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ORIGINAL RESEARCH

Aberrant DNA methylation of *ESR1* and *p14ARF* genes could be useful as prognostic indicators in osteosarcoma

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Abstract: Osteosarcoma (OS) is the eighth most common form of childhood and adolescence cancer. Approximately 10%–20% of patients present metastatic disease at diagnosis and the 5-year overall survival remains around 70% for nonmetastatic patients and around 30% for metastatic patients. Metastatic disease at diagnosis and the necrosis grade induced by preoperative treatment are the only well-established prognostic factors for osteosarcoma. The DNA aberrant methylation is a frequent epigenetic alteration in humans and has been described as a molecular marker in different tumor types. This study evaluated the DNA aberrant methylation status of 18 genes in 34 OS samples without previous chemotherapy treatment and in four normal bone specimens and compared the methylation profile with clinicopathological characteristics of the patients. We were able to define a three-gene panel (*AIM1*, *p14ARF*, and *ESR1*) in which methylation was correlated with OS cases. The hypermethylation of *p14ARF* showed a significant association with the absence of metastases at diagnoses, while *ESR1* hypermethylation was marginally associated with worse overall survival. This study demonstrated that aberrant promoter methylation is a common event in OS and provides evidence that *p14ARF* and *ESR1* hypermethylation could be useful as a prognostic indicator for this disease.

Keywords: DNA methylation, *ESR1,* hypermethylation, osteosarcoma, *p14ARF*

Introduction

Osteosarcoma (OS) is the eighth most common form of childhood and adolescence cancer, comprising 2.4% of all malignancies in pediatric patients, and approximately 20% of all primary bone cancers.[1] Characteristically, OS is found in the metaphyseal regions of long bones in the appendicular skeleton, with the majority of patients developing the disease during the period of active bone growth in early adolescence. More than 15% of patients present clinically detectable pulmonary metastases.[2]

The implementation of combined treatments (neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy) and the use of multi-agent chemotherapy have improved prognosis over the last several decades, reaching 5-year survival rates of up to 70% for patients without metastatic disease at diagnosis.[3,4] Despite these advances, for patients that present with metastases at diagnosis or those with tumors showing a poor response to chemotherapy, the prognosis is still unsatisfactory (5-year survival rates, 20%–40%), even with dose-intensive or high-dose chemotherapy.[5–9] This suggests that even in tumors with the same histologic type, different genetic and epigenetic mechanisms may be operating, altering the response to chemotherapy and the metastatic capability.

The best prognostic factor for OS is the presence of metastatic disease at diagnosis and the necrosis grade induced by chemotherapy.[10] At the molecular level,

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OS is characterized by frequent genomic instability, highly heterogeneous karyotypes, gross changes in gene expression, and recurrent epigenetic alterations.[11–16]

Genetic alterations caused either by loss of heterozygosity or by mutations have been reported in OS. Besides this, most of the available data suggests that this bone tumor arises as a result of the inactivation of different tumor suppressor genes.[17] However, none of these alterations can characteristically reflect the biologic nature or clinical features of all OS cases. Therefore, assessment of more genetic and epigenetic data from OS tumors could provide important insights concerning both OS oncogenesis and molecular alterations governing differential clinical response to treatment.

Aberrant DNA methylation (hypermethylation) of gene promoter regions is the most widely epigenetic abnormality studied in human malignancies and is an important epigenetic mechanism of gene transcription regulation. This process is catalyzed by DNA methyltransferases and involves the addition of a methyl group to the carbon 5 position of the cytosine ring in CpG dinucleotides.[18] It is associated with several changes in chromatin structure, including the regulation of histone methylation and acetylation and the recruitment of proteins to the methylated sites. The methylation usually leads to the obstruction of the promoter region, hindering gene transcription and subsequently causing gene silencing.[19] In addition to genetic aberrations, there is increasing evidence that epigenetic processes also play a major role in carcinogenesis. Hypermethylation of the gene promoter region acts as an alternative to mutations in disrupting tumor suppressor gene function.[20]

Aberrant promoter methylation has been found in several genes in various malignant diseases, and each tumor type may have its own distinct pattern of methylation.[21] It was reported previously that hypermethylation of different genes may occur in OS cases, among them are *CDKN2A*, *CDKN2B*, *RASSF1A*, *DAPK*, *MGMT*, *TIMP3*, and *RB1*.[22–26] All of these studies focused on a few genes and most of them were conducted using conventional methylation-specific (MSP) polymerase chain reaction (PCR) technique.

Therefore, we sought to evaluate the presence of aberrant DNA methylation in promoter regions of 18 candidate genes in 34 OS specimens as well as four normal bone samples by a real-time quantitative MSP (QMSP) PCR approach. Thus, the aims of our study were (1) to determine the methylation profile of a panel of genes in OS; (2) to correlate the molecular data with the clinicopathological characteristics of the patients; and (3) to identify epigenetic biomarkers that may be useful for diagnosis and/or as prognostic factors for OS cases.

Materials and methods

Patients, sample collection, and DNA preparation

The study involved 34 patients with OS treated between 1996 and 2004 at A C Camargo Hospital, São Paulo, Brazil and at the Pediatric Oncology Institute, GRAACC/Federal University of São Paulo, São Paulo, Brazil. Fresh OS biopsies were collected at diagnosis, without previous chemotherapy treatment. Additionally, four fresh normal bone specimens (distal femurs) from patients without bone related sarcomas who underwent inferior member amputation during radical surgery treatment at Barretos Cancer Hospital, Barretos, Brazil were included as normal controls.

Tissue sections of all samples were stained with hematoxylin and eosin according to standard procedures and reviewed by a senior pathologist in order to confirm the diagnosis of OS. Clinical information was collected from the patients' medical records. The study was approved by the Ethics Committees of all the institutions.

DNA was isolated from bone specimens using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendations.

Bisulfite treatment

Bisulfite treatment of DNA converts unmethylated cytosines to uracil, while the methylated ones remain as cytosines. Sodium-bisulfite conversion of 2 μg of DNA was performed according to a previously described method with modifications.[27] In brief, 2 μg of DNA from each sample was denatured in 0.2 M of NaOH for 20 minutes at 50°C (in a total volume of 20 μL). The denatured DNA was diluted in 500 μL of bisulfite solution (2.5 M of sodium metabisulfite, 125 mM of hydroquinone, 350 mM of sodium chloride, pH 5.0) and incubated for 3 hours at 70°C in the dark. Bisulfite-modified DNA was purified using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions and eluted in 45 μL of 80°C water. After treatment with NaOH (final concentration 0.3 M) for 10 minutes at room temperature, the treated DNA was precipitated by the addition of 75 μL of ammonium acetate, 2.5 volumes of ethanol, and 2 μL of glycogen (5 mg/mL). Each resulting DNA pellet was washed with 70% ethanol, dried, dissolved in 110 μL of water, and stored at −80°C.

Gene selection

After a literature examination and mining in different public databases, 18 genes were selected for the evaluation of methylation abnormalities. All genes analyzed in this study were previously reported as targets for epigenetic silencing in different human cancers. The majority of these genes present tumor suppressor activities and their silencing could contribute to the tumorigenesis process. Among these genes are *CCNA1*, *CDKN2A*, *HIC1*, *p14ARF*, *RB1*, and *SOCS1* which are involved in cell cycle control; *CDH1* in cell adhesion; *ESR1*, *APC*, *DAPK*, *RASSF1A*, *RARβ*, and *THBS1* in signal transduction processes; *GSTP1* in cell detoxification; *MLH1* in DNA repair; *CALCA* in cell-cell signaling processes; and *SFRP1* in cell differentiation and proliferation. The methylation pattern of *AIM1* was also examined, but its function is not yet well understood. It has been previously shown that these genes are affected by aberrant promoter methylation in association with transcription silencing in different types of human malignancies.[28–34]

QMSP PCR analyses

The QMSP PCR analyses were conducted as previously described.[35] Basically, bisulfite-modified DNA was used as a template in fluorogenic QMSP assays carried out in a final volume of 20 μL in a 7500 Real Time PCR System (Life Technologies). PCR was done in separate wells for each primer/probe set and each sample was run in triplicate. The final reaction mixture contained 3 μL of bisulfite-modified DNA, 1.2 μM of forward and reverse primers, 200 nM of probe, 0.6 U of platinum Taq polymerase (Life Technologies), 200 μM of dNTPs, 16.6 mM of ammonium sulfate, 67 mM of Tris-HCl pH 8.0, 6.7 mM of magnesium chloride (2.5 mM for CDKN2A), 10 mM of mercaptoethanol, 0.1% DMSO and 1× ROX dye (Life Technologies). PCR was conducted with the following conditions: 95°C for 2 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Each plate included DNA samples, multiple water blanks, a negative control (normal leukocyte DNA), and serial dilutions (90–0.009 ng) of a positive control for constructing the calibration curves. Leukocyte DNA from a healthy individual was methylated in vitro with SssI methyltransferase (New England Biolabs Inc, Ipswich, MA, USA) to generate completely methylated DNA at all CpG and used as a positive control.

Primers and probes were designed to specifically amplify the promoter regions of the 18 genes of interest and the internal reference gene, *ACTB* (Supplementary Table S1). The relative level of methylated DNA of each gene in each sample was determined as a ratio of MSP PCR-amplified gene to *ACTB* and then multiplied by 100 for easier tabulation (average value of triplicates of specific gene divided by the average value of triplicates of *ACTB* × 100). Cases were scored as positive if a percentage value of ≥0.1% was obtained. This cutoff was chosen for being clinically relevant and also to exclude very low-level background readings that can occur in certain individual for certain genes.[36]

Statistical analysis

SPSS 13.0 (IBM Corporation, Armonk, NY, USA) for Windows was used for all statistical analyses. Descriptive statistics were used to summarize study data. For all analysis we considered statistical significance when P-value <0.05. Comparisons between clinical-demographic variables and methylation patterns were performed using the chi-square test or Fisher's exact test. Survival curves were estimated using the Kaplan–Meier method. Survival data were censored for patients alive at the last observation. The log-rank test was used to compare survival outcomes. The univariate Cox regression model was used to evaluate the methylation level influence in the overall survival.

Results

Patient characteristics and clinical predictors

Clinical characteristics of the patients are summarized in Table 1. The age at diagnosis ranged from 7 to 29 years (median of 14 years). The most frequent site of the primary tumor was the femur (52.9%), followed by tibia (26.5%), humerus (8.8%), and fibula (5.9%). Metastasis at diagnosis was detectable in 29.4% of the cases. Osteoblastic (61.8%) was the most common histological subtype, followed by chondroblastic (17.6%) and telangiectasic (5.9%).

The necrosis grade (Huvos Grade) was evaluated in the surgical specimens after neoadjuvant chemotherapy and 29.4% of patients were classified as Huvos I (less than 50% of tumor necrosis), 29.4% as Huvos II (51% to 90% of necrosis), 20.6% as Huvos III (90% to 99% of necrosis), and 20.6% as Huvos IV (100% of necrosis). Recurrent disease was observed in 52.9% of patients with 17.7% showing combined local and lung relapses, 8.8% local recurrence alone, 20.6% isolated pulmonary relapses, and 5.8% bone relapse at a different site from the primary one (data not shown).

QMSP PCR in osteosarcoma

In the first series, the promoter methylation status of 18 genes was evaluated in DNA from 13 OS biopsy samples, collected

Table 1 Clinical characteristics of the osteosarcoma patients included in this study

Patient characteristics	Number of patients (%)
Total	34 (100)
Gender	
Male	16 (47.1)
Female	18 (52.9)
Age (years)	
Median age	14
Range	7–29
Primary site	
Femur	18 (52.9)
Tibia	9 (26.5)
Humerus	3 (8.8)
Fibula	2 (5.9)
Others	2 (5.9)
Metastasis at diagnosis	
Yes	10 (29.4)
No	24 (70.6)
Histology	
Osteoblatic	21 (61.8)
Chondroblastic	6 (17.6)
Telangiectasic	2 (5.9)
Not evaluated	5 (14.7)
HUVOS	
I	10 (29.4)
II	10 (29.4)
III	7 (20.6)
IV	7 (20.6)
Recurrence	
Yes	18 (52.9)
No	16 (47.1)

at diagnosis, without any previous treatment. This analysis showed that *CDKN2A*, *CCNA1*, *GSTP1*, *THBS1*, *RB1*, and *DAPK* were unmethylated in the tested samples and the hypermethylation of *RASSF1A* (7.6%), *GSTP1* (10.0%), *RARβ* (15.3%), *APC* (23.0%), *SOCS1* (23.0%), and *MLH1* (23.0%) were rare events. Conversely, *ESR1* (30.7%), *AIM1* (30.7%), *p14ARF* (61.5%), *SFRP1* (61.5%), *CALCA* (76.0%), *CDH1* (76.0%), and *HIC1* (92.0%) were found to be frequently methylated (Table 2). Representative examples of QMSP results are shown in Supplementary Figure S1. The most appropriate genes for the additional analyses were those that were shown to be frequently methylated in the OS samples evaluated in the first series. Thus, in the second series, the presence of promoter methylation of seven genes, namely, *AIM1*, *CALCA*, *CDH1*, *ESR1*, *HIC1*, *p14ARF*, and *SFRP1*, was tested in 21 additional cases and four normal bone samples.

By the end, *CALCA* was methylated in 79.4% of all analyzed cases (27/34), *SFRP1* and *HIC1* in 76.5% (26/34), *CDH1* in 61.8% (21/34), *AIM1* in 38.2% (13/34), *p14ARF*

Table 2 Promoter methylation frequency for the 18 genes analyzed in osteosarcoma samples (pilot group n = 13 and total group n = 34) and in the normal control group (n = 4)

Genes	Osteosarcoma n (%)		Normal control n (%)
	Pilot group	Total group	
HIC1	12 (92.0)	26 (76.5)	3 (75.0)
CDH1	10 (76.0)	21 (61.8)	2 (50.0)
CALCA	10 (76.0)	27 (79.4)	3 (75.0)
SFRP1	8 (61.5)	26 (76.5)	2 (50.0)
p14ARF	8 (61.5)	8 (23.5)	0 (0.0)
AIM1	4 (30.7)	13 (38.2)	0 (0.0)
ESR1	4 (30.7)	5 (14.7)	0 (0.0)
MLH1	3 (23.0)		
APC	3 (23.0)		
SOCS1	3 (23.0)		
RARB	2 (15.3)		
RASSF1A	1 (7.6)		
CDKN2A	0 (0.0)		
CCNA1	0 (0.0)		
GSTP1	0 (0.0)		
RB1	0 (0.0)		
DAPK	0 (0.0)		
THBS1	0 (0.0)		

in 23.5% (8/34), and *ESR1* in 14.7% (5/34) (Table 2). Although *CALCA*, *HIC1*, *CDH1*, and *SFRP1* were found to be methylated in OS samples, they were also methylated in the normal bone samples used as the control (75%, 75% 50%, and 50%, respectively) and, for this reason, they were not good candidates for tumor markers. On the other hand, the methylation of *AIM1*, *ESR1*, and *p14ARF* seems to be specific to tumor samples because no hypermethylation was detected in the control samples (Table 2).

Methylation levels and clinical-pathologic correlations

The methylation patterns of *AIM1*, *ESR1*, and *p14ARF* were analyzed for potential correlations with clinical characteristics of patients with OS, including age, gender, primary tumor site, histologic subtype, Huvos Grade, presence of metastasis at diagnosis, and recurrence. Hypermethylation of *p14ARF* was significantly associated with the absence of metastasis ($P = 0.041$), while unmethylation of *AIM1* was associated with the chondroblastic OS histological subtype ($P = 0.038$) (Table 3). No significant correlation was observed between the other clinical features and methylation status of the remaining genes tested.

The 5-year overall survival for all OS patients included in this study was 64%. There was no significant difference in overall survival by gender ($P = 0.184$), age ($P = 0.690$), primary tumor site ($P = 0.096$), histological subtype

Table 3 Correlations between hypermethylation pattern and clinicopathological parameters of OS patients evaluated

Patient characteristics	*AIM1*			*ESR1*			*p14ARF*		
	M (%)	U (%)	*P*-value	M (%)	U (%)	*P*-value	M (%)	U (%)	*P*-value
Age (years)									
$<$10	1 (25.0)	3 (75.0)	0.384	0 (0)	4 (100)	0.591	2 (50.0)	2 (50.0)	0.369
10 to 19	11 (37.9)	18 (62.1)		5 (17.9)	23 (82.1)		6 (20.7)	23 (79.3)	
$>$19	1 (100.0)	0 (0)		0 (0)	1 (100.0)		0 (0)	1 (100.0)	
Gender									
Female	7 (38.9)	11 (61.1)	0.607	2 (11.8)	15 (88.2)	0.47	4 (22.2)	14 (77.8)	0.583
Male	6 (37.5)	10 (62.5)		3 (18.8)	13 (81.3)		4 (25.0)	12 (75.0)	
Primary site									
Femur	8 (44.4)	10 (55.6)	0.778	1 (5.6)	17 (94.4)	0.184	6 (33.3)	12 (66.7)	0.537
Tibia	3 (33.3)	6 (66.7)		3 (33.3)	6 (66.7)		2 (22.2)	7 (77.8)	
Fibula	0 (0)	2 (100)		0 (0)	2 (100)		0 (0)	2 (100)	
Humerus	1 (33.3)	2 (66.7)		0 (0)	2 (100)		0 (0)	2 (100)	
Metastasis at diagnosis									
No	10 (41.7)	14 (58.3)	0.406	4 (16.7)	20 (83.3)	0.582	8 (33.3)	16 (66.7)	0.041
Yes	3 (30.0)	7 (70.0)		1 (11.1)	8 (88.9)		0 (0)	10 (100)	
Histology									
Osteoblatic	10 (47.6)	11 (52.4)	0.038	3 (14.3)	18 (85.7)	0.41	6 (28.6)	15 (71.4)	0.776
Chondroblastic	0 (0)	6 (100.0)		1 (20.0)	4 (80.0)		1 (16.7)	5 (83.3)	
Telangiectasic	2 (100)	0 (0)		1 (50.0)	1 (50.0)		0 (0)	2 (100)	
HUVOS									
I and II	7 (41.2)	10 (58.8)	0.475	4 (23.5)	13 (76.5)	0.344	3 (17.6)	14 (82.4)	0.693
III and IV	5 (45.5)	6 (54.5)		1 (91.9)	10 (90.9)		3 (27.3)	8 (72.7)	
Recurrence									
Yes	6 (33.3)	12 (66.7)	0.393	3 (17.6)	14 (82.4)	0.53	3 (16.7)	13 (83.3)	0.276
No	7 (43.8)	9 (56.3)		2 (12.5)	14 (87.5)		5 (31.3)	11 (68.8)	
Status									
Alive	10 (43.5)	13 (56.5)	0.301	2 (9.1)	20 (90.9)	0.193	6 (26.1)	17 (73.9)	0.481
Dead	3 (27.3)	8 (72.7)		3 (27.3)	8 (72.7)		2 (18.2)	9 (81.8)	

Abbreviations: M, methylated; OS, osteosarcoma; U, unmethylated.

(P = 0.343), and Huvos Grade (P = 0.407). On the other hand, as expected, the presence of metastasis at diagnosis (16.7% metastatic at diagnosis versus 86.8% nonmetastatic; P = 0.001) and tumor recurrence after pre-adjuvant chemotherapeutic treatment (41.3% with recurrence versus 92.9% nonrecurrence; P = 0.024) influenced the overall survival of OS patients (Table 4).

There was no significant association between the overall survival and the hypermethylation profile of the genes investigated. However, although the difference in overall survival between patients with and without *ESR1* hypermethylation was not significant (70.4% hypermethylated versus 30% non-methylated; P = 0.059), the patients with methylated *ESR1* seem to have had a worse prognosis when compared with OS patients with unmethylated *ESR1* (hazard ratio = 3.554; confidence interval = 0.873–14.475; P = 0.077) (Table 4 and Figure 1).

Discussion

Many studies have demonstrated the importance of DNA hypermethylation in the extinction of tumor suppressor gene activity in different human cancers. Considerable variations exist in promoter methylation profiles of different cancers, such that, individual tumor types have characteristic methylation profiles.[21]

To date, few studies have attempted to assess the OS methylation pattern. All but one of them relied on the conventional MSP PCR approach and evaluated a few genes in a small number of cases.[15,16,26] Thus, the present study is pioneering in its use of the QMSP approach to conduct an extensive analysis of the OS methylation profile. Given the sensitivity of the QMSP technique used to detect the presence of methylated alleles in a background of normal cells at a threshold of 1/1000 to 1/10,000, this strategy allowed us to define methylated genes that were highly specific for tumor, and rarely or never present in normal bone.[37]

In the present study, we evaluated the hypermethylation pattern of 18 genes in 34 OS cases. Of note, this panel of 18 genes included genes already reported to be methylated in OS (*CDKN2A*, *DAPK*, *RASSF1A*, and *p14ARF*) and genes not yet evaluated in this neoplasia (*AIM1*, *APC*, *CALCA*,

Table 4 Univariate analysis of selected factors for overall survival

Patient characteristics	Number of patients	Deaths (n)	5-year survival (%)	*P*-value (log-rank)	Hazard ratio for local recurrence (95% CI)	*P*-value
Age (years)						
<10	4	2	50.0	0.690	Reference	
10 to 19	29	9	65.2		0.628 (0.135–2.918)	0.553
>19	1	0	n/a		n/a	0.991
Gender						
Female	18	8	56.5	0.184	Reference	
Male	16	3	75.0		0.418 (0.111–1.578)	0.198
Primary site						
Femur	18	3	88.9	0.096	Reference	
Tibia	9	5	40.0		3.954 (0.937–16.676)	0.061
Fibula	2	0	n/a		n/a	0.990
Humerus	3	1	50.0		2.257 (0.230–22.168)	0.485
Others	2	2	0.0		7.158 (1.173–43.679)	0.033
Metastasis at diagnosis						
No	24	4	86.8	0.001	Reference	
Yes	10	7	16.7		6.995 (1.788–27.358)	0.005
Histology						
Osteoblatic	21	5	78.3	0.343	Reference	
Chondroblastic	6	3	40.0		3.151 (0.702–14.156)	0.134
Telangiectasic	2	1	0.0		4.126 (0.448–37.964)	0.211
Not evaluated	5	2	60.0		1.970 (0.359–10.797)	0.435
HUVOS						
I and II	17	7	54.6	0.407	Reference	
III and IV	11	2	77.9		0.364 (0.075–1.773)	0.211
Not evaluated	6	2	80		1.004 (0.207–4.873)	0.996
Recurrence						
No	16	2	92.9	0.024	Reference	
Yes	18	9	41.3		4.995 (1.067–23.385)	0.041
AIM1 status						
U	21	8	60.2	0.391	Reference	
M	13	3	70.7		0.563 (0.149–2.129)	0.397
ESR1 status						
U	28	8	70.4	0.059	Reference	
M	5	3	30.0		3.554 (0.873–14.475)	0.077
p14ARF status						
U	26	9	53.9	0.390	Reference	
M	8	2	87.5		0.514 (0.110–2.406)	0.398

Abbreviations: CI, confidence interval; M, methylated; U, unmethylated.

CCNA1, *CDH1*, *ESR1*, *GSTP1*, *HIC1*, *MLH1*, *THBS1*, *RARβ*, *SFRP1*, and *SOCS1*).

OS is the most commonly diagnosed primary malignancy of bone, particularly among children and adolescents; however, it is rare, representing less than 1% of all cancers. Due to restrictions in the available amount of DNA from some cases, we first investigated the panel of 18 genes in 13 tumor samples and then the seven most frequently methylated genes in a second series of cases (n = 21) and in four normal bone samples. We were able to define a three-gene panel (*AIM1*, *p14ARF*, and *ESR1*) for which methylation was correlated with OS cases. Worth mentioning, the clinical and pathological characteristics of the cohorts analyzed in the first and second series were similar.

AIM1 (*absent in melanoma 1*) is one of the newest cancer-associated genes discovered and its function is poorly understood. It was found to be involved in melanoma tumorigenesis and in calcium binding.[38] Ray et al described a possible role of *AIM1* in processes of stress response, differentiation, and changes in cell morphology through interactions with the cytoskeleton.[38] This gene was found to be methylated in different tumors such as lung, bladder, and nasopharyngeal carcinoma,[39–41] but there is no previous report of *AIM1* methylation in OS.

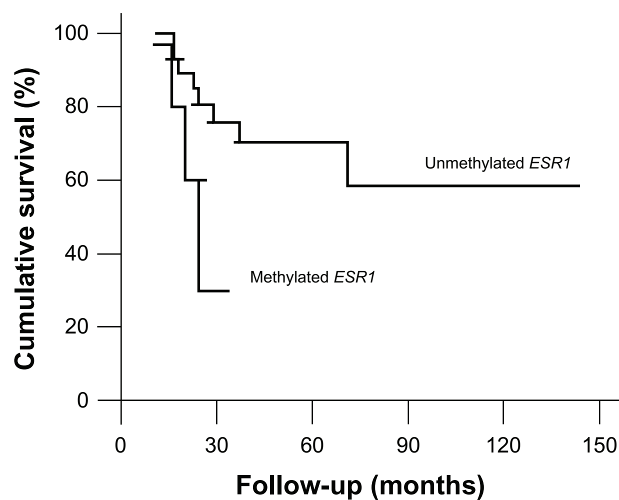

Figure 1 Kaplan–Meier curve comparing the probability of survival in patients with positive or negative hypermethylation of *ESR1* in osteosarcoma samples.
Note: Kaplan–Meier survival curve according to *ESR1* hypermethylation ($P = 0.059$).

According to our results, the absence of methylation in the *AIM1* promoter region is significantly correlated with the OS chondroblastic histological subtype. Worth mentioning, *AIM1* is localized in chromosome 6 (6q21) and this locus is described as frequently deleted in OS.[42,43] Thereby, not only hypermethylation, but also chromosomal deletion, is contributing to *AIM1* silencing in OS, corroborating its role as a tumor suppressor gene.

The *p14ARF* gene is encoded in the INK4a/ARF locus, situated on chromosome 9p21, which also encodes the cyclin inhibitor *CDKN2A*. The *p14ARF* protein is translated from an alternative reading frame of *CDKN2A*.[44] The *p14ARF* gene induces growth arrest and acts as a negative regulator of cell proliferation.[45] It has been demonstrated that *p14ARF* binds to MDM2 and inhibits the ubiquitination of p53, thereby stabilizing p53.[46] *p14ARF* is regulated mainly at the transcriptional level by DNA hypermethylation of its promoter region, which has come to the forefront of many studies.[47] Oh et al evaluated the hypermethylation profile of the *p14ARF* promoter region in 32 OS samples using conventional MSP.[16] They were able to detect *p14ARF* methylation in 47% of the samples and this alteration was correlated to worse overall survival (79% of survival for unmethylated versus 31% of survival for methylated, $P = 0.03$). Controversially, in our study, *p14ARF* was found to be methylated in 23.5% of the OS cases and this methylation was associated with the absence of metastases at diagnosis ($P = 0.04$), a clinical indicator for favorable outcome. So the hypermethylation of *p14ARF* seems to be correlated with a favorable prognosis for OS patients. Other studies with different tumor types have also identified the aberrant methylation of *p14ARF* as a favorable prognostic factor. Sailasree et al analyzed 116 oral tumor samples and concluded that methylation of *p14ARF* is related to a low rate of local recurrence and a better prognosis compared with patients who present this gene unmethylated.[48]

ESR1 encodes the estrogen receptor 1, a protein that can initiate or enhance gene transcription in response to estrogen stimulation. Estrogen has a multifunctional role, influencing the growth, differentiation, and function of different tissues. In bone tissue, estrogen has an important role in regulating bone growth during puberty and bone remodeling in adults.[49] Some authors have suggested that the estrogen effect mediated by *ESR1* is involved with bone mineralization and the homozygous deletion of this gene could be associated to severe osteoporosis and increased bone turnover.[50] In addition, several studies have shown the presence of these receptors in osteoblasts and osteoclasts, but with different activities.[51] This gene was found to be methylated in 14.7% of the samples and this group of patients showed a worse 5-year overall survival (non-methylated: 60%; methylated: 30%, $P = 0.059$). Despite the fact that this difference was not statistically significant, the survival curves show a clear trend to separate the two groups. We do not exclude the possibility that the trend to significant association observed between aberrant methylation of *ESR1* and poor prognosis could be due to the small sample size. Susman analyzed the expression of *ESR1* in tumor samples from 110 OS patients by immunohistochemistry and found that *ESR1* expression was associated with localized disease at presentation and improved outcomes.[52] According to Susman, high levels of *ESR1* inhibited tumor proliferation and *ESR1* expression could be associated with event-free survival. Issa et al have already demonstrated that hypermethylation is an important mechanism of transcriptional repression and gene silencing for the *ESR1* gene.[53] So, taking that into account, our findings are in complete concordance with Susman's because both studies found that the OS patients expressing *ESR1* (unmethylated cases) presented better overall survival rates.

The data generated in this study do not allow us to know if the aberrant methylation of *ESR1* could be useful only as a marker of tumor progression or could serve to disrupt directly a gene that is critical to the biology of OS. But, for the first time, it was suggested that *ESR1* aberrant methylation is a relevant molecular alteration related with poor prognosis of OS patients. We trust that this study shed some light on this field and more analyses are needed to dissect the association

between the *ESR1* pathway and OS. Besides, our results, together with other findings, provide some evidence that aberrant methylation is a common event in OS and suggest that patients with this bone neoplasia could also have benefited by demethylating drug treatments. However, this hypothesis needs to be proved by in vitro functional studies before using demethylating agents in OS clinical trials.

Histologic response to chemotherapy is currently the best prognostic parameter in OS, but it can be evaluated only after several weeks of chemotherapy. Thus, a prognostic parameter known at the time of diagnosis would be of great clinical benefit. We do not exclude the possibility that the limited number of cases available for analysis could bias our findings; however our data suggest that *p14ARF* and *ESR1* methylation status may be useful as prognostic markers for OS. Of note, a larger patient cohort needs to be evaluated in support of our findings. In the future, preoperative tests could be performed and the methylation status of these genes could help in the choice of the best therapy scheme to be adopted.

Conclusion

This study represents the largest quantitative evaluation of the methylation profile of OS. We demonstrated that aberrant methylation is a frequent event in OS and our data provide further evidence that aberrant methylation of *p14ARF* was significantly associated with the absence of metastasis at diagnosis and *ESR1* methylation could be correlated with poor prognosis.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Table S1 Primers and probes used in the quantitative methylation-specific assays

Gene	Sequence (5′–3′)		Reference
ACTB	Forward	TGGTGATGGAGGAGGTTTAGTAAGT	Eads et al[28]
	Reverse	AACCAATAAAACCTACTCCTCCCTTAA	
	Probe	ACCACCACCCAACACACAATAACAAACACA	
AIM1	Forward	CGCGGGTATTGGATGTTAGT	Carvalho et al[29]
	Reverse	CCGACCCACCTATACGAAAA	
	Probe	GGGAGCGTTGCGGATTATTCGTAG	
APC	Forward	GAACCAAAACGCTCCCCAT	Eads et al[28]
	Reverse	TTATATGTCGGTTACGTGCGTTTATAT	
	Probe	CCCGTCGAAAACCCGCCGATTA	
CALCA	Forward	GTTTTGGAAGTATGAGGGTGACG	Eads et al[28]
	Reverse	TTCCCGCCGCTATAAATCG	
	Probe	ATTCCGCCAATACACAACAACCAATAAACG	
CCNA1	Forward	TCGCGGCGAGTTTATTCG	Carvalho et al[29]
	Reverse	CCGACCGCGACAAACG	
	Probe	CGTTATGGCGATGCGGTTTCGG	
CDH1	Forward	AATTTTAGGTTAGAGGGTTATCGCGT	Eads et al[28]
	Reverse	TCCCCAAAACGAAACTAACGAC	
	Probe	CGCCCACCCGACCTCGCAT	
CDKN2A	Forward	TTATTAGAGGGTGGGGCGGATCGC	Harden et al[30]
	Reverse	GACCCCGAACCGCGACCGTAA	
	Probe	AGTAGTATGGAGTCGGCGGCGGG	
DAPK	Forward	GGATAGTCGGATCGAGTTAACGTC	Harden et al[30]
	Reverse	CCCTCCCAAACGCCGA	
	Probe	TTCGGTAATTCGTAGCGGTAGGGTTTGG	
ESR1	Forward	AGTTGGCGGAGGGCGTTC	Eads et al[28]
	Reverse	GACACGCGAACTCTAACCCCG	
	Probe	CGATAAAACCGAACGACCCGACGA	
GSTP1	Forward	AGTTGCGCGGCGATTTC	Jeronimo et al[31]
	Reverse	GCCCCAATACTAAATCACGACG	
	Probe	CGGTCGACGTTCGGGGTGTAGCG	
HIC1	Forward	GTTAGGCGGTTAGGGCGTC	Eads et al[28]
	Reverse	CAACAACTACCTAAAATAACCGAACG	
	Probe	CAACATCGTCTACCCAACACACTCTCCTACG	
MLH1	Forward	CGTTATATATCGTTCGTAGTATTCGTGTTT	Eads et al[28]
	Reverse	CTATCGCCGCCTCATCGT	
	Probe	CGCGACGTCAAACGCCACTACG	
p14ARF	Forward	ACGGGCGTTTTCGGTAGTT	Eads et al[28]
	Reverse	CCGAACCTCCAAAATCTCGA	
	Probe	CGACTCTAAACCCTACGCACGCGAAA	
RARB	Forward	GGGATTAGAATTTTTTATGCGAGTTGT	Hoque et al[32]
	Reverse	TACCCCGACGATACCCAAAC	
	Probe	TGTCGAGAACGCGAGCGATTCG	
RASSF1A	Forward	GCGTTGAAGTCGGGGTTC	Lehmann et al[33]
	Reverse	CCCGTACTTCGCTAACTTTAAACG	
	Probe	ACAAACGCGAACCGAACGAAACCA	
RB1	Forward	TTAGTTCGCGTATCGATTAGCG	Eads et al[28]
	Reverse	ACTAAACGCCGCGTCCAA	
	Probe	TCACGTCCGCGAAACTCCCGA	
SFRP1	Forward	GAATTCGTTCGCGAGGGA	Weisenberger et al[34]
	Reverse	AAACGAACCGCACTCGTTACC	
	Probe	CCGTCACCGACGCGAAAACCAAT	
SOCS1	Forward	GCGTCGAGTTCGTGGGTATTT	Müller et al[54]
	Reverse	CCGAAACCATCTTCACGCTAA	
	Probe	ACAATTCCGCTAACGACTATCGCGCA	

(*Continued*)

Table S1 (*Continued*)

Gene	Sequence (5′–3′)		Reference
THBS1	Forward	CGACGCACCAACCTACCG	Eads et al[28]
	Reverse	GTTTTGAGTTGGTTTTACGTTCGTT	
	Probe	ACGCCGCGCTCACCTCCCT	

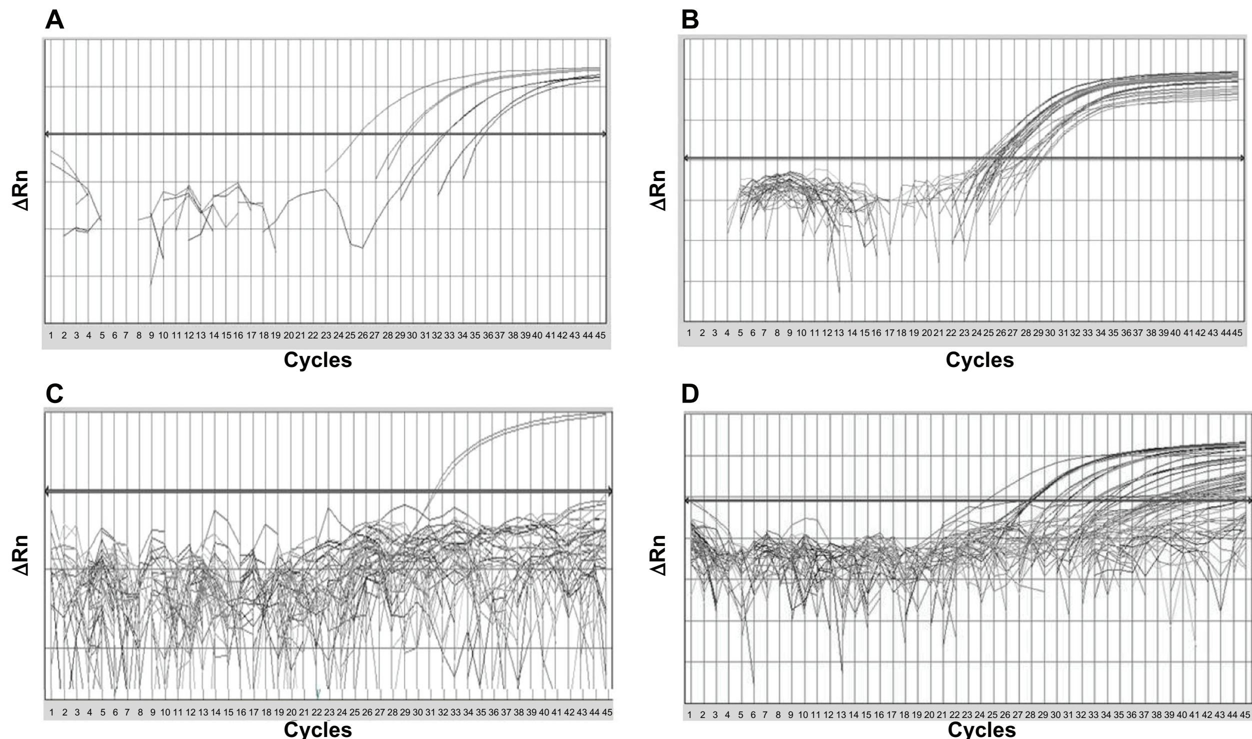

Figure S1 Amplification plots representing the quantitative methylation-specific results.

Notes: (**A**) Calibration curves constructed by serial dilutions (90–0.009 ng) of a leukocyte DNA methylated in vitro. (**B**) Amplification of all samples by the reference gene *ACTB*. (**C**) A frequently unmethylated gene showing the amplification of the positive control only (leukocyte DNA methylated in vitro). (**D**) A frequently methylated gene.

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