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ORIGINAL ARTICLE

Insights on *PRAME* and osteosarcoma by means of gene expression profiling

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Abstract
Background Osteosarcoma (OS) is the most frequent bone tumor in children and adolescents. Tumor antigens are encoded by genes that are expressed in many types of solid tumors but are silent in normal tissues, with the exception of placenta and male germ-line cells. It has been proposed that antigen tumors are potential tumor markers.
Objectives The premise of this study is that the identification of novel OS-associated transcripts will lead to a better understanding of the events involved in OS pathogenesis and biology.
Methods We analyzed the expression of a panel of seven tumor antigens in OS samples to identify possible tumor markers. After selecting the tumor antigen expressed in most samples of the panel, gene expression profiling was used to identify osteosarcoma-associated molecular alterations. A microarray was employed because of its ability to accurately produce comprehensive expression profiles.
Results PRAME was identified as the tumor antigen expressed in most OS samples; it was detected in 68% of the cases. Microarray results showed differences in expression for genes functioning in cell signaling and adhesion as well as extracellular matrix-related genes, implying that such tumors could indeed differ in regard to distinct patterns of tumorigenesis.
Conclusions The hypothesis inferred in this study was gathered mostly from available data concerning other kinds

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of tumors. There is circumstantial evidence that *PRAME* expression might be related to distinct patterns of tumorigenesis. Further investigation is needed to validate the differential expression of genes belonging to tumorigenesis-related pathways in *PRAME*-positive and *PRAME*-negative tumors.

Introduction

There is considerable interest in genes coding for tumor-associated antigens or CGGs (cancer germ-line genes) that have been identified in the last decade, including the melanoma antigen families *MAGEA*, *BAGE*, *GAGE/PAGE* and *LAGE/NY-ESO-1* [1, 2]. These antigens are encoded by genes that are expressed in many types of solid tumors but are silent in normal tissues from adults, with the exception of placenta and male germ-line cells [2]. They consist of an antigenic peptide 8–10 amino acids in length which is presented to cytolytic T lymphocytes (CTL) by HLA class I molecules [1, 2]. Unlike the first category of antigens (*MAGEA*, *BAGE*, *GAGE/PAGE* and *LAGE/NY-ESO-1*), *PRAME* is expressed in some normal tissues, such as adrenal tissue, ovaries and endometrium, although at very low levels [3]. It is possible that these normal cells do not present enough *PRAME* peptide to ensure recognition by CTL [3, 4]. The *PRAME* gene encodes a 509-amino acid protein and is located on chromosome 22 (22q11.22), in contrast to the *MAGEA*, *BAGE*, *GAGE/PAGE* and *LAGE/NY-ESO-1* genes, which are located on chromosome Xp [2–5].

PRAME (preferentially expressed in melanoma), is a gene that encodes an HLA-A24-restricted antigenic peptide presented to an autologous CTL clone in melanoma patients [3, 4, 6]. The question of whether activation of *PRAME* is causally implicated in oncogenic transformation or whether it is a mere reporter of progressive disease remains unanswered. Recent data indicate that *PRAME* may be instrumental in disease progression as it interferes with retinoic acid (RA) receptor (RAR) signaling [7–9]. RA signaling is essential for development, cell fate determination, and tissue homeostasis. RA induces the transcription of a set of target genes by binding to and activating its receptor, resulting in differentiation and cell cycle arrest in responsive cells. Loss of RA responsiveness is therefore beneficial to cancer cells [8, 9].

Besides melanoma, *PRAME* is frequently expressed in many different cancers, and its expression correlates with prognosis and survival [8]. For instance, *PRAME* is expressed in non-small-cell lung carcinomas, breast carcinomas, renal cell carcinomas, head and neck cancers, Hodgkin's lymphomas, and sarcomas [3, 8]. The expression of *PRAME* is also significant in the acute and chronic phases of both myelocytic and lymphocytic leukemias, with reported frequencies of expression ranging from 17–42% in acute lymphoblastic leukemia (ALL) to 30–64% in acute myelogenous leukemia (AML) [4, 7]. A comparison of the gene signatures of the chronic, accelerated, and blast phases of chronic myelogenous leukemia (CML) revealed that early in the accelerated phase, before the accumulation of increased numbers of leukemia blast cells, new gene expression patterns occur, including an increase in *PRAME* expression [4, 7].

A number of studies have shown significant involvement of the tumor antigens in the pathogenesis of different cancers, including pediatric solid tumors [10, 11]. However, little information is available regarding the expression of tumor antigens in pediatric tumors. Jacobs et al. [10] measured the expression levels of 8 *MAGEA* genes and the genes *LAGE-2/NY-ESO-1* and *GAGE-1, 2, 8* in 9 OSs, 10 neuroblastomas, 12 rhabdomyosarcomas and 18 Ewing's sarcomas using quantitative real-time PCR. All OSs and 80% of the neuroblastoma samples expressed several tumor antigens at high levels. Six of 12 rhabdomyosarcomas and 11 of the 18 Ewing's sarcomas expressed at least one tumor antigen. No correlation was found between the level of tumor antigen expression in the tumor and the clinicopathological parameters of the patient.

The same group also used qPCR to quantify the expression of the genes *MAGEA1*, *MAGEA2*, *MAGEA3*, *MAGEA4*, *MAGEA6*, *MAGEA10*, *MAGEA12*, *MAGEC2*, *NY-ESO-1* and *GAGE-1, 2, 8* in 50 pediatric brain tumors with different histological subtypes. Fifty-five percent of the medulloblastomas ($n = 11$), 86% of the ependymomas ($n = 7$), 40% of the choroid plexus tumors ($n = 5$), and 67% of the astrocytic tumors ($n = 27$) expressed one or more tumor antigens. Except for a minority of such tumors, the overall level of tumor antigen expression in pediatric brain tumors was low and high expression of at least one tumor antigen was observed in 32% of the samples [11].

Osteosarcoma (OS) is the most common primary bone tumor in children and young adults [12]. Approximately 10–20% of patients with OS have metastatic disease at diagnosis. The most frequent site of metastasis is the lung. However, a smaller percentage of patients have bone and soft tissue metastases. The presence of metastasis at diagnosis is a prognostic factor, with a strong impact on the overall survival of these patients. Patients without metastasis at diagnosis have a 5-year overall survival rate of 70%, whereas overall survival falls to 32% in patients with metastasis at diagnosis [13–15]. In Brazil, studies from the Brazilian Osteosarcoma Treatment Group (BOTG) have shown that 21% of patients have metastatic disease at diagnosis—twice the rate observed in developed countries [15].

Despite the dramatic advances made in OS treatment, patient survival has reached a plateau. Recent clinical trials

which have attempted to improve outcome through the intensification of therapy or the incorporation of new agents have not been widely successful. Therefore, increasing focus has been placed on achieving a greater understanding of the basic biology of OS, with the goal of using that information to improve treatment [16].

The premise of this study is that the identification of novel OS-associated transcripts will lead to a better understanding of the events involved in OS pathogenesis and biology. We analyzed the expression of a panel of tumor antigens in OS samples to identify possible tumor markers. After identifying the *PRAME* gene as the tumor antigen expressed in most samples of the panel, we used the microarray platform CodeLink (GE/Amersham, Piscataway, NJ, USA; now Applied Microarrays, Tempe, AZ, USA) to identify OS-associated molecular alterations. This microarray was employed because of its ability to accurately produce comprehensive expression profiles. Finally, we selected the *EPCAM* (epithelial cell adhesion molecule) gene to validate the microarray results by quantitative PCR (qPCR).

Materials and methods

Patient tumor samples

All 51 flash-frozen OS samples of the 48 patients utilized in this study were obtained from patients attending the Pediatric Oncology Institute GRAACC/UNIFESP (Grupo de Apoio ao Adolescente e a Criança com Câncer/Federal University of São Paulo) on the basis of clinical, imaging and laboratory findings. Tumor tissues were taken from typical and viable tumor areas with >80% tumor-cell contents and immediately frozen in liquid nitrogen and preserved at −80°C. Five normal bone samples were used as controls. These samples were obtained from healthy individuals without genetic and/or musculoskeletal diseases who underwent orthopedic surgery due to trauma. Samples from each primary tumor and normal bone were collected after informed consent was obtained from patients/guardians according to the University's IRB (CEP/UNIFESP no. 0050).

Twenty-nine OS tumors—16 biopsy and 13 metastasis samples from 26 different patients—were used to identify the expression pattern of the tumor antigen gene panel tested by semi-quantitative RT-PCR. Of the total 26 patients included, only three had paired biopsy and metastasis samples (patients 5, 9 and 19 in Additional File 3 of the "Electronic supplementary material"). After identifying the *PRAME* gene as the tumor antigen expressed in most OS tumor samples, we selected 6 of these OS biopsy samples that had previously been tested for PRAME expression by RT-PCR for the microarray technique. To validate the microarray results by qPCR, we used 30 OS biopsy samples (Additional File 3 of the "Electronic supplementary material").

RNA extraction and cDNA synthesis

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was recovered from the aqueous phase by ethanol precipitation, and the pellets were dissolved in RNase-free water. First-strand cDNA synthesis, primed with oligo (dT) and 1 μg of RNA template, was catalyzed with Superscript III reverse transcriptase (Invitrogen), following the manufacturer's instructions.

Semi-quantitative RT-PCR for analysis of the CT antigen expression panel

We evaluated the expression of mRNA of the tumor antigens *MAGEA1*, *MAGEA4*, *MAGEA10*, *MAGEA12*, *BAGE*, *LAGE* and *PRAME* on 29 OS samples. The RT-PCR (reverse transcriptase-polymerase chain reaction) amplification was performed using 1/10 of cDNA, and the quality of the RNA and cDNA was evaluated by concomitant amplification of the *ACTB* (actin beta) mRNA. Primers used for amplification are presented in Table 1. The sequences were positioned in different exons of each gene to avoid false-positive results caused by DNA contamination of the RNA preparation. Assessment of the PCR product was performed visually on an ethidium bromide-stained agarose gel by comparing the intensity of the band with that resulting from a semi-quantitative RT-PCR performed on serial dilutions (1:1, 1:3, 1:9 and 1:27) of the RNA from the tumor cell lines MZ2-MEL and LB373 (kindly gifted by Dr. P.G. Coulie from the Ludwig Institute for Cancer Research, Brussels, Belgium) or K562, used as positive controls for antigen expression. MZ2-MEL was used as control for the expression of *MAGEAA2*, *A10* and *GAGE*, the LB373 line as control for *MAGEA4*, *A12*, *LAGE 1*, and *PRAME*, whereas the leukemia cell line K562 was used as control for the expression of *MAGEA1*. Samples were scored ++++, +++, ++ or + if the amount of the amplified product was equal to or greater than that obtained with the 1:1, 1:3, 1:9 and 1:27 dilutions of the reference RNA, respectively. Lower levels of expression were scored negative. An expression level of the *ACTB* gene comparable to that of the positive control was obtained with each sample.

Microarrays, data acquisition and statistical analysis

We used gene expression profiling to compare 6 biopsy tumor samples grouped according to *PRAME* expression.

Table 1 Primers for detecting gene expression of the antigen tumors investigated

Gene	Primers
ACTB	Forward 5′-GGCATCGTGATGGACTCCG-3′
	Reverse 5′GCTGGAAGGTGGACAGCGA-3′
MAGEA1	Forward 5′-CGGCCGAAGGAACCTGACCCAG-3′
	Reverse 5′-GCTGGAACCCTCACTGGGTTGCC-3′
MAGEA4	Forward 5′-GAGCAGACAGGCCAACCG-3′
	Reverse 5′-AAGGACTCTGCGTCAGGC-3′
MAGEA10	Forward 5′-CACAGAGCAGCAACTGAAGGAG-3′
	Reverse 5′-CTGGGTAAAGACTCACTGTCTTGG-3′
MAGEA12	Forward 5′-CGTTGGAGGTCAGAGAACAG-3′
	Reverse 5′-GCCCTCCACTGATCTTTAGCAA-3′
BAGE	Forward 5′-TGGCTCGTCTCACTCTGG-3′
	Reverse 5′-CCTCCTATTGCTCCTGTTG-3′
LAGE	Forward 5′-GCAGGATGGAAGGTGCCC-3′
	Reverse 5′-CTGGCCACTCGTGCTGGGA-3′
PRAME	Forward 5′-CTGTACTCATTTCCAGAGCCAGA-3′
	Reverse 5′TATTGAGAGAGGGTTTCCAAGGGGTT-3′

The *PRAME* gene expression statuses of these six samples were defined by semi-quantitative RT-PCR. Microarray experiments were carried out using the microarray platform CodeLink (GE/Amersham; now Applied Microarrays). This platform utilizes bioarrays consisting of a 30-base single pre-validated oligonucleotide probe per gene target. CodeLink UniSet Human I bioarrays containing 10,000 human transcripts were used for all experiments. Hybridization procedures strictly followed protocols provided by the manufacturer. A total of 12 arrays were hybridized, including 6 tumor samples (3 *PRAME*-positive and 3 *PRAME*-negative) and their respective technical replicates. Arrays were scanned following the recommended scanning procedure and settings for CodeLink bioarrays on GenePix 4000B Array Scanner/GenePix Pro 4.0 software (Axon Instruments). Generated data were batch normalized using CodeLink Software v.2.3. Statistical analysis for the identification of differentially expressed genes was carried out using the BayBoots tool [17]. The Bayes error rate (*P*) and fold-change are shown simultaneously in a volcano plot. Functional annotations were performed for differentially expressed genes ($P < 0.05$ and fold-change ≥ 2) with the program Database for Annotation, Visualization and Integrated Discovery (DAVID; http://niaid.abcc.ncifcrf.gov/content.jsp?file=functional_annotation.html) using the parameters Gene Ontology (GO) molecular process term level 4 and SP_PIR_Keywords in the Functional Category section. The array design and gene expression results are publicly available in the GEO database under the accession number GSE8079.

qPCR for validating the microarray results

The *EPCAM* gene was chosen to validate the microarray results. Oligonucleotide for *EPCAM* was used from the predesigned assay Hs00158980_m1, TaqMan Gene Expression Assays (Applied Biosystems; Foster City, CA, USA). Thermal cycling comprised initial steps at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The fluorescence of the double stranded products was monitored in real time. A standard curve was constructed with serial dilutions of a mix of a few samples. The cDNA was amplified and quantified using an ABI 7500 sequence detection system (SDS) (Applied Biosystems). To exclude variations arising from different inputs of total mRNA to the reaction, data on *EPCAM* were normalized to an internal housekeeping *GAPDH* gene (glyceraldehyde 3-phosphate dehydrogenase), for which data were obtained using TaqMan *GAPDH* control reagents (TaqMan Gene Expression Assays, Applied Biosystems, huGAPDH 4326317E). All the reactions for 30 biopsy samples were performed in triplicate. The data were averaged from the values obtained in triplicate for each reaction.

Statistical analyses

Data analysis was performed using GraphPad Prism software, version 4 (San Diego, CA, USA). Comparisons between the median of the gene expression profile of the tumor samples and normal bone controls were evaluated using Wilcoxon's signed rank test. Continuous data (age and gene expression) were evaluated and compared using the Mann–Whitney test. Categorical data (gender, histological OS subtype, and presence of metastasis at diagnosis) were studied using chi-square or Fisher's exact tests. Statistical significance was taken as $p < 0.05$.

Results

We analyzed the expression of *MAGEA1*, *MAGEA4*, *MAGEA10*, *MAGEA12*, *BAGE*, *LAGE* and *PRAME* on 29 OS samples, 16 biopsies and 13 metastases by RT-PCR. After identifying the *PRAME* gene as the antigen expressed in the most samples, we selected 6 OS biopsy samples to use on the microarray platform CodeLink (GE/Amersham; now Applied Microarrays) in order to identify OS-associated molecular alterations. To validate the microarray results, we selected the *EPCAM* (epithelial cell adhesion molecule) gene and analyzed the mRNA expression level of this gene on 30 OS biopsy samples by quantitative PCR (qPCR). A summary of the clinicopathological characteristics is detailed in Table 2.

Table 2 Clinical parameters of the OS patients investigated

No. of patients	Age (median)	Sex (%)	Presence of metastasis (%)	No. of samples	Type of sample (%)	Histology (%)	No. of samples/ methodology
48	5–34 years	Male	Meta	51	Biopsies	Osteo	RT-PCR 29
	(15 years)	32 (67)	26 (54)		39 (76)	30 (59)	Microarray 6
						Chond	qPCR 30
						10 (20)	
		Female	NMeta		Metastases	Telang	
		16 (33)	22 (46)		12 (24)	4 (8)	
						Others	
						7 (14)	

Meta metastatis at diagnosis, *NMeta* nonmetastasis at diagnosis, *Osteo* osteoblastic, *Chond* chondroblastic, *Telang* telangiectatic

Table 3 Summary of tumor antigen gene expression in OS samples

	MAGEA1	*MAGEA4*	*MAGEA10*	*MAGEA12*	*BAGE*	*LAGE*	*PRAME*
0	19	22	21	19	17	26	9
+	3	2	0	1	3	0	4
++	0	2	0	0	0	0	0
+++	1	1	4	4	5	1	3
++++	5	1	3	4	3	1	12
Positive cases	9	6	7	9	11	2	19

Semi-quantitative RT-PCR for analysis of the CT antigen expression panel

We used semi-quantitative RT-PCR to analyze the expression of *MAGEA1*, *MAGEA4*, *MAGEA10*, *MAGEA12*, *BAGE*, *LAGE* and *PRAME* in 29 OS samples. The gene expression results are summarized in Table 3. There was no difference in the pattern of gene expression between paired biopsy and metastasis samples. Of the 16 biopsy samples, 75% expressed *PRAME*; of the 13 metastasis samples, 70% expressed *PRAME*. Twenty-four (86%) of the OS samples expressed at least one of the tumor antigens. *MAGEA1* was detected in 9 (32%) samples, *MAGEA4* was detected in 6 (21%), *MAGEA10* was detected in 7 (25%), *MAGEA12* was detected in 9 (32%), *BAGE* was detected in 11 (39%), *LAGE* was detected in 2 (7%), and *PRAME* was detected in 19 (68%). In 12 (58%) of the 19 *PRAME*-positive samples, mRNA expression scored ++++, and 7 (25%) of the samples were positive only for *PRAME*. Results for *MAGEA1* and *MAGEA12* were similar, with 5 (55%) out of 9 positive samples and 4 (44%) out of 9 samples, respectively, scoring ++++. Only four samples (14%) were negative for the expression of all genes.

Microarrays, data acquisition and statistical analysis

In order to contribute to the current knowledge on *PRAME* and its relation to OS physiology, we used the technique of gene expression profiling to compare six tumor samples grouped according to expression. Figure 1 shows that neither the usual clustering analysis (Fig. 1a) nor the principal component analysis (Fig. 1b) were able to clearly separate both groups of tumors, suggesting that there is no underlying global signature or difference between *PRAME*-positive and *PRAME*-negative tumors.

When *PRAME*-expressing osteosarcoma samples were compared with samples that did not express this antigen, 86 genes were considered to be differentially expressed (fold-change >2; $P < 0.05$; see the highlighted region in the volcano plot of Fig. 2). *PRAME*-expressing samples overexpressed 40 genes. A certain number of cell signaling-related genes presented overexpression in *PRAME*-positive samples (see the "Electronic supplementary material"). Of particular interest is the overexpression of *EPCAM*.

PRAME-negative samples overexpressed 46 genes, including extracellular remodeling-related genes, as well as cell signaling (including notch signaling pathway genes) and transport-related genes (see the "Electronic supplementary

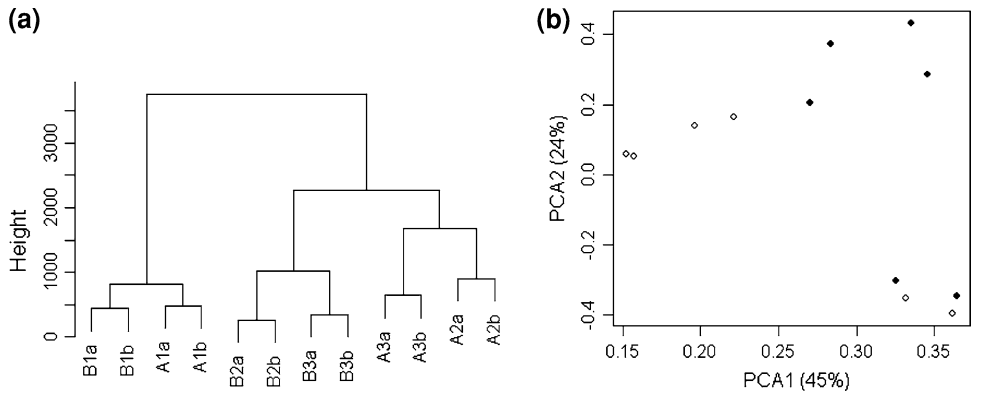

Fig. 1 Clustering and principal component analysis. **a** Clustering analysis performed with Euclidian distance and the hclust algorithm. Label *A* represents PRAME-negative samples. Label *B* represents PRAME-positive samples. Numbers *1–3* represent patient labels. Labels *a* or *b* indicate each technical replicate. **b** Principal component analysis showing the projection of the transcriptome measurements over the first two principal components enclosing 45 and 24% of the total variance, respectively. *Filled dots* represent the PRAME-negative samples and *empty dots* represent PRAME-positive samples. It is clear that there is no obvious separation between both tumor classes using the whole transcriptional profile

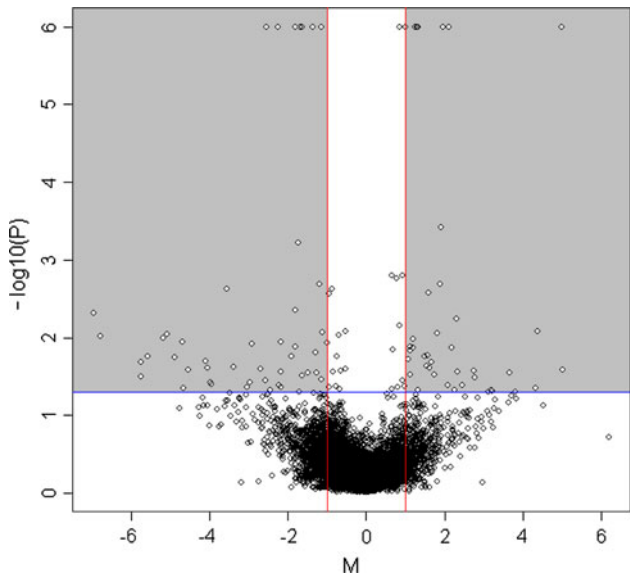

Fig. 2 Volcano plot summarizing the results of the PRAME-positive vs PRAME-negative comparison. The *highlighted region* defines the genes considered to be differentially expressed (fold-change greater than 2 and significance cutoff $P < 0.05$). *M* is the usual notation for log2 (PRAME-positive/PRAME-negative). The statistical significance *P* is measured via the Bayesian error rate (Vêncio et al. [17])

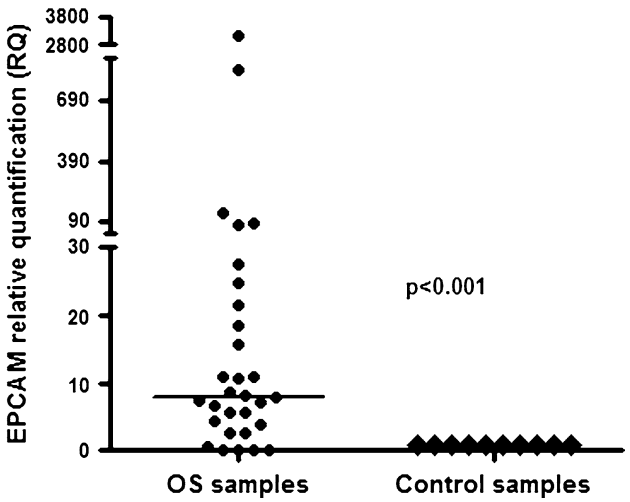

Fig. 3 EPCAM gene expression levels. Expression profile for the *EPCAM* gene on biopsy samples of osteosarcoma patients compared to that for control normal bone samples. The median is represented by a *black bar*

material"). Together with these genes, a series of additional glycoproteins and adhesion molecules (*CNTN1*, *VEGF*, *PCDHB9*, *B4GALT5*, *GRIN2C*, *NGFB*, *SLC12A3*, *IDUA*, *THBS4*, *TSPAN5*, *SCRG1*, *EPHB2*, *ISLR*) were overexpressed in *PRAME*-negative tumor samples.

qPCR for validating the microarray results

We selected the *EPCAM* gene from microarray data and quantified the mRNA expression, by qPCR, on 30 OS biopsy samples. Twenty-five (83%) of the 30 samples showed high expression of *EPCAM* and 5 (17%) samples showed low expression of *EPCAM* (cutoff ≥ 2). Comparison of the *EPCAM* expression levels in OS samples versus normal bone showed a significant difference ($P < 0.001$) (Fig. 3).

Discussion

There are only scarce data on the expression of tumor antigens in OS that can be specially recognized by CTL in primary and metastatic tumors. In the present study, we show that OS frequently expresses genes encoding tumor-

specific antigens. The identification of tumor-specific antigens is an essential step in the development of therapeutic cancer vaccines. The importance of antigens encoded by tumors as vaccine targets has led to detailed studies of their expression in various tumors [18]. Little information is available regarding the expression of tumor antigens in pediatric tumors [10, 11]. In this work, we report *MAGEA1*, *MEGEA4*, *MAGEA10*, *MAGEA12*, *BAGE*, *LAGE* and *PRAME* tumor antigen expression in OS. Our data demonstrated that 86% of the OS samples expressed at least one antigen, 64% of the cases are positive for two or more antigens, and only 14% of samples were negative for the expression of at least one of the CT antigen genes tested. This phenomenon has also been described for other tumors, and is most likely a consequence of a global demethylation of the genome in the tumor [10, 11]. It is also possible that the activation of a single tumor antigen leads to the activation of other tumor antigens [6].

As described above, there is a tendency for the expression of CT antigens to be clustered, because certain tumor specimens are found to express multiple CT antigens simultaneously whereas other tumor specimens will be totally negative [19, 20]. This phenomenon is related to the activation/derepression process for these genes, and suggests that in cancer, the expression of the *MAGE* family of genes and other tumor antigen genes that we studied is the result rather than the cause of tumorigenesis. Studies have shown that *MAGE* gene activation may be regulated by promoter demethylation, and that both tumor and normal cells contain the transcription factors that activate the *MAGE* gene family [19–21]. This is probably true of other antigens as well, so that the molecular mechanism responsible for the abnormal expression of these antigens in malignant tissues seems to be a widespread change in gene methylation patterns [19–22].

In this study, *PRAME* was the antigen most frequently expressed in OS, as it was detected in 68% of cases, followed by *MAGEA1*, *A4*, *A10*, *A12* and *BAGE* with almost equivalent frequencies, and it was the only antigen in 25% of the cases tested by semi-quantitative RT-PCR. The expression of this antigen has been reported in a variety of human neoplasias, including melanoma, multiple myeloma, myeloid and lymphoid leukemias, renal carcinoma, non-small-cell lung carcinomas, breast carcinomas, renal cell carcinomas, head and neck cancers, Hodgkin's lymphomas, Wilms' tumors, medulloblastomas and sarcomas [3, 4, 8, 10, 11]. It thus seems particularly interesting as a target for immunotherapy, since it is expressed in a wide spectrum of human neoplasias, to which we add OS. Further studies are needed to evaluate whether tumor antigen gene derived immunogens can serve as the basis for inducing CTL response in patients with OS or other malignant diseases.

In order to contribute to the current knowledge of *PRAME* and its relation to OS physiology, we used gene expression profiling to compare 6 tumor biopsy samples grouped according to *PRAME* gene expression. A microarray was employed because of its ability to accurately produce comprehensive expression profiles.

PRAME-expressing genes overexpressed 40 genes. In agreement with recent findings associating *PRAME* and cell death regulation, two apoptosis-related genes were overexpressed in the *PRAME*-positive samples: *EAF2*, an androgen-response gene that was downregulated in advanced human prostate cancer specimens and whose overexpression can markedly induce apoptosis in prostate cancer cells, and *CASP5*, an apoptosis-related cysteine peptidase that is potentially involved in cancer [23]. No apoptosis-related genes were overexpressed in *PRAME*-negative samples.

A certain number of cell signaling-related genes presented overexpression in *PRAME*-positive samples (see the "Electronic supplementary material"). Of particular interest is the overexpression of *EPCAM* (cellular adhesion molecule), also known as epithelial *TACSTD1* (tumor-associated calcium signal transducer). A confirmatory qPCR analysis was performed for the *EPCAM* gene selected in a set of 30 samples. Our qPCR results showed that *EPCAM* was overexpressed in OS at significant levels compared to all control normal bone samples ($P < 0.05$). *EPCAM* is a membrane-bound glycoprotein involved in signaling that promotes gene transcription and cell proliferation [24–26]. The high level of overexpression of *EPCAM* in a plethora of carcinomas has led to the use of it as a marker with prognostic quality and as a target for therapeutic strategies [26]. *EPCAMs* have been historically considered a target of passive immunotherapy using monoclonal antibodies and, more recently, a first Pox-vector-based cancer vaccine [25]. The next step is to amplify the number of cases investigated and to relate the molecular results to clinicopathological parameters.

Also related to cancer immunotherapy: members of the *NKG2* gene family, here represented by *KLRC3*, encode natural killer (NK) cell-specific lectin-like molecules that may have receptor function. NK cells are lymphocytes that can mediate the lysis of certain tumor cells without previous activation. *NKG2* receptors are expressed predominantly on NK cells, and they have been shown to play an important role in regulating responses to tumorigenic cells. OS cell lines vary greatly in their susceptibility to NK cell lysis in vitro; the expression of CD58 adhesion molecules on their surfaces appears to influence their vulnerability [27]. *KLRC3* and CD58 overexpression in *PRAME*-positive samples may reflect different patterns of interaction between the studied tumors and cells of the immune system.

PRAME-negative samples overexpressed 46 genes, including notch signaling pathway genes and transport-related genes (see the "Electronic supplementary material"). The notch pathway is a gene regulatory pathway involved in multiple differentiation processes, which is often repressed in cancer. Notch signaling that inhibits secretory cell differentiation is oncogenic in gastric cancer and colorectal cancer, while notch signaling that promotes keratinocyte differentiation is anti-oncogenic in esophageal squamous cell carcinoma [28]. In OS, the notch pathway was identified as a new invasion and metastasis-regulating pathway with a novel function: the regulation of metastasis [28]. In this study, the notch ligands *DLL1*, *Notch 1* and *2*, and the notch target gene *HES1* were expressed in OS cells, and the expression of *HES1* was associated with invasive and metastatic potential. Furthermore, blocking notch pathway signaling with a small molecule inhibitor of gamma secretase eliminated invasion in matrigel without affecting cell proliferation, survival, or anchorage-independent growth, and the manipulation of notch and *HES1* signaling demonstrated that *HES1* plays a crucial role in OS invasiveness and metastasis in vivo. Since the notch pathway can be inhibited pharmacologically, these findings point toward possible new treatments to reduce invasion and metastasis in OS [28]. Additionally, another study investigated the expression of the notch pathway molecules in OS biopsy specimens, and examined the effect of notch pathway inhibition. qPCR revealed the overexpression of *Notch2*, *Jagged1*, *HEY1*, and *HEY2*, and *Notch1* and *DLL1* were downregulated in biopsy specimens. In addition, notch pathway inhibition using gamma-secretase inhibitor and *CBF1* siRNA slowed the growth of OS in vitro, and gamma-secretase inhibitor-treated xenograft models exhibited significantly slower OS growth [29]. These findings suggest that inhibiting the notch pathway suppresses OS growth by regulating cell cycle regulator expression, and inactivation of the notch pathway may be a useful approach to the treatment of patients with OS [29].

Extracellular matrix (ECM) remodeling is critical for many developmental processes, and remodeled ECM contributes to tumorigenesis [30]. ECM is composed of a network of secreted proteins and glycoproteins. The matrix-associated genes *COL2A1*, *COL9A3* and *ASPN* are ECM components expressed in cartilage. Together with those genes, a series of additional glycoproteins and adhesion molecules (*CNTN1*, *VEGF*, *PCDHB9*, *B4GALT5*, *GRIN2C*, *NGFB*, *SLC12A3*, *IDUA*, *THBS4*, *TSPAN5*, *SCRG1*, *EPHB2*, *ISLR*) were overexpressed in *PRAME*-negative tumor samples. Adhesion molecules play a critical role in tumor invasion and metastasis. Taken together, these findings characterize distinct tumor microenvironments for *PRAME*-positive and *PRAME*-negative osteosarcoma samples. Recent research indicates that the tumor microenvironment—where proliferation, survival and migration actually take place—is an indispensable participant in the neoplastic process.

Osteosarcoma is the most common malignant bone tumor in children, and despite significant discoveries concerning functional changes in cancer cells over the past few decades, no significant impact has been observed on the long-term survival of OS patients. Literature is poor concerning the molecular aspects of OS. Thus, the hypothesis inferred in this study was based mostly on available data on other kinds of tumor. There is circumstantial evidence that *PRAME* expression may be related to distinct patterns of tumorigenesis. Further investigation is needed to validate the differential expression of genes belonging to tumorigenesis-related pathways in *PRAME*-positive and *PRAME*-negative tumors.

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