Expression of MAGE genes in neuroblastoma cell lines and the detection of an alternatively spliced transcript

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Abstract

Human neuroblastoma (NB), a solid tumor cancer, arises from neural crest tissue of the developing sympathetic nervous system (SNS). NB accounts for about 9 % of all childhood cancers, with about 500-700 new cases diagnosed each year. Three phenotypically distinct cell types compose NB cell lines: neuroblastic (N), substrate-adherent (S), and intermediate (I). A subfamily of human melanoma antigen (MAGE) genes (MAGE-A,-B and –C) encodes tumor-specific antigens and MAGE genes have been shown to be expressed in various tumor types. Here, the expression of MAGE-C1, -C2, and –D2 was studied by RT-PCR in the different NB cell lines represented here by LAI-55(N), LAI-5(S), BE(2)-C (I) and JMN (I) cells. RT-PCR yielded an alternatively spliced transcript variant of MAGE-D2. Sequencing verified the transcript variant retained the 79bp intron one found in MAGE-D2 transcript variant three cDNA. No significant differences were observed in the expression levels of MAGE-C1, and –C2, between the cell lines.

Introduction

Human neuroblastoma (NB), a solid tumor cancer, is an embryonic cancer arising from neural crest tissue (Ross and Spengler, 2007), more specifically from the postganglionic, sympathetic nervous system (SNS) of embryonic neural crest (Thiele, 1998). Many cases of NB begin in the sympathetic nerve ganglia along the SNS. NB accounts for about 9 % of all childhood cancers (Thiele, 1998), with about 500-700 new cases diagnosed each year.

Biochemical distinctions and differences in malignancy help characterize three different NB cell types: neuroblastic (N); substrate-adherent cells (S); and intermediate type cells (I), which are stem cell precursors to N- and S-type cells. N cells are small, weakly substrate-adherent cells that have cytoplasmic extensions (neurites) and grow as clumps of cells (pseudoganglia) (Ross and Spengler, 2007). S-type cells are substrate-adherent, and form a contact –inhibited monolayer when grown in culture; they are considered non-malignant and unable to form tumors (Ross and Spengler, 2007). I-type cells are considered the most malignant, followed by N-type cells. At its discovery, I-type cells were termed as such because their growth characteristics and morphology resembled an "intermediate" between N- and S-type cells; later research recognized I-type cells to be malignant and most closely resemble cancer stem cells (Ross and Spengler, 2007). Very little is known about what triggers the formation of the actual cancer or what causes the differences in its regression or growth potential.

Human melanoma antigen (MAGE) genes have been shown to be expressed in both normal tissues and in various tumors and tumor related cells such as neoplasia (Jungbluth et al., 2002), melanoma (Caballero et al. 2010), lung carcinomas and synovial sarcomas (Jungbluth et al., 2000) . Two types of MAGE genes have been characterized based on their expression: type-I members are silent in all normal tissues except for in the male germ line and placenta while type-II members are expressed ubiquitously in both tumor and normal cells. MAGE-C subfamily

members are type-I genes expressed in various tumor types; their proteins are tumor-specific antigens that can be recognized by cytolytic T lymphocytes. MAGE-D subfamily members are type-II members – they do not encode for those tumor-specific antigens seen in type-I MAGE and are also expressed ubiquitously in normal adult tissues (Chomez et al., 2001). While MAGE genes could be targets for immunotherapy, the function and expression pattern of MAGE-C and MAGE–D genes, however, remain largely unknown. Studies have suggested that MAGE genes may play a role in the function of p53, a tumor suppression gene (Kendall et al., 2002; Kuwako et al., 2004; Monte et al, 2005; Papageorgio et al., 2007). Analysis of the gene expression of type-I and type-II MAGE genes in various histological tumors may lead to improved diagnoses and the development of MAGE-based anti-cancer immunotherapies.

In this study, the expression levels of two MAGE-C genes, -C1 and –C2, and one MAGE-D2 family gene, were examined in different NB cell lines. MAGE-D2 has three transcript variants (TV), characterized by differences at exon one and intron one between the TVs. RT-PCR using variant-specific primers was performed and the products were analyzed on agarose gels.

Type	Subfamily	Number of members	Location	Expression
Type-I	MAGE-A	15	Xq28	Completely silent in normal tissues, except male germ line and placenta
	MAGE-B	17	Xp21	
	MAGE-C	7	Xq26-27	
Type- II	MAGE-D	4	Xp11	Expressed in normal adult tissues
	MAGE-E	3	Xq13.3	
	MAGE-F	1	3q13	
	MAGE-G	1	15q13.1	
	MAGE-H	1	Xp11.21	
	MAGE-L	1	15q11-q12	

Table 1. Human MAGE gene family

Materials and Methods

Neuroblastoma Cell Lines

LAI-55N, LAI-5S, BE(2)-C, and JMN cell lines were kindly provided by Dr. Robert Ross of the Laboratory of Neurobiology, Department of Biological Sciences, Fordham University. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's Nutrient F 12 medium supplemented with 10% Fetal Bovine Serum (FBS) at 37° C.

Primers

Primers specific for the reported mRNA sequences of MAGE-C1 (GenBank Accession Number NM_005462.4), and MAGE-C2 (GenBank Accession Number NM_016249.3) were designed. Transcript variant specific primers for MAGE-D2 transcript variants one (GenBank Accession Number NM_014599.4), two (GenBank Accession Number NM_177433.1) and three (GenBank Accession Number NM_201222.1) were also designed. Primer pair for MAGE-C1

(F 5'-GGAGGAGACTTATAGACCTATCCAG-3', R 5'-CCATGATGGCTCTGACAAAGG-3') amplified exons 1-3. Primer pair for MAGE-C2 (F 5'-CATCTTGGGAATCTGACG-3', R 5'- GCAGGTAAACGTATCAACAG-3') amplified exons 1-3. Primer pair for MAGE-D2 variant one (F 5' CTCGGGAATTGTAGGAGGAC-3', R 5' CTTGGTGTCAGCTGCTAGAC-3') amplified exons 1-3. Primer pair for MAGE-D2 variant two

(F 5'-TTCGAGAGGGACTTAGAGAAGGCAG-3', R 5'-CTTGGTGTCAGCTGCTAGAC-3')

amplified exons 1-3. Primer pair for MAGE-D2 variant three

(F 5'-TCCTGTATCTGAGAACG-3', R 5'-CTTGGTGTCAGCTGCTAGAC-3')

amplified exons 1-3. Primers specific to T7 (for forward sequences 5'-TAATACGACTCACTATAGGG-3') and SP6 (for reverse sequences 5'-GATTTAGGTGACACTATAG -3') promoters were added to the 5' end of each forward and reverse MAGE primer pair, respectively.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for this study (Forward sequence 5'-GAAGGTGAAGGTCGGAGT-3', Reverse sequence 5'-GAAGATGGTGATGGGATTTC-3') expecting to yield a product of 226 bp.

RNA extraction

Total RNA was extracted from the cell lysates using the RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions and with minor modifications. The RNA was eluded twice with 50μl of dH$_2$O.

RT-PCR

RT-PCR was performed using QIAGEN® One-Step RT-PCR Kit following the instructions. GAPDH was used as the loading control. Ten nanograms of RNA were amplified in 25μl RT-PCR reactions (5μl 5×RT buffer, 1μl 10mM dNTPs, 1μl enzyme mix, 1μl 10pmol/μl forward primer, 1μl 10pmol/μl reverse primer, 1 μl 10ng/μl RNA and 15μl dH$_2$O). Temperature cycles for MAGE genes are as follows: one cycle each of 50° for 30min and 95° C for 15min; 50 cycles of 94° C for 30sec, 57° C for 30sec, and 72° C for 30sec; followed by a final extension of 72° C for 10min and a final hold at 4°C. For GAPDH, temperature cycles remained the same but with modified amplification performed for 27 cycles.

Agarose Gel Electrophoresis

2.5μl of loading dye was added to each RT-PCR product. 5μl of each product was then added to 1-2% agarose gels containing ethidium bromide (EtBr), and electrophoresis was performed at 170V for 1 hour. Band intensities for the RT-PCR products were then visualized in an UV trans-illuminator (BioRad).

PCR Purification, Gel Extraction and Sequencing

Representative RT- PCR products from each primer pair were purified using the QIAquick® PCR Purification Kit (QIAGEN). Bands from the MAGE-D2 variant three RT-PCR products were cut out and purified using the QIAquick Gel Extraction Kit (QIAGEN). The samples were then sent out for sequencing by Genewiz Inc. The results were compared by BLAST to confirm product identities.

Results

To study the expression of MAGE-C1, -C2, -D2 TV1, -D2 TV2, and –D2 TV3, RT-PCR using variant specific primers was performed on mRNA isolated from four neuroblastoma cell lines—LAI-55N, LAI-5S, BE(2)-C, and JMN. RT-PCR was also performed with GAPDH as a loading control to monitor the amount of mRNA in the samples. RT-PCR products were then analyzed on 2% agarose gels. RT-PCR of MAGE-D2 TV3 shows the expression of two splice variants with the MAGE-D2 TV3 primer pair. Sequencing confirmed the retention of intron 1 in transcript variant 3. Band intensities for MAGE-C1, MAGE-C2, MAGE-D2 TV1, MAGE-D2 TV2, and MAGE-D2 TV3 were similar between the cell lines. Products were confirmed by sequencing and all experiments were replicated at least three times.
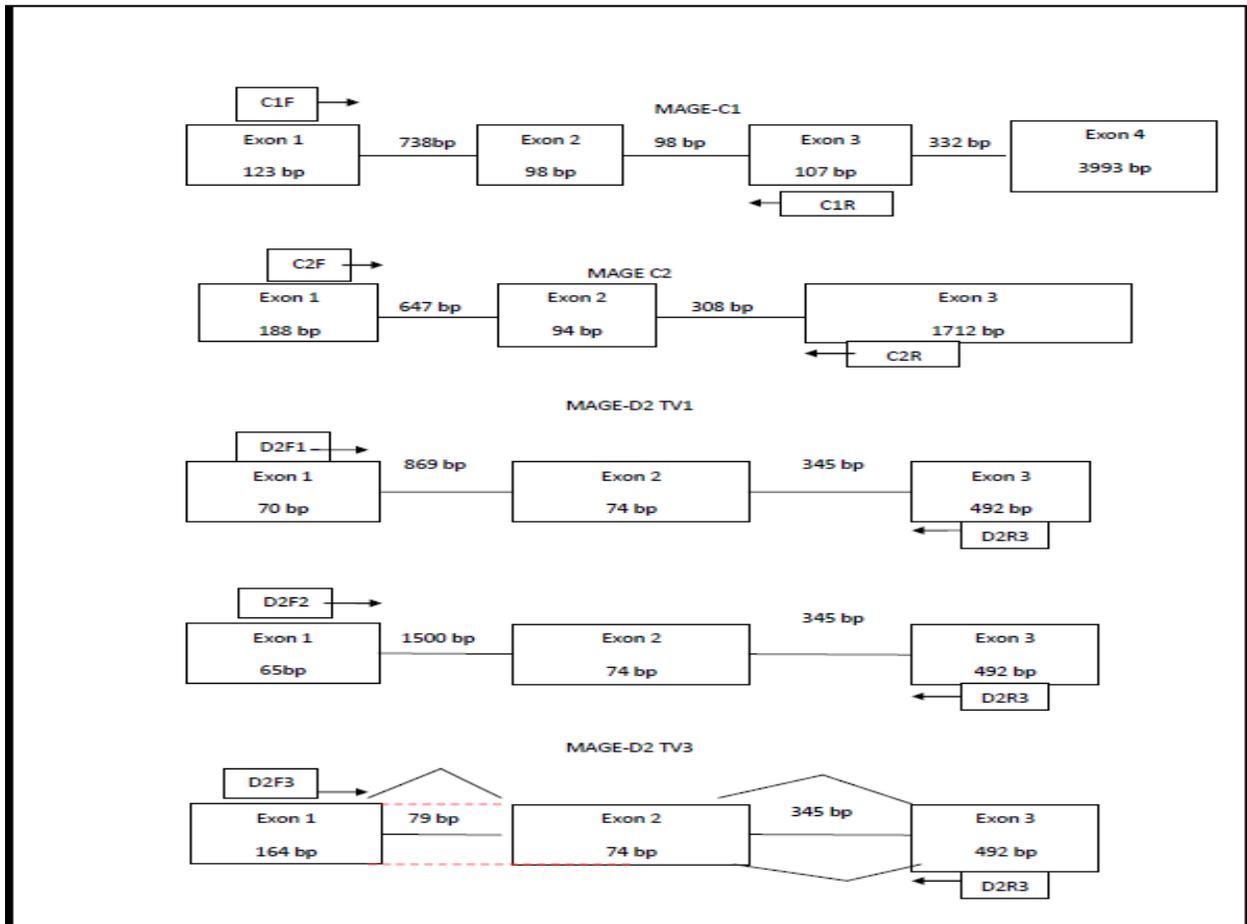

Figure 1.

Figure 1. MAGE-C1, MAGE-C2, MAGE-D2 TV 1, MAGE-D2 TV2, and MAGE-D2 TV3 with primer placements shown. All MAGE-D2 genes have 13 exons total, but differ in exon 1. Exons 2-12 and introns 2-11 are identical in all MAGE-D2 genes. For MAGE-D2 TV3, normal splicing is shown in black while an alternative splicing pathway, which contains the retention of the 79 bp intron one, is shown in red.
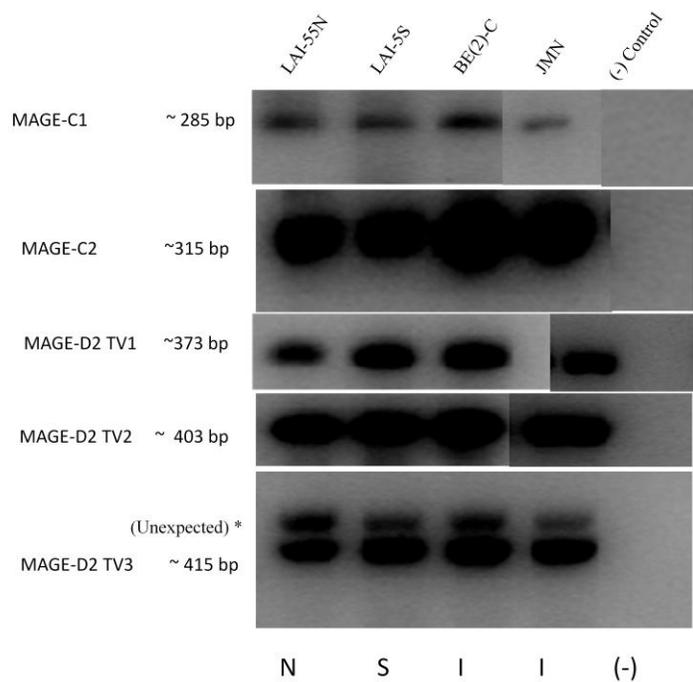

Figure 2a. RT-PCR products of MAGE-C1, MAGE-C2, MAGE-D2 TV1, MAGE-D2 TV2, MAGE-D2 TV 3, and GAPDH. MAGE-D2 TV 3 reaction yielded two bands when separated on agarose gel.
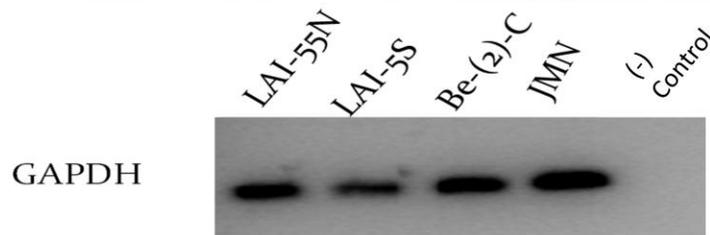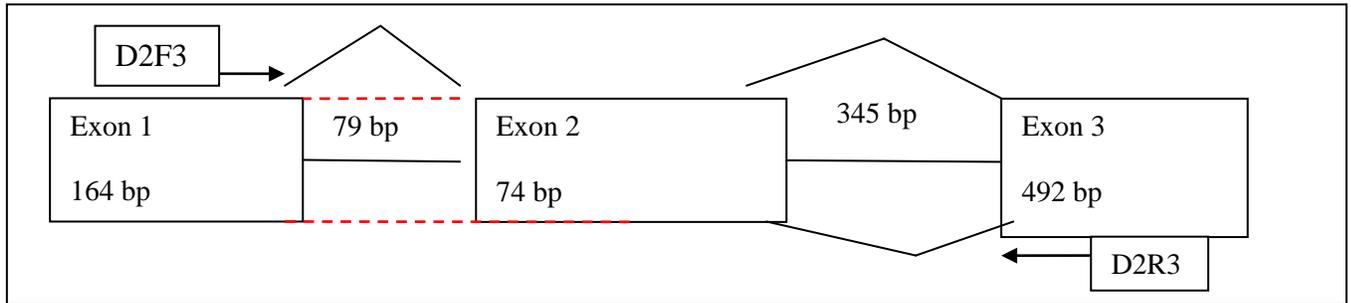
Figure 2b. GAPDH was used as a loading control

Fig 3a.

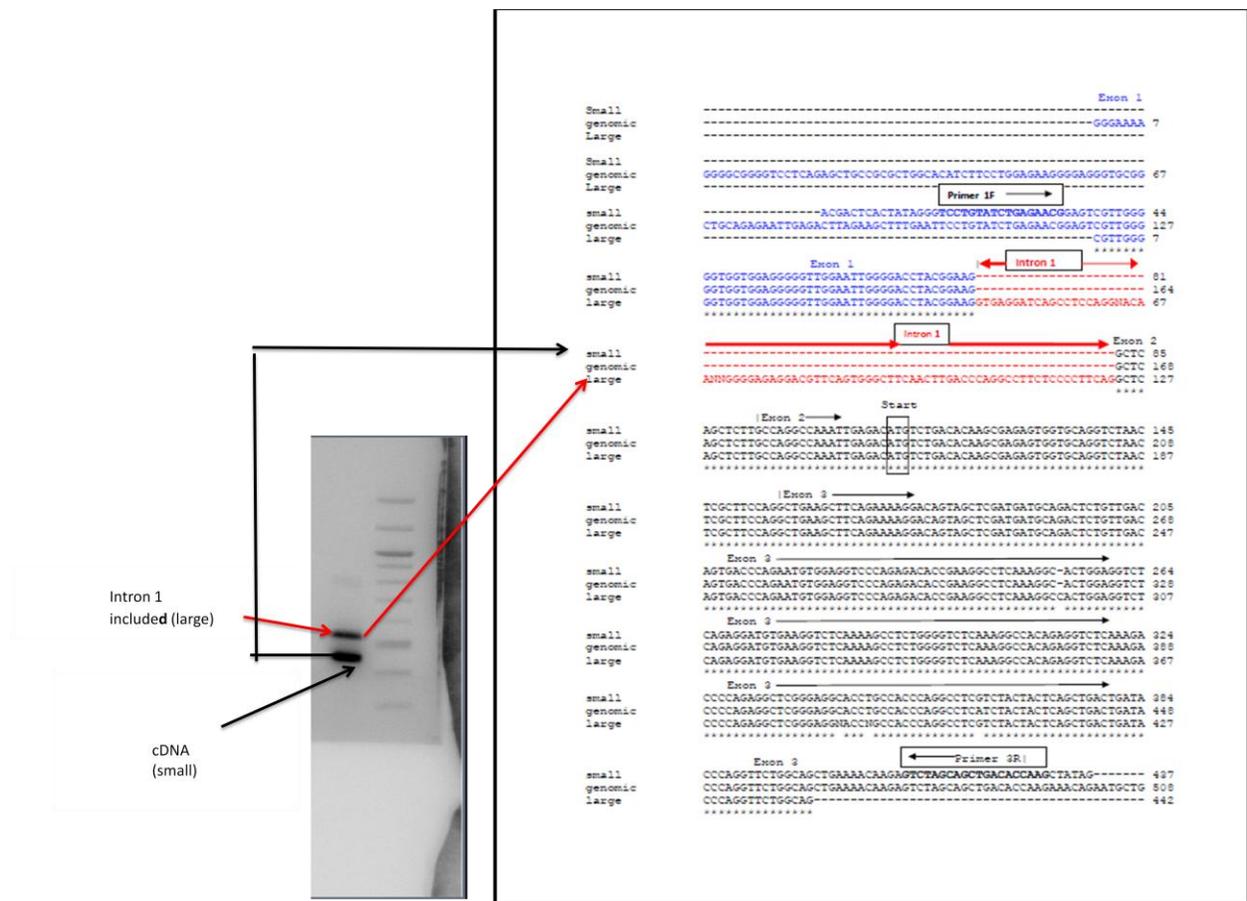**Fig. 3b**   **Fig. 3c**

Figure 3. Alternative splicing in MAGE-D2 TV 3. **3a.** Alternative splicing schematic of MAGE-D2 TV3.**3b.** MAGE-D2 TV3 RT-PCR generated two bands. **3c.** Blast partial alignment of MAGE-D2 TV3. Retention of intron one is shown in the larger RT-PCR product.

Discussion

      Results show the discovery of a new MAGE-D2 variant that retains intron one. The significance of this variant with the retained intron remains unknown. Papageorgio et al. 2007 suggests MAGE-D2 is a potential dissociator of p53, a tumor suppressing gene, but whether or not MAGE genes play a significant role in cell cycle regulation and subsequent cancer prognosis remains to be seen. Subsequent analysis of MAGE-D2 sequencing data –with a focus on splice sites and possible mutations within those sites—may provide further insight.

      There were no significant differences observed in expression levels of MAGE-C1 or MAGE-C2 between the three NB cell lines. Further studying of differential expression between different tumor and normal cell lines should be done to conclusively state that there is no difference in gene expression. Studies examining other cell lines could provide clues as to the function of the inclusion of intron one in the MAGE-D2 variant, and could also provide further understanding of the role of the MAGE gene family in neuroblastoma, other tumor cells and in normal tissues. Monte et. al. (2005) write that MAGE-A1, a type-I MAGE, has shown resistance to chemotherapeutic agents, but whether this is universal in all MAGE family genes has yet to be established, and further studies on MAGE type-I and –II genes could be done to aid in the development of possible MAGE-based anti-cancer immunotherapies.

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