO-1 (CTAG-1B) have shown clinical benefits in patients with melanoma [26, 27]. 2. CTAs Expression in Multiple Myeloma. Oncogenic cancer/testis immunotherapy. antigens: prime.Â Moreover, immune targeting of oncogenic cancer/testis antigens in combination with conventional cytotoxic therapies or novel immunotherapies such as checkpoint blockade or adoptive transfer, represents a highly synergistic approach with the potential to improve patient survival. INTRODUCTION. immune surveillance of cancer by virtue of their ability to detect quantitative and qualitative differences of presented antigens on transformed cells. Indeed, carcinogenic alterations result in an altered protein repertoire. Among the different types of tumor antigens, cancer/testis (CT) antigens represe