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MAGE-A1 expression is associated with good prognosis in neuroblastoma tumors

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Abstract

Purpose Neuroblastoma is an embryonal tumor of neuro-ectodermal cells. Patients with metastatic neuroblastoma have a poor survival rate, which has led to numerous efforts to develop prognostic markers. Cancer/testis-specific antigens MAGE-A1 and MAGE-A3 genes were proposed as minimal residual disease (MRD) markers in neuroblastoma, but its usefulness for this purpose is rather limited.

Methods We studied 47 primary neuroblastoma tumors. RNA was extracted and cDNA was prepared by reverse transcription. Detection of the MAGE-A1 expression was done by hybridization of the RT-PCR products. We used methylation-specific-PCR to perform the epigenetic studies.

Results We studied the MAGE-A1 and MAGE-A3 expressions, and the MAGE-A1 expression showed significant association with tumor stage, absence of bone marrow infiltration and survival. A multivariate analysis enabled us to conclude that the MAGE-A1 expression represents a new independent predictive factor, which is independent of N-Myc amplification (*P* value = 0.000), age at diagnosis (*P* value = 0.002) or tumoral stage (*P* value = 0.024). Considering the epigenetic regulation of MAGE-A1, we analyzed its methylation profile, and found a significant association with its expression in tumor cells. Moreover, we found tumors that failed to show the MAGE-A1 expression despite the hypomethylated sequence, and corresponded to advanced neuroblastoma that might share another mechanism involved in MAGE-A1 silencing. Given the association described between genome-wide hypomethylation and microsatellite instability, we determined the MSI status of tumor samples, finding a significant correlation with the MAGE-A1 expression and, more specifically, with the hypomethylated status of this gene only in female patients.

Conclusion We conclude that the MAGE-A1 expression is associated with good prognosis in neuroblastoma.

Keywords Neuroblastoma · MAGE-A1 · Prognosis factor · Cancer/testis antigens · Hypomethylation

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Introduction

Neuroblastoma is an embryonal tumor of neuroectodermal cells, derived from the neural crest whose cells migrate to the adrenal medulla and sympathetic nervous system (Bown 2001) It is characterized by a diversity of clinical behaviors ranging from spontaneous remission to rapid tumor progression and death. Neuroblastoma tumor cells show complex combinations of acquired genetic aberrations, including ploidy changes, amplifications of the N-Myc oncogene, gains of chromosomal arm 17q and deletions of chromosome arms 1p and 11q (Maris and Matthay 1999; Attiyeh et al. 2005).

Among the various molecular markers that have been proposed for detecting minimal residual disease in neuroblastoma, there are two cancer/testis antigens, MAGE-A1 and MAGE-3 (Cheung and Cheung 2001). MAGE genes are a family of clusters situated in the X chromosome. The MAGE-A cluster is located on the q28 region of the X chromosome (Rogner et al. 1995), and is expressed, as other family members are, in tumors of different histological types; however, it is not expressed in normal adult tissues, except in germ-line cells (De Plaen et al. 1994). On the other hand, the functions of the MAGE-A group of proteins are largely unknown. The subcellular localization of MAGE-A proteins can vary from one family member to another. MAGE-A1 and MAGE-A3 are reported to be located in the cytosol of melanoma cells. Moreover, MAGE-A1 has also been detected in both the nucleus and cytoplasm of spermatogonias (Schultz-Thater et al. 1994). MAGE-A10 and MAGE-A11 have been predominantly detected in the nucleus of tumor cells (Rimoldi et al. 1999; Jurk et al. 1998).

The MAGE-A1 gene (melanoma antigen A1) encodes a human antigen, which is recognized by cytolytic T lymphocytes (natural killer T cells) (Rogner et al. 1995). MAGE-A1 gene has been studied in neuroblastoma tumors by several groups, and is expressed in 44–66% of tumoral samples. This expression correlates with the absence of N-Myc amplification and normal serum ferritin levels (Soling et al. 1999; Wolfl et al. 2005). In general, the MAGE-A expression (MAGE-A1–4) does not correlate with the tumor stage, and is not stably expressed throughout progression, therefore its usefulness as a neuroblastoma MRD marker is rather limited (Cheung and Cheung 2001).

In cancer, one of the most common epigenetic phenomena is the global hypomethylation of cytosines, which leads to an activation of genes that are silenced under normal conditions. Thus, the promoter and the first exon demethylation of the MAGE-A1 gene strongly correlate with its expression in male germinal cells, certain melanoma cell lines, and skin and lymph nodes melanoma metastasis. In normal cells of the adult organism, this gene is methylated and not expressed (De Smet et al. 1999).

Previous studies have demonstrated that methylation of the MAGE-A1 promoter in vitro inhibits the binding of the transcription factors and its transcription, so there is no expression of this cancer/testis gene (Cho et al. 2003). In contrast, to remark the stochastic nature of epigenetic changes, it is worth noting that some studies have found melanoma cell lines that did not express MAGE-A1 in spite of their generalized global hypomethylation (De Smet et al. 1996).

Considering these results, we have performed an analysis of MAGE-A1 and MAGE-3 in neuroblastoma primary tumor samples to assess the possible role of this gene as a predictor of the clinical outcome. We have also analyzed the mechanisms that lead to the MAGE-A1 variable ectopic expression.

Materials and methods

Clinical samples

Tumor samples were collected from 47 NB patients at diagnosis before treatment. Fourteen samples come from stage 4 patients, 11 from stage 3, 6 from stage 2, 14 from stage 1, and 2 from stage 4S. NB staging was established according to the International Neuroblastoma Staging System (INSS) (Brodeur et al. 1988). NB tumors, classified as stages 1 and 2 were grouped as localized, while those at stages 3 and 4 were considered advanced, in accordance with the INSS. Stage 4S was classified within the localized group, since it is known to exhibit good metastatic behavior and due to the fact that the primary tumor by the 4S definition in the INSS should be stage 1 or 2. There were 24 tumoral samples for female patients and 23 for male patients. The mean age for neuroblastoma patients was 26 months (range: 1–126 months), and 21 patients were above 18 months of age. Ten of our series of tumors showed N-Myc amplification. Tumoral samples were studied by FISH in order to analyze N-Myc amplification in the Reference Pathology Laboratory of the Medicine University of Valencia for neuroblastoma according to the ENQUA guidelines (Noguera et al. 2003).

Preparation of total RNA

Frozen tumor samples were lysed in RLT after mechanical desegregation. Total RNA was isolated with the RNasy kit (Qiagen) following the manufacturer's recommendations.

RT-PCR of MAGE-A1 and MAGE-A3

Total RNA was reverse transcribed in a total volume of 40 μL, following the manufacturer's guidelines (Geneamp Gold RNA PCR Core Kit, Applied Biosystems). The reaction mixture was incubated at 25°C for 15 min and at 42°C for 30 min. The integrity of the mRNA isolated from these samples was confirmed by PCR using the specific primers for human β-microglobulin with an amplified DNA fragment of 333 bp. The oligonucleotides for MAGE-A1 and MAGE-A3 are based on those published by Oltra et al. (2004). In brief, 2 μL cDNA samples were amplified under standard conditions in a volume of 25 μL. Each reaction set included both negative (blank amplification) and positive controls (IMR-32 cDNA). In addition, a sensitivity control was added to the electrophoresis, corresponding to a

1:10,000 dilution of a reference PCR product (from a tumoral cDNA) in a dilution buffer containing herring sperm DNA (50 ng/μL). Amplification conditions were an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 95°C for 45 s, 58°C for 1 min, and 72°C for 30 s, ending with a final elongation step at 72°C for 10 min.

Expression analysis of MAGE-A1 and MAGE-A3 by Southern blot analysis

The RT-PCR products were electrophoresed on 1% agarose and transferred to positively charged membranes (Boehringer Mannheim). DNA was cross-linked to the membranes at 120°C for 30 min. Once fixed, the PCR products were hybridized with a digoxigenin-labeled probe. This probe was obtained by PCR amplification, as described above, and checked by direct sequencing. Digoxigenin labeling was done in a second round of amplification of the diluted PCR product by adding digoxigenin-11-dUTP. Chemiluminescent detection was carried out according to the Roche protocol.

Sodium bisulfite modification

Sodium bisulfite modification was carried out using the standard method (Herman et al. 1996). Genomic DNA from neuroblastoma primary tumor was extracted by following a phenol–chloroform standard protocol. The quality and quantity of the DNA were evaluated in terms of the $A_{260/280}$ ratio. Genomic DNA (0.5 μg) was denatured at 37°C for 10 min in 0.3 M NaOH. Unmethylated cytosines were sulfonated by incubation in 3 M sodium bisulphite, 1 M hydroquinone (pH 5) at 55°C overnight. The resulting sulphonated DNA was purified using the GeneClean II cleanup system (Q-BioGene) according to the manufacturer's instructions, except that DNA was eluted with distilled water (50 μL) at room temperature. Following elution, DNA was desulfonated in 0.3 M NaOH for 15 min at 37°C, then the DNA was precipitated with sodium acetate (5 μL of 3 M) and ethanol (125 μL of 100%), and was resuspended in 50 μL distilled water.

Methylation-specific PCR

Methylation-specific PCR (MSP) analysis was performed using the primers described by Zhang et al. (2004) to produce a 274 bp product from the unmethylated sequence (U), and a 275 bp product from the methylated sequence (M). PCR amplification in a 25 μL volume with 50 ng template DNA, or less was carried out under the following conditions: 12 min at 95°C followed by 40 cycles of 95°C for 30 s and 55°C for 30 s, and a final extension of 10 min of 72°C. Genomic DNA purified from peripheral blood of a healthy voluntary donor was used as a positive control for the unmethylated reaction. A blank control containing all the PCR components, except template DNA was also included in all the experiments. Normal lymphocyte DNA treated in vitro with SssI methyltransferase (New England Biolabs) was used as a positive control for methylated alleles. Reaction products were separated by electrophoresis on a 12% acrylamide gel, stained with silver nitrate.

HDAC2 mutation screening

The microsatellite sequence amplification of the HDAC2 gene, located within exon 1, was performed using primers described by Ropero et al. (2006). The PCR conditions included an initial denaturation step at 95°C for 12 min, followed by 30 cycles consisting of denaturation at 95°C for 30 s, an annealing step at 60°C for 30 s and an extension step of 72°C for 30 s. A final extension cycle was performed at 72°C for 10 min. The PCR product (3 μL) was denatured by heating for 5 min at 95°C in loading buffer and then cooled in ice. Then it was electrophoresed on a polyacrylamide gel (12%) and run at room temperature for 12–14 h at 700 V. Gels were visualized by silver staining and the presence of sequence alterations was based on the detection of different migration pattern variants.

DNA sequencing

DNA fragments displaying an abnormal single-strand conformational polymorphism (SSCP) pattern were sequenced with the ABI PRISM Big- Dye™ Terminator Cycle Sequencing Kit 1.1 (Applied Biosystems). The products of sequencing reactions were precipitated and resuspended in Template Suppression Reagent TSR (Applied Biosystems), and electrophoresed on an ABI PRISM 310 automated fluorescent sequencer unit (Applied Biosystems) according to ABI protocols. Sequence alterations were detected using the associated software and by visual inspection. Samples were sequenced in both the forward and reverse directions to confirm the location of sequence changes.

Microsatellite instability

BAT-26 amplification was performed using the reaction mixture and the PCR conditions described in Grau et al. (2005) with primers previously described by (Alonso et al. (2001). The PCR products were electrophoresed under the conditions described above.

Statistical analyses

Statistical analyses were performed using the SPSS 10.0 software for Windows XP. Frequency analyses were per-

formed using either the Fisher's exact test or the Chi-square test. Survival analyses were performed according to Kaplan–Meier. The stepwise Cox proportional hazards model (successive inclusion of significant covariates with $P < 0.05$) was applied to test the independent significant influence of different genetic and clinical parameters on patient outcome.

Results

Expression analysis of MAGE-A1 and MAGE-A3

Forty-seven neuroblastoma (NB) primary tumors were analyzed for the ectopic expression of MAGE-A1 and MAGE-A3 genes by RT-PCR/hybridization.

The MAGE-A1 gene was expressed in 17/47 tumors (36%) and the MAGE-A3 gene in 23/47 tumors (49%). We found a significant correlation between the MAGE-A1 expression and the tumor stage (P value = 0.017), with a predominant expression of this gene in localized neuroblastoma tumors. In contrast, the MAGE-A3 expression was not seen to significantly correlate with the tumor stage (Table 1).

We analyzed the MAGE-A1 and MAGE-A3 expressions in relation with other biological features of neuroblastoma tumors, such as bone marrow infiltration at diagnosis and N-Myc amplification. None of these genes showed significant correlations with N-Myc amplification. However, the MAGE-A1 expression showed a significant inverse correlation with bone marrow infiltration at diagnosis (P value = 0.0251).

In the same way, the MAGE-A1 expression was significantly correlated with event-free survival (EFS) (P value = 0.0305) and overall survival (OS) (P value = 0.0042) (Fig. 1), while the MAGE-A3 gene did not display such a correlation. The MAGE-A1 expression also correlated with overall survival in females (P value = 0.0400) (Fig. 2), and with event-free survival (P value = 0.0417) and overall survival (P value = 0.0198) in the group of tumors that showed no amplification of the N-Myc oncogen (Fig. 3), suggesting that these are independent factors. The MAGE-A1 expression also showed a significant association with survival

when age at diagnosis was less than 18 months (P value = 0.0146) (Data not shown).

In order to test the independence of the MAGE-A1 expression as a prognostic factor in relation to the remaining variables, a multivariate analysis was performed according to the Cox regression analysis. Significant results were obtained for this expression against N-Myc amplification (P value = 0.000), age at diagnosis (P value = 0.002) and the tumoral stage (P value = 0.024). In this way, we could conclude that the MAGE-A1 expression represents a new independent predictive factor.

As presented above, there is a significant correlation between the MAGE-A1 expression and overall survival in localized tumors. In the group of advanced tumors, however, although the result for this association is not statistically significant, a similar tendency to the behavior displayed by localized tumors could be observed (Fig. 4a). Furthermore, the MAGE-A1 expression clearly correlated with overall survival in those tumors which showed no N-Myc amplification. A separate analysis of the group of advanced tumors without N-Myc amplification did not reach significance; once again, however, the same tendency could be observed (Fig. 4b).

Epigenetic studies with the MAGE-A1 gene

Given these results obtained in the expression analysis of the MAGE-A1 gene, we performed an epigenetic study of the MAGE-A1 methylation status in our series of neuroblastoma primary tumors. For two samples of our series, we have no methylation results. By MSP of the promoter region of this gene, we found that 42.2% (19/45) displayed an abnormally demethylated pattern. Methylation status of the MAGE-A1 gene correlated significantly with its ectopic expression (P value = 0.0006).

In particular, we found that the association established by previous studies between the demethylated status of MAGE-A1 and its ectopic expression was supported in 73.3% of the samples (33/45). In spite of this, we also found some neuroblastoma primary tumors that express this gene at variable levels, even though they lacked hypomethylation of the MAGE-A1 promoter (6/45), and others that did not express this gene despite its hypomethylated status

Table 1 MAGE-A1 and MAGE-A3 expressions versus tumor stage

	MAGE-A1			MAGE-A3		
	Localized tumors	Advanced tumors	Total	Localized tumors	Advanced tumors	Total
Absence of expression	7 (23.3%)	23 (76.7%)	30	8 (33.3%)	16 (66.7%)	24
Expression	10 (58.8%)	7 (41.2%)	17	9 (39.1%)	14 (60.1%)	23
	P value 0.0174		$n = 47$	P value 0.4165		$n = 47$

Fig. 1 Kaplan–Meier plots of event-free survival (**a**) and overall survival (**b**) based on the detection of MAGE-A1 and MAGE-A3 (**c**, **d**) expression
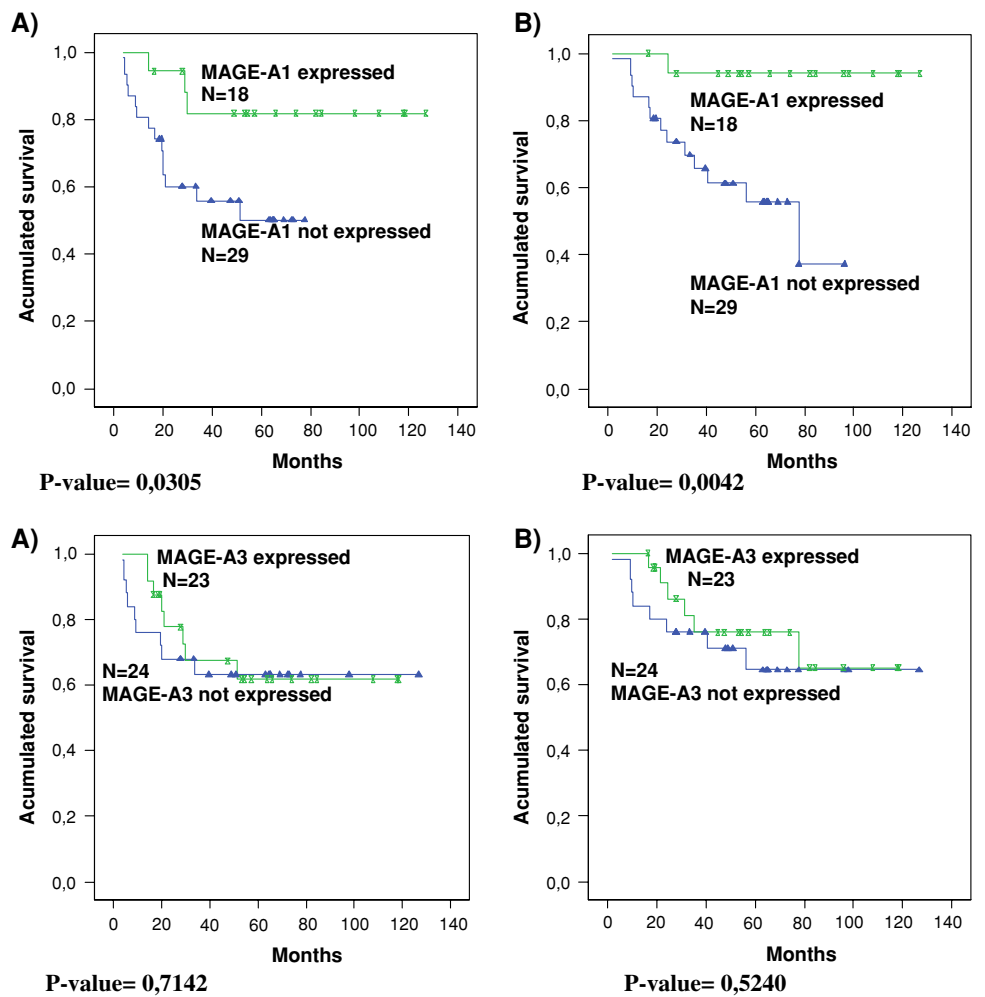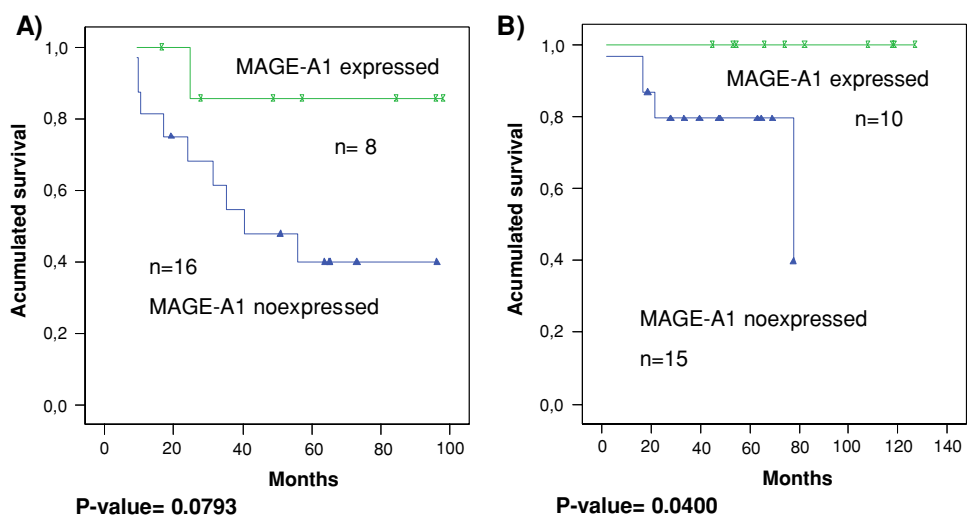

Fig. 2 Kaplan–Meier plots of overall survival based on the detection of MAGE-A1 expression in tumors from male (**a**) and female (**b**) patients

(7/45). Neither event-free survival nor overall survival correlated statistically with methylation pattern.

To clarify the apparent discrepancy between methylation status and gene expression, we analyzed the data considering tumor stage. We found that localized tumors showed a significant correlation between the MAGE-A1 methylation pattern and the MAGE-A1 expression (*P* value = 0.0104). In contrast, in the advanced stages we found that most tumors (58%) with hypomethylated MAGE-A1 gene revealed absence of ectopic expression (Table 2).

Fig. 3 Kaplan–Meier plots of event-free survival (**a**) and overall survival (**b**) based on the detection of MAGE-A1 expression in non-amplified N-Myc tumors
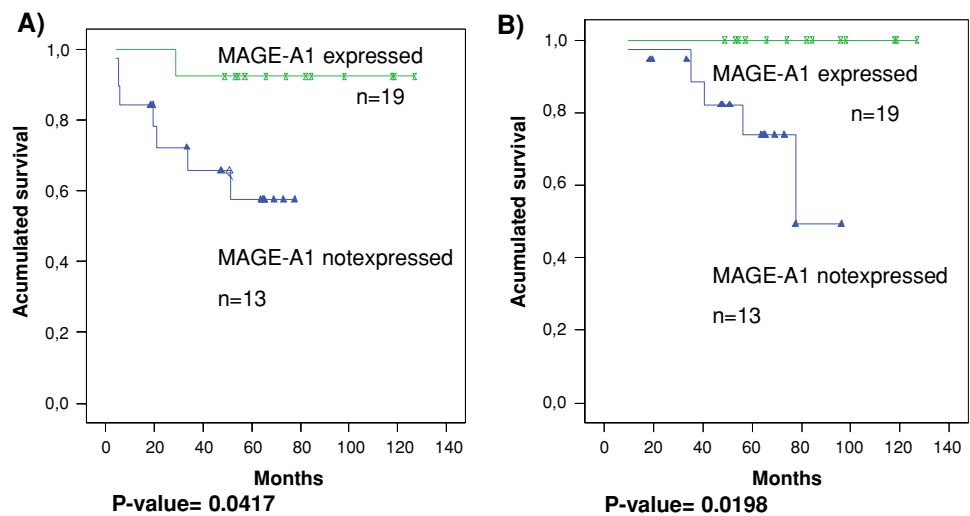

Fig. 4 Kaplan–Meier plots of overall survival based on the detection of MAGE-A1 expression in advanced tumors (**a**) and advanced tumors without N-Myc amplification (**b**)
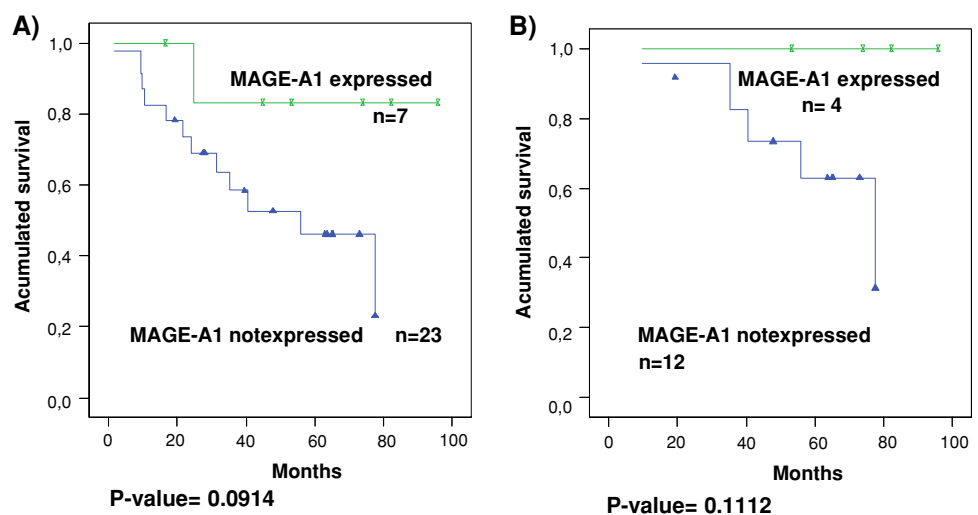

Table 2 MAGE-A1 methylation pattern versus expression in localized and advanced tumors

MAGE-A1	Localized tumors			Advanced tumors		
	Absence of expression	Expression	Total	Absence of expression	Expression	Total
Unmethylated	0 (0%)	7 (100%)	7	7 (58.3%)	5 (41.7%)	12
Methylated	6 (66.7%)	3 (33.3%)	9	15 (88.2%)	2 (11.8%)	17
	P value 0.0104		$n = 16$	P value 0.0795		$n = 29$

In an attempt to discover the mechanisms responsible for the MAGE-A1 expression, besides hypomethylation of its promoter, we followed two approaches. We decided to analyze the microsatellite instability (MSI) in our series of primary neuroblastoma tumors, as well as the truncating mutation in a microsatellite region of HDAC2, described for a subset of sporadic carcinomas causing epigenetic changes independent of the methylation status.

We found that microsatellite instability is associated to the ectopic expression of MAGE-A1 (P value = 0.028), but not to the expression of MAGE-A3 (P value = 0.536). On

the other hand, microsatellite instability appears to be more specifically associated with the unmethylated pattern of the MAGE-A1 gene (P value = 0.007).

Conversely, the search for microsatellite frameshifts in the coding region of HDAC2 failed to show a pathological mutation, which rules out this alteration in the coding sequence of this enzyme as the putative link between microsatellite instability and cancer/testis antigens expression.

Due to the different behaviors of localized and advanced tumors at MAGE-A1 methylation status and its ectopic expression, we decided to analyze these data attending to

the advanced status of the tumor, as well as to patient gender, acknowledging the X-linked condition of these genes.

With this analysis, we found a significant correlation between the epigenetic status of MAGE-A1 and the presence of microsatellite instability in localized tumors (P value = 0.0192). Moreover, this association seems to be more evident in female patients (P value = 0.0005).

No correlation was found between methylation status of the MAGE-A1 gene or MSI and survival (data not shown).

Discussion

The cancer/testis antigen genes MAGE-A1 and MAGE-A3 have been analyzed in a series of neuroblastoma primary tumors. We have performed an expression analysis for both the genes, and the MAGE-A1 expression showed a significant association between tumor stage and the absence of bone marrow infiltration at diagnosis. More importantly, its expression was significantly correlated with survival, mainly in those tumors that had no amplification of the oncogen N-Myc.

MAGE-A1 is mainly expressed in localized tumors. The fact that these tumors had a high rate of the MAGE-A1 expression, in contrast with advanced tumors, supports the significant association with survival, more specifically in those tumors that did not show N-Myc amplification. Separate analysis of advanced neuroblastoma did not reach statistical significance (probably due to the small sample size), but confirms a better prognosis of MAGE-A1 expressing tumors (Fig. 4).

This association might be stronger in female patients given the location of this gene in the X chromosome (Rogner et al. 1995) that could affect the expression of this molecular marker by some indirect mechanism. In fact, we have proved that microsatellite instability is associated with abnormal hypomethylation of this gene specifically in females (see below).

We found a random expression of the MAGE-A3 gene for neuroblastoma primary tumors, with no correlation with survival or the main biological features for this tumor type. As a matter of fact, the MAGE-A1 and MAGE-A3 genes belong to the same cancer/testis gene family (Rogner et al. 1995), but only the expression of the MAGE-A1 gene correlates with survival, which could be related with their different molecular roles. In this respect, previous studies demonstrated the interaction between the MAGE-A1 protein and HDAC1 through the SKIP molecule, played a role as a transcriptional factor that could influence the activation or repression of gene expression through chromatin remodeling (Laduron et al. 2004).

The MAGE-A1 gene displays a CpG island in its promoter region, and previous studies in melanoma cell lines demonstrated that this gene is hypermethylated in normal adult cells in order to repress its expression. This epigenetic regulation of the MAGE-A1 expression led us to analyze the methylation profile of this gene, in order to asses whether the absence of expression in advanced tumors is due to hypermethylation of the promoter sequence (De Smet et al. 2004). We found a partial, albeit significant association between the methylation pattern of MAGE-A1 and its expression in neuroblastoma tumor cells. However, no correlation with survival was found.

Notwithstanding the above, we found tumors that failed to show expression of this gene in spite of its hypomethylated sequence. Meanwhile, other tumors displayed different levels of gene expression despite the hypermethylated promoter. The behavior of the latter might be due to a structural modification of the chromatin, which may open the gene region up to transcription factors, despite being hypermethylated. In fact, the tumoral samples that show expression of MAGE-A1 and hypermethylation corresponded to both localized and advanced stages in a minor proportion in both the subgroups.

Conversely, all those cases with absence of expression and hypomethylation of the MAGE-A1 gene corresponded to advanced neuroblastoma tumors that might share another mechanism involved in this gene-silencing event. This putative event is apparently related to the relative lack of detectable expression levels of the MAGE-A1 gene in advanced primary tumors, therefore this gene silencing mechanism could play an important role in tumoral progression. In this way, the fact that the MAGE-A1 expression could be associated with good prognosis in neuroblastoma tumors is further supported, because only in advanced tumors MAGE-A1 was silenced by mechanisms other than primordial hypermethylation. This mechanism would have arisen during tumor progression as an alternative way to compensate for genome-wide hypomethylation.

Previous studies in melanoma cell lines had similar results, finding that the MAGE-A1 activation in tumor cells was basically correlated with a genome-wide hypomethylation. However, there were also some cases where generalized hypomethylation did not correlate with the MAGE-A1 expression (Brodeur et al. 1988).

In addition to DNA methylation, changes in histone proteins could affect DNA organization and gene expression. Changes in chromatin structure also influence gene expression because genes are inactivated when the chromatin is condensed, and they are expressed when the chromatin is open (Rodenhiser and Mann 2006). These dynamic chromatin states are controlled by histone modifications, involving the histone deacetylase (HDACs) family of enzymes in this process. Active promoter regions normally have unmethylated DNA and high levels of acetylated histones, whereas inactive regions of chromatin contain methylated DNA and deacetylated histones.

Given the important role these enzymes play in activating or silencing gene expression, the cDNA sequence of the HDAC1 gene has been screened for mutation in sporadic carcinomas, obtaining no evidence of mutations in this gene. On the other hand, truncating mutations were found in one of the primary human histone deacetylases, HDAC2, in sporadic carcinomas with microsatellite instability. The presence of the HDAC2 frameshift mutation in a microsatellite sequence of its first exon causes a loss of the HDAC2 protein expression and its enzymatic activity (Ropero et al. 2006). For this reason, we decided to study these specific mutations in those tumors without the MAGE-A1 expression and hypomethylated MAGE-A1 sequence, in order to assess whether this could affect silencing in these neuroblastoma cells. However, we found no significant results.

Previous studies have associated genome-wide hypomethylation with microsatellite instability in cancer cells, mainly in colon carcinoma cells (Raptis and Bapat 2006). Thus, although no mutational event repressing HDAC2 was found in our neuroblastoma primary tumors; there could be an alteration in the DNA damage repair system leading to microsatellite instability that might affect the MAGE-A1 gene expression.

We have analyzed the MSI status of our tumoral samples and a significant association with the MAGE-A1 expression was found. By comparison, no association with the MAGE-A3 expression was found, suggesting that MAGE-A3 is not affected by the same mechanism that affects the MAGE-A1 expression. On the other hand, we found that MSI highly correlates with the MAGE-A1 hypomethylation pattern, specifically in localized tumors but not in advanced tumors. This association can be interpreted as further evidence of the relationship between MSI and genome-wide hypomethylation in neuroblastoma.

The fact that the MAGE-A1 gene is located on the X chromosome led us to analyze our results paying attention to patient gender. Upon doing this, we found a highly significant association between MSI and the hypomethylated status of the MAGE-A1 gene only in the female patient subgroup. It is well known that lesion-containing DNA is less efficiently methylated than lesion-free DNA, and that an increase in DNA strand breaks precedes DNA hypomethylation; therefore hemy-methylated DNA is progressively replaced by double-stranded unmethylated DNA. Supposedly, DNA lesions would compete with hemymethylated CpG islands as binding sites in the DNA for HDAC molecules (James et al. 2003). In this way, DNA lesions may favor the disruption of normal DNA methylation patterns in the cell, and this situation would be more evident in chromosomes with a higher proportion of hypermethylated CpG islands, the inactive X chromosome, which is exclusive for females. Accordingly, female tumoral cells with MSI would be especially prone to hypomethylation of X-linked genes such as MAGE-A1. For this reason, hypomethylation of the MAGE-A1 gene might in some instances be a consequence of microsatellite instability. Furthermore, due to the higher demethylation rate of MAGE-A1 in female samples of advanced stages (10/14 females vs. 4/18 males), this relationship may explain the reason why the MAGE-A1 expression seems to be a slightly better prognostic marker in females.

Likewise, there are studies showing that MAGE, GAGE and LAGE families are co-expressed in a large proportion of pediatric solid tumors, so we could suppose that, as well as the MAGE-A1 gene, there must be other cancer/testis genes that demonstrate similar behavior (Jacobs et al. 2007).

In conclusion, if the putative protective effect of the expression of the MAGE-A1 gene against tumoral progression of neuroblastoma was confirmed, the elucidation of the underlying mechanisms regulating the MAGE-A1 gene expression could provide a new and promising therapeutic approach for this disease.

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