Differential expression of MAGE genes in Neuroblastoma cell lines and a possible regulatory mechanism

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Abstract

The human MAGE gene family is characterized by a conserved domain. A subset of MAGE genes (MAGE-A, -B, -C) encode tumor-associated antigens, which can be recognized by CTL in conjunction with MHC class I molecules on the tumor cell surface. Many studies have demonstrated the overexpression of MAGE-A genes in various tumor types; however, their biological function and expression pattern are still largely unknown. Here we exam the expression of MAGE-A2,-A3, -A12 and MAGE-D4 in six neuroblastoma cell lines, and find coordinative expression between MAGE-A2, -A3, and –A12, but not MAGE-D4. We also find that the CSAG1 gene, which is located proximate to the MAGE-A genes, has a similar expression pattern. In conclusion, our data show that the regulation of MAGE-A gene expression could be due to promoter demethylation or histone acetylation.

Introduction

Human MAGE family

The first member of the human MAGE family was identified as a gene encoding a tumor-specific antigen in melanoma cells(van der Bruggen et al., 2007). Since then, the MAGE gene family has extensively increased in complexity with the conservation of the typical signature domain (Mage Homology Domain; MHD) (Monte et al., 2005). The MAGE family has been divided in two big subfamilies: type-I and type-II MAGE family. Type-I MAGE family includes MAGE-A, B, and C, which are located as clusters on the X chromosome. They are completely silent in normal tissues, with the exception of male germ cells and placenta (Chomez. et al., 2001). Some of them are expressed in tumor cells of various histological types, where they code for antigens recognized by cytolytic T lymphocytes (CTL) in a major histocompatibility complex (MHC)-restricted way. Type-II MAGE family includes genes which encode different proteins containing MHD with undefined chromosome clustering. These genes differ from the type-I MAGE family by their genomic structure: they do not encode the antigenic peptide encoded by type-I MAGE and therefore will not trigger an immune response (Chomez. et al., 2001). This characteristic also gives them a different expression pattern compared to type-I MAGE: they are expressed in all normal tissues.

The functions of various MAGE proteins remain to be elucidated. Recent studies suggest some necdin-related type-II MAGE genes are involved in cell cycle arresting and neuronal apoptosis through interaction with E2F1 (Kuwako, Taniura &

Yoshikawa, 2004). In addition, several MAGE-A genes, such as MAGE-A1, -A2, -A4, and –A11 are involved in transcription regulation through specific binding to transcription complexes (Bai, He & Wilson, 2005; Laduron et al., 2004; Monte et al., 2005; Sakurai et al., 2004). The broad range of expression of type-I MAGE in various tumor types (e.g., melanoma, lung, breast, bladder gastric carcinoma, and neuroblastoma (Inoue et al., 1995; Ishida et al., 1996; Kufer et al., 2002; Otte et al., 2001)) makes them possible targets for anticancer immunotherapy. However, their heterogeneous expression leads to loss of recognition of tumor cells and their subsequent destruction by CTL (Kufer et al., 2002).

Control of type-I MAGE expression

The expression of type-I MAGE in tumors is known to be activated by promoter demethylation, as well as histone acetylation (Honda et al., 2004; Wischnewski, Pantel & Schwarzenbach, 2006). Global DNA demethylation has been observed in carcinomas of the breast, liver, colon, and neuron, and is considered to occur in the early stages of tumor development. MAGE gene expression is epigenetically repressed by promoter region methylation in most cells and CD117 receptor tyrosine kinase activation may allow MAGE gene expression by suppressing the methylation of MAGE promoter regions in addition to global demethylation (Yang et al., 2007).

MAGE-A expression in neuroblastoma cells

Neuroblastoma (NB) is a neuroectodermal tumor that affects children and is the most common cancer in infancy. NB cells have been classified into three major cell types: I-type (intermediate), N-type (neuroblastic), and S-type (substrate-adherent).

I-type cells are the malignant NB stem cells and express features of both N cells and S cells(Ross & Spengler, 2007).

Past studies showed that NB cells express MAGE-A genes, but lack constitutive expression of co-stimulatory molecules and surface HLA class I and II molecules. As a result, NB cells are likely to be ignored by CTL, since expression of HLA and costimulatory molecules on antigen presenting cells are an essential condition for efficient peptide presentation to CTL and for the subsequent activation and clonal expansion of the latter cells (Prigione et al., 2004). The expression of MAGE-A genes in NB cells is unrelated to disease staging or histology. However, study in mouse embryonic stem cells (mES) showed mES can induce demethylation of MAGE-A1 (Loriot et al., 2007). This leads us to the assumption that the expression level of type-I MAGE genes may increased in I-type NB cells.

In this study, we focused on the expression pattern of both type-I and type-II MAGE genes in different NB cell lines. Result showed the differential expression patterns of MAGE A2, A3, and A12 (type-I MAGE) among the six different cell lines are very similar to each other, but D4 (type-II MAGE) is expressed equally in all six cell lines. This expression pattern is independent of cell types. To confirm the coexpression between MAGE-A genes is due to promoter demethylation or histone acetylation, I further investigated the expression pattern of the CSAG1 gene in these six NB cell lines. CSAG1 is located at Xq28 and is proximate to the MAGE-A genes, but is not related to the MAGE family. Result showed the expression of the CSAG1 gene in the six different cell lines is similar to that of MAGE A2, A3, and A12.

Materials and Methods

Neuroblastoma cell lines

IMR-32, SH-SY5Y, BE(2)-C, CB-JMN, LA1-5S, SH-EP1 cell lines were kindly provided by the Laboratory of Neurobiology, Department of Biological Sciences, Fordham University. The six cell lines were cultured in a mixture of 45% Eagle's Minimum Essential Medium with non-essential amino acids, 45% Ham's Nutrient Mix F12 (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum without antibiotics.

Primers:

Primer sequences used were: MAGE-A2 forward (5'-ATG ACC TTG TTT TCA GAA GG-3') and MAGE-A2 reverse (5'-GGA ACT CGG ATT GTC TCC-3') for MAGE-A2; MAGE-A3 forward (5'-TCT TCA GCA AAG CTT CCA G-3') and MAGE-A3 reverse (5'-TCC ACA TAG CTG GTT TCA AC-3') for MAGE-A3; MAGE-A12 forward (5'-ACT TGA GCA GAG GAG TCA GC-3') and MAGE-A12 reverse (5'-CTA CTT GGA AGC TCG TCT CC-3') for MAGE-A12; MAGE-D4 forward (5'-GCA TCA CAG CCC TCA TTG-3') and MAGE-D4 reverse (5'-GTC ATC TGT GAT GAG CTT CC-3') for MAGE-D4; CSAG1 forward (5'-AGG AAA CCA CGA GCC TCC-3') and CSAG1 reverse (5'-GAA TGC CCA AGG AAG CAC-3') for CSAG1GAPDH forward (5'-GAA GGT GAA GGT CGG AGT-3') and GAPDH reverse (5'-GAA GAT GGT GAT GGG ATT TC-3') for GAPDH.

RNA extraction

The total RNA was extracted using RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions.

RT-PCR

RT‐PCR was performed using QIAGEN® One-Step RT‐PCR Kit following the instructions. GAPDH was used as the loading control.Twenty nanograms of RNA was amplified in 20μl RT-PCRs (4μl 5×RT buffer, 0.8μl 10mM dNTPs, 0.8 μl 10pmol/μl forward primer, 0.8 μl 10pmol/μl reverse primer, 0.8μl enzyme mix, 2μl 10ng/μl RNA and 10.8μl ddH2O). Temperature cycles as follow: one cycle of 50℃ for 30min and 95℃ for 15min, 94℃ for 30s, 56℃ for 30s, and 72℃ for 30s, and a final extension of 72℃for 10min followed by a final hold at 4°C. For MAGE-A2, -A3, -A12, and CSAG1, amplification was performed for 50 cycles. For MAGE-D4, amplification was performed for 33 cycles. For GAPDH, amplification was performed for 27 cycles.

Electrophoresis

5μl of loading dye was added to each RT-PCR product. 5μl of each product was then added to a 1.5% agarose gel, and electrophoresis was performed at 130V. Band intensities were visualized by ethidium bromide in a UV trans-illuminator (BioRad)

PCR product purification, gel extraction and sequencing

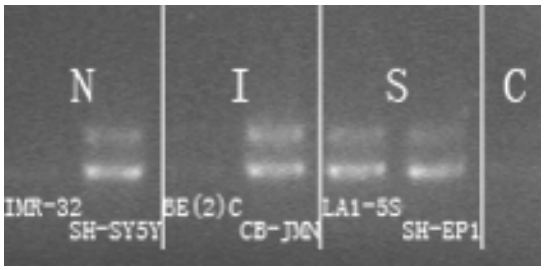
The remaining PCR products were purified using the QIAquick® PCR Purification Kit and QIAquick Gel Extraction Kit(QIAGEN) following the manufacturer’s instructions and subsequently sequenced in order to confirm the identity of the PCR products.

Results

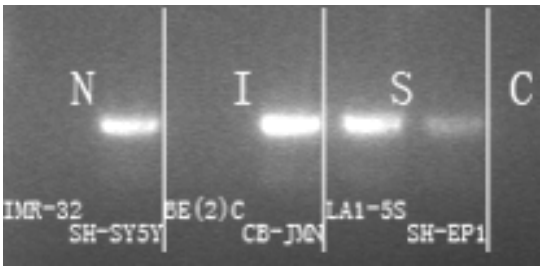
To study the expression of MAGE-A2, -A3, A12, and D4 in six NB cell lines, RT-PCR of mRNA isolated from these three samples was performed using specific primers for each gene. RT-PCR amplification of GAPDH was used to monitor the amount of RNA in the samples. PCR products were then analyzed on 1.5% agarose gel (Figure 1). In different NB cell lines, the expression patterns of MAGE-A2, -A3, and –A12 are very similar to each other (Figure.1 A-C). This expression pattern is not related to the cell types. In the two N-type NB cell lines, IMR-32 does not express this three genes. In the two I-type NB cell lines, BE(2)-C does not express MAGE-A2, -A3, and A12, but CB-JMN does. Both S-type cell lines express all tested MAGE-A genes. The difference of mRNA amount between cell lines that that did express these genes was slight. In contract, MAGE-D4 is expressed in all six cell lines, with a greater amount of mRNA in S-type cells (Figure.1D). RT-PCR with CSAG1 specific primers confirmed similar differential expression patterns with MAGE-A2, A3, and A12 (Figure.1E).

In order to confirm the sequence identity of the genes, PCR products were purified and sequenced by Genewiz. Sequencing results confirm that the RT-PCR products are of the correct genes. Sequencing results also confirm the two bands in figure 1A are the splice variants of MAGE-A2, and the two bands in figure 1E are the splice variants of CSAG1.

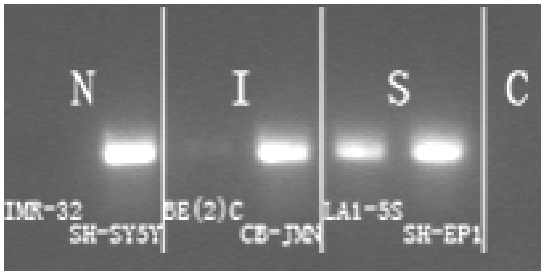
A. MAGE-A2



B. MAGE-A3

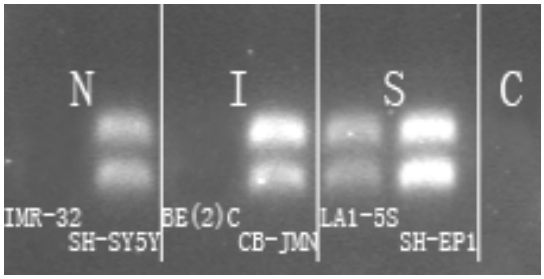


C. MAGE-A12



D. MAGE-D4

E. CSAG1



F. GAPDH

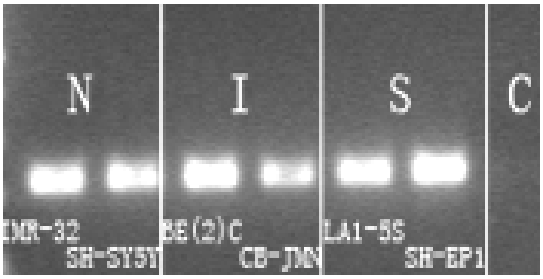
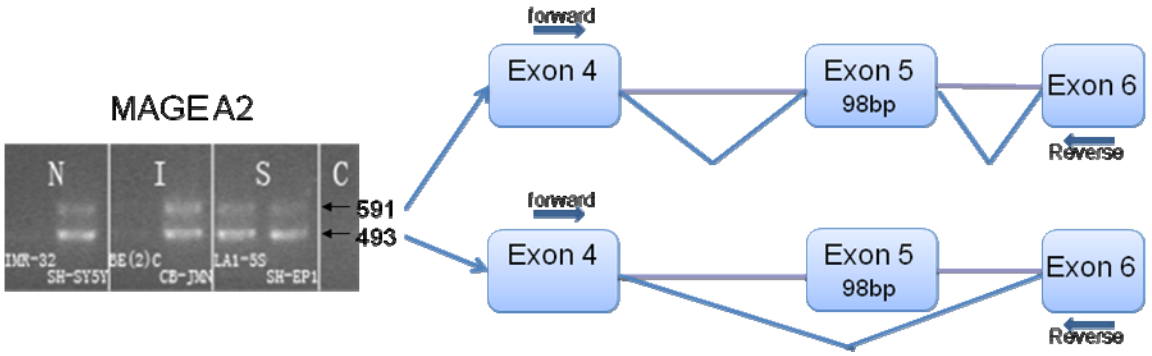


Figure 1 RT-PCR of six NB cell lines; (A), RT-PCR of MAGE-A2; (B), RT-PCR of MAGE-A3; (C), RT-PCR of MAGE-A12; (D), RT-PCR of MAGE-D4; (E), RT-PCR of CSAG1; (F), RT-PCR of GAPDH; C=negative control; Lanes: IMR-32; SH-SY5Y; BE(2)-C; CB-JMN; LA1-5S; and SH-EP1.

A



B

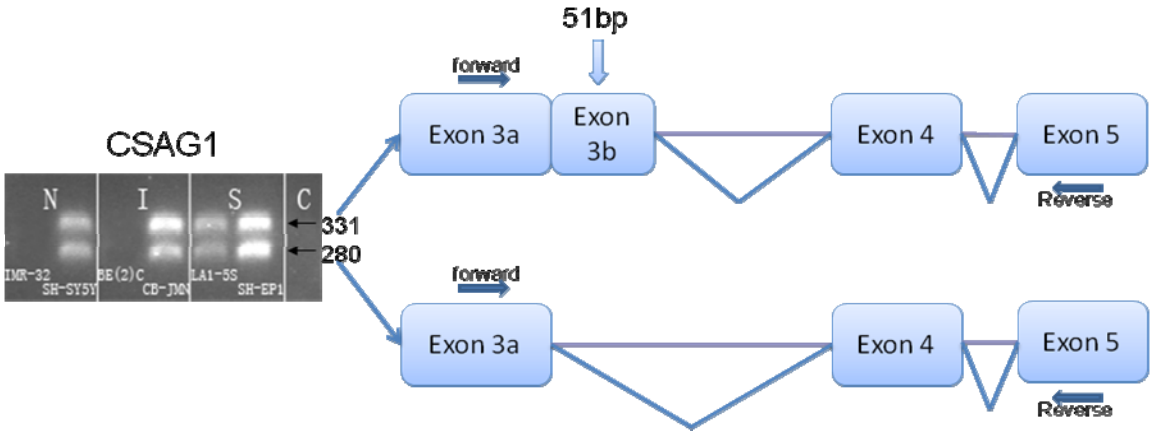


Figure 2. Splicing variants of MAGE-A2 and CSAG1 genes. (**A**) Splicing of the MAGE-A2 transcript. The large MAGE-A2 isoform is due to removal of introns 4 and 5 and in retention of exon5. The small MAGE-A2 isoform comes from the removal of introns 4 and 5 and exon 5. The primers are indicated as arrows. (**B**) Splicing of the CSAG1 transcript. The large CSAG1 isoform is due to removal of introns 3 and 4 and in retention of exon3b and exon 4. The small CSAG1 isoform comes from the removal of introns 3, 4 and exon 3b and in retention of exon 4. The primers are indicated as arrows.

Discussion

The heterogeneous intratumor expression of type-I MAGE genes is always a drawback in anticancer immunotherapy. Identification of the expression patterns of type-I MAGE genes may lead to more effective diagnoses and improved MAGE-based anticancer immunotherapy. In this study, MAGE-A2, A3, and A12 coexpressed in different NB cell lines, but did not express in the IMR-32 and BE(2)-C cell lines. This expression pattern was not related to cell type (there was no consistency in the expression in I-type and N-type cells) and was not shared by MAGE-D4.

The coexpression of MAGE-A genes could be due to simultaneous promoter methylation. Even though the promoters of MAGE-A genes are less homologous compared to their coding region, they still share high homology. In the three MAGE-A genes tested, MAGE-A2 and -A12 have a promoter identity of ~90%, MAGE–A3 has a promoter identity of ~72% compare to the others. The promoters of MAGE-A genes contain binding sites for the transcription factor Ets, which is responsible for the high transcriptional activation. In normal tissues, DNA methylation of CpG sites located in the Ets element inhibits the binding of Ets transcription factors, and therefore inhibits the expression of MAGE-A genes (Wischnewski et al., 2006). In contrast, the CpG island of MAGE-D4 genes does not show any homology to MAGE-A genes, which is correlated to their expression in normal cells.

At the same time, this coexpression of MAGE-A genes could also be due to a

global DNA hypomethylation. To test this assumption, we also examined the expression pattern of CSAG1 gene in NB cell lines. CSAG1 is located at Xq28 which is proximate to the MAGE-A genes (Figure 3). CSAG1 is not related to MAGE-A genes, and does not share homology with them at the gene CpG island. Therefore, it is less possible that the similar expression pattern of MAGE-A and CSAG1 is due to promoter demethylation. A more possible explanation is histone acetylation, which may affect more genes' expression at the same time. This means, the expression of genes in the area of Xq28 could be regulated in a coordinated manner. Future study with an inhibitor to histone deacetylase and an inhibitor to DNA methylase treated IMR-32 and BE(2)-C may induce the expression of MAGE-A genes.

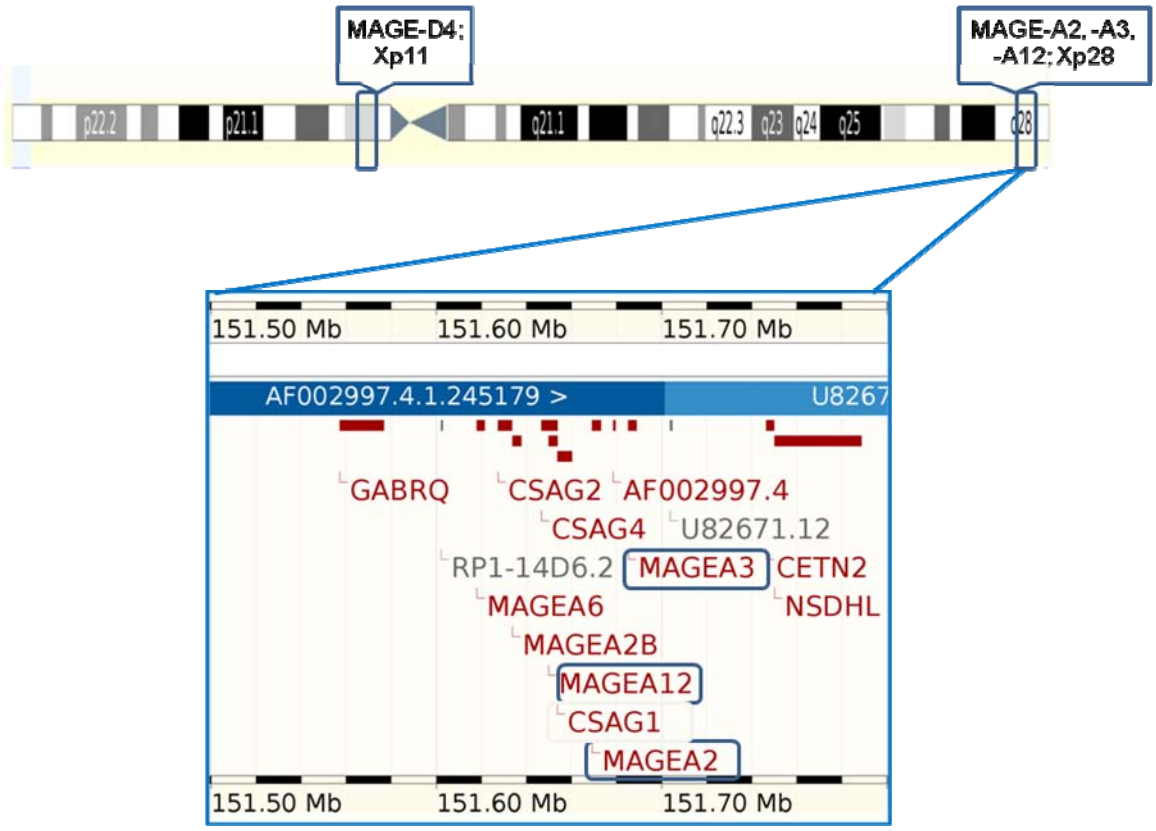


Figure 3. Location of MAGE-A2, -A3, -A12, MAGE-D4, and CSAG1.

In contrast to MAGE-A genes, the MAGE-D4 gene was expressed in all six NB

cell lines, with a slight increase of expression in the S-type cells LA1-5S and SH-EP1. S-type cells resemble nonneuronal precursor cells, display a glial-like morphology, and show contact inhibition of cell growth. This may due to MAGE-D4's necdin-related neuronal apoptosis function. The expression of MAGE-D4 could be down-regulated in tumor cells. However, more data is needed to support this cell type related expression pattern of MAGE-D4.

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