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RASSF2 methylation is a strong prognostic marker in younger age patients with Ewing sarcoma

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Ras-association domain family of genes consist of 10 members (*RASSF1–RASSF10*), all containing a Ras-association (RA) domain in either the C- or the N-terminus. Several members of this gene family are frequently methylated in common sporadic cancers; however, the role of the *RASSF* gene family in rare types of cancers, such as bone cancer, has remained largely uninvestigated. In this report, we investigated the methylation status of *RASSF1A* and *RASSF2* in Ewing sarcoma (ES). Quantitative real-time methylation analysis (MethyLight) demonstrated that both genes were frequently methylated in Ewing sarcoma tumors (52.5% and 42.5%, respectively) as well as in ES cell lines and gene expression was upregulated in methylated cell lines after treatment with 5-aza-2'-deoxycytidine. Overexpression of either RASSF1A or RASSF2 reduced colony formation ability of ES cells. *RASSF2* methylation correlated with poor overall survival ($P = 0.028$) and this association was more pronounced in patients under the age of 18 y ($P = 0.002$). These results suggest that both *RASSF1A* and *RASSF2* are novel epigenetically inactivated tumor suppressor genes in Ewing sarcoma and *RASSF2* methylation may have prognostic implications for ES patients.

Introduction

Ewing sarcoma (ES) is characterized by the t(11;22)(q24;q12) chromosomal translocation. This translocation fuses the *EWSR1* gene on chromosome 22 to the *FLI1* gene on chromosome 11 and encodes the EWS/FL1 fusion protein, which contributes to pathogenesis of ES by modulating the expression of target genes.[1] Ewing sarcoma is a rare but highly malignant tumor of children and young adults with approximately 15–25% of patients present with metastasis. After osteosarcoma, Ewing sarcoma is the most common pediatric bone cancer. Cure rates for patients with localized tumors are approximately 70%, but survival rates for patients with metastasis/relapse are poor and require aggressive treatment. Therefore, elucidation of molecular pathways that play important roles in these tumors and development of biomarkers for diagnostic/prognostic purposes are required for improved patient outcome. One such avenue of research that has in recent years come to the forefront is epigenetic regulation of gene expression in relation to cancer development and establishment of methylation profiles that are specific for a particular tumor type. DNA methylation studies can lead to the development of biomarkers as well as understanding the processes involved in tumorigenesis.

Inactivation of tumor suppressor genes can occur by genetic and/or epigenetic mechanisms. Tumor-specific hypermethylation of gene promoters has been described for an increasing number of tumor suppressor genes. The recently discovered tumor suppressor gene, *RASSF1A*, is almost exclusively inactivated by tumor-specific hypermethylation of its promoter region.[2,3] Genes silenced by promoter hypermethylation can be reactivated by treatment with the demethylating drugs such as 5-aza-2'-deoxycytidine, either alone or in combination with histone deacetylases (HDAC) inhibitors such as trichostatin A. *RASSF1A* methylation has been analyzed in many frequently occurring cancers, but other members of the RASSF gene family have not been studied as widely and there is dearth of knowledge on the methylation status of this gene family in rarer types of cancer including bone cancer.[4,5] Therefore, we analyzed the methylation status of first two members of the Ras-association domain family of genes (*RASSF1A* and *RASSF2*) in a cohort of Ewing sarcomas and determined any association with clinical outcome.

Results and Discussion

We analyzed the methylation status of *RASSF1A* and *RASSF2* in Ewing Sarcoma (ES) cell lines and tumor samples using

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MethyLight, a very sensitive and quantitative assay. Mesenchymal stem cells (MSC) are thought to be the origin of Ewing sarcoma, hence human bone marrow derived MSC (hBMSC) were included in the MethyLight assay as controls. hBMSC were found to be unmethylated for both *RASSF1A* and *RASSF2* genes. Among the RASSF gene family, *RASSF1A* is the only gene that has been previously shown to be methylated in Ewing sarcoma.[6] In agreement with the previous report *RASSF1A* was completely methylated in 2 of the 3 ES cell lines analyzed, and *RASSF1A* gene was shown to be hypermethylated (PMR value > 100%) and re-expressed in methylated cell lines after treatment with 5-azaDC (**Fig. 1A and C**). *RASSF2* was partially methylated in 2/3 ES cell lines, qRT-PCR demonstrated upregulation of *RASSF2* gene expression in the methylated ES cell lines after treatment with 5-aza-DC (**Fig. 1A and B**; **Fig. S1A and B**).

We analyzed the methylation status of *RASSF1A* and *RASSF2* in a cohort of Ewing sarcomas (n = 55) (**Table S1**). *RASSF1A* and *RASSF2* were methylated in 52.5%, 42.5% of ES, respectively (**Fig. 2A**). Furthermore expression of RASSF2 was investigated in methylated and unmethylated ES samples by qRT-PCR. Hypermethylated samples showed significant reduction in the expression of RASSF2 in comparison to unmethylated samples (**Fig. 2B**). Overexpression of RASSF2 or RASSF1A in methylated ES cell lines caused a significant reduction in colony formation ability (**Fig. 2C**).

In our cohort we did not find any association of *RASSF1A* with overall survival. *RASSF2* methylation was associated with worst overall survival (P = 0.028) and this was much more pronounced in younger age patients (<18 y) (P = 0.002) (**Fig. 3**).

The RAS-association domain family (RASSF) of proteins consists of ten members, all containing a RA-domain (RAS-association domain) at either their C-terminus (classical *RASSF1–6*) or N-terminus (N-terminal *RASSF7–10*).[4,5] This domain enables RASSF family members to bind RAS. The classical RASSF members also possess a C-terminal SARAH domain (SALVADOR-RASSF-HIPPO). In *Drosophila melanogaster* these proteins can homo- or hetero-dimerize resulting in modifications to the Hippo signaling pathway and subsequent effects on growth and apoptosis while in humans several components of the Hippo pathway (WW45, LATS) are implicated as tumor suppressors.[7] *RASSF2* is the second member of the Ras-association domain family of proteins and contains a RA domain in the C-terminus. *RASSF2* has previously been shown to be methylated in various cancers including breast, colorectal, gastric, lung, oral and thyroid cancer.[8-12] Expression of *RASSF2* was found to be an independent prognostic factor in gastric cancer patients[10] and *RASSF2* methylation could be detected in fecal DNA from patients with gastrointestinal tumors.[13]

In functional terms RASSF2 is a novel pro-apoptotic effector of K-Ras and acts as a tumor suppressor gene.[14] Recently it was shown that loss of RASSF2 in lung cancer cells enhanced their tumorigenic potential and conferred resistance to chemotherapy.[15] We have previously demonstrated that RASSF2 associates with and stabilizes the pro-apoptotic kinase MST2 and contains a functional nuclear localization (NLS) signal that is important for its tumor suppressor gene function.[8,16] RASSF2 also forms a complex with and controls the prostate apoptosis response protein 4 (PAR-4) tumor suppressor.[17] Global gene expression analysis revealed that RASSF2 inhibits expression of genes involved in immune responses, angiogenesis and metastasis.[12] Song H et al.[18] demonstrated that RASSF2 depletion in mice lead to bone defects and subsequent hematopoietic anomalies and that RASSF2 regulates differentiation of osteoblasts and osteoclasts by inhibiting NF-KB signaling. Our preliminary data indicates that *RASSF2* (as well as *RASSF1A* and *RASSF10*) is also frequently methylated in osteosarcoma cell lines (**Fig. S1C**). Although the present study concentrated on *RASSF1A* and *RASSF2* methylation analysis in Ewing sarcoma, we also analyzed other members of the RASSF gene family in Ewing sarcoma cell lines *RASSF3–RASSF10* (*RASSF9* does not have a 5' CpG island hence it was not analyzed for methylation). *RASSF6* was found to be frequently methylated in ES cell lines and expression was upregulated in methylated ES cell lines after 5-azaDC treatment (**Fig. S2**). MethyLight analysis in ES patient cohort demonstrated frequent methylation of *RASSF6* (35%; **Fig. S2C**). *RASSF6* methylation was not associated with clinical outcome. In summary we have demonstrated that *RASSF2* is frequently methylated in Ewing sarcoma and methylation is associated with poor prognosis particularly in younger age patients. Furthermore we have shown that RASSF2 expression suppresses growth of ES cells and hence is biologically relevant. Further studies using a larger cohort of patients will be required to validate our findings.

Material and Methods

Cell culture and-5-aza-2'-Deoxycytidine treatment. The ES cell lines and 5-aza-2'-deocycytidine treatments were previously described in references 6 and 19.

Isolation and culture of hBMSCs. hBMSCs were purified from aliquots of heparinized bone marrow aspirates obtained from healthy patients undergoing osteotomy, after written consent and Institutional-Review Board authorization from IRCCS Istituto Ortopedico Galeazzi. In order to isolate the hBMSCs, Ficoll-Hypaque gradient (1.077 g/ml) (Sigma-Aldrich) was used.[20] Briefly, nucleated cells were collected at the interface, washed twice, suspended in DMEM with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine (Sigma-Aldrich), counted and plated at a concentration of 10^4 cells/cm^2. After 48 h non-adherent cells were removed and the adherent hBMSCs expanded in vitro for further experiments.

Patient DNA and RNA samples, cell lines and bisulfite modification. ES tumors (n = 55; **Table S1**) were grounded in liquid nitrogen and DNA was isolated using DNA isolation and purification kit (Roche Diagnostics Ltd). Total RNA was isolated from ES cell lines, tumors and hBMSC using TRIzoL reagent (Invitrogen) and DNase treated with DNA-Free, Ambion as previously described.[21] Bisulfite modification of genomic DNA (0.5–1 μg) from 55 ES tumors, three ES cell lines and two hBMSC, was performed using Qiagen Epi Tect kit (Qiagen) according to the manufacturer's instructions. The study was approved by the relevant Institutional Review Board/Ethics committees and is in

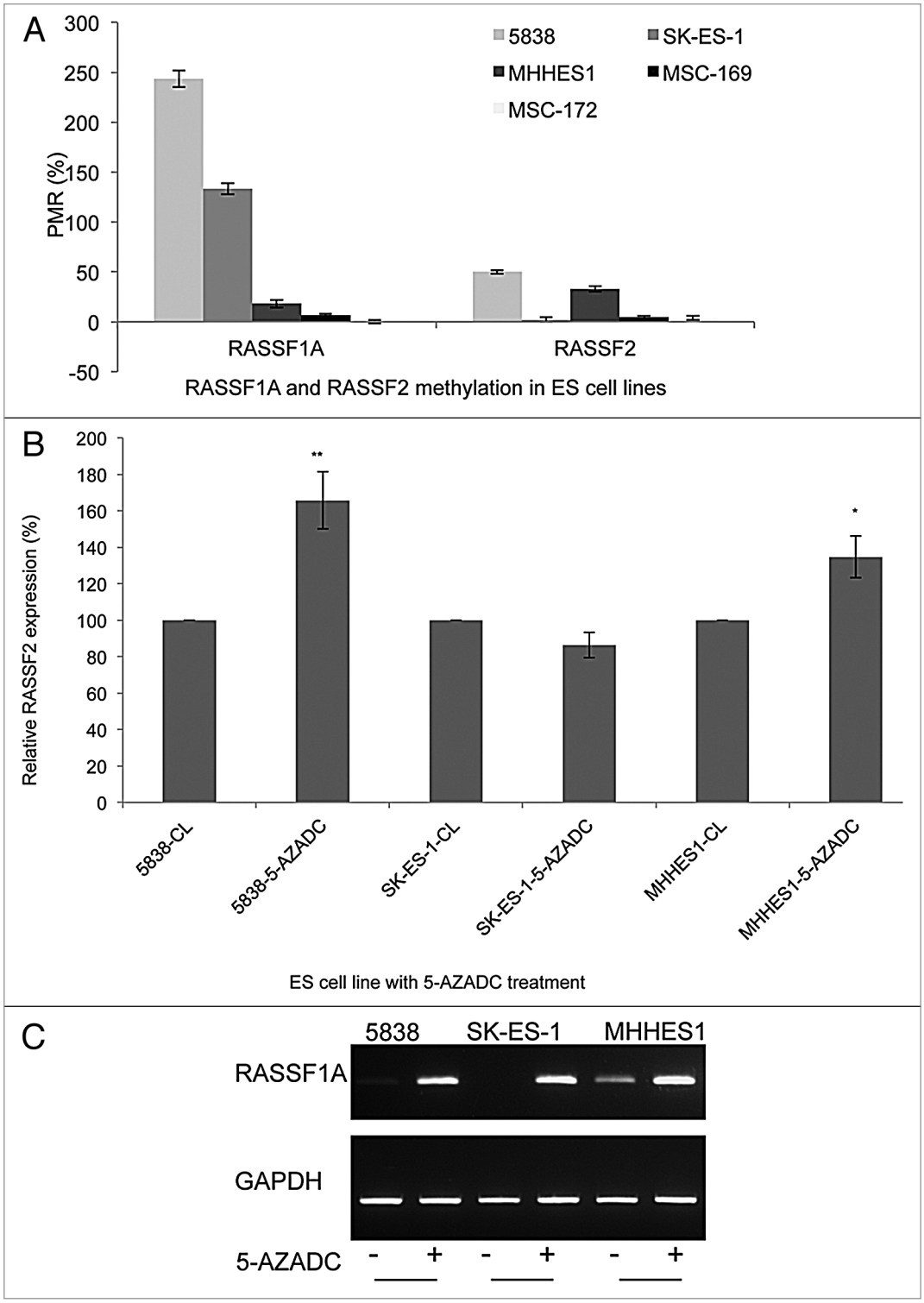

Figure 1. Methylation and expression profiling of *RASSF1A* and *RASSF2* in Ewing Sarcoma cell lines. (**A**) Methylation analysis of *RASSF1A* and *RASSF2* in ES cell lines and two hBMSC-control samples by MethyLight. A percentage of fully methylated reference (PMR) > 10 was considered methylated. n > 3; n, number of independent runs using at least 2 sets of bisulfite modified DNA. (**B**) Quantitative real time PCR analysis of *RASSF2* gene using SYBR green in 5-azaDC treated and untreated ES cell lines, Beta actin is used as reference. T-test: $*P < 0.05$; $**P < 0.01$ n = 4; n, number of independent runs using at least 3 RNA preparations. (**C**) RT-PCR analysis of *RASSF1A* expression in ES cell lines with and without 5-azaDC treatment. GAPDH is used as reference. n = 3; n, number of independent runs using at least 3 RNA preparations.

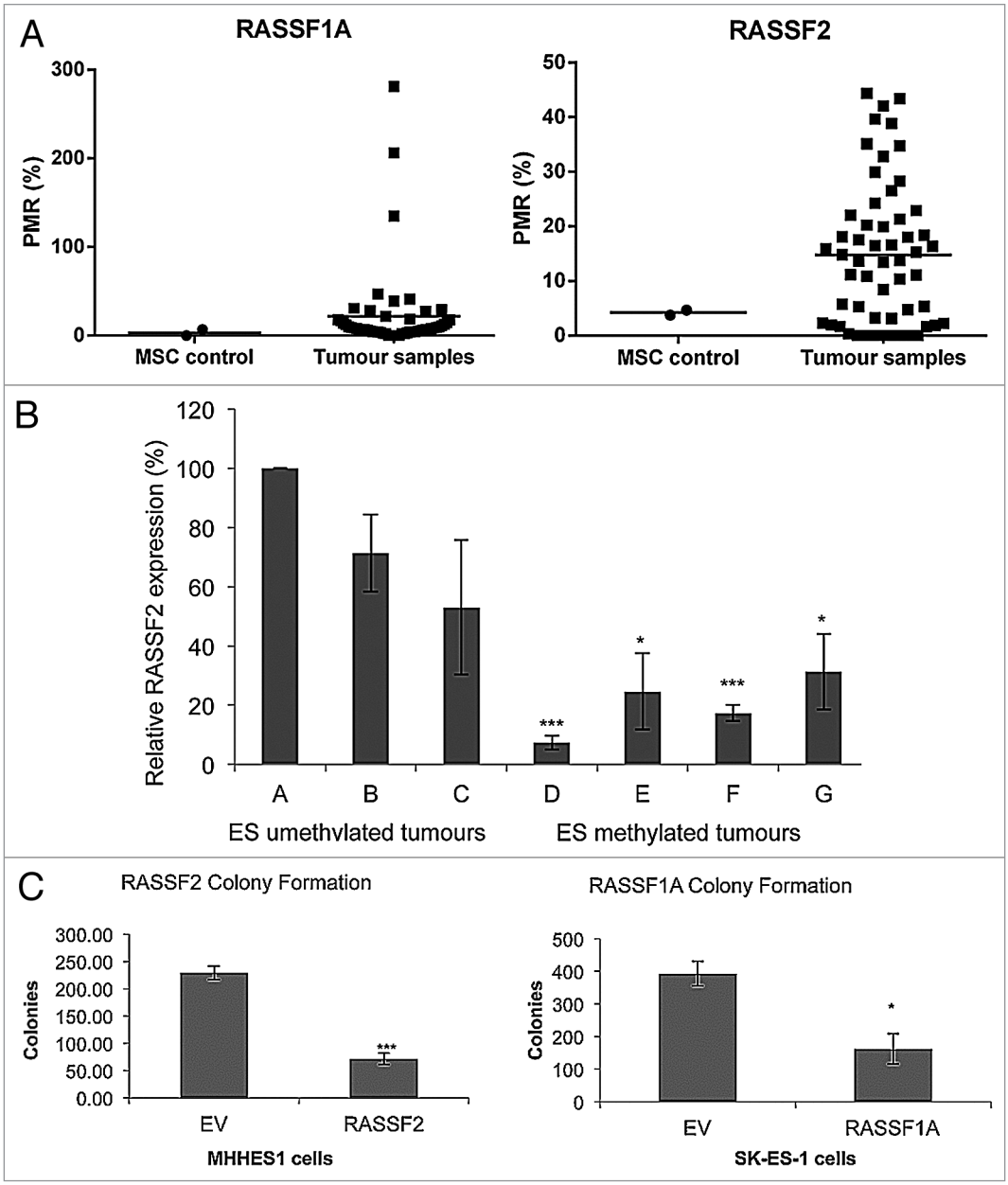

Figure 2. DNA methylation status of *RASSF1A* and *RASSF2* in ES samples and their effect on expression and cell growth. (**A**) Scatter plots of DNA methylation of *RASSF1A* and *RASSF2* in a cohort of 55 ES samples and in hBMSC controls. (**B**) Quantitative real time PCR analysis of *RASSF2* gene using SYBR green in ES methylated and unmethylated ES samples, Beta actin is used as reference. One of the unmethylated samples A was used as a control for ΔΔCT calculations. T-test: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n = 3; n, number of independent runs using at least 3 RNA preparations. (**C**) Exogenous re-expression of RASSF1A and RASSF2 genes in ES cell lines resulted in a significant reduction in in vitro colony formation compared with ES cell lines transfected with an empty vector (EV). Equal amounts of empty vector or the gene of interest were transfected into SK-ES-1 or MHHES1 cell lines. The experiments were performed in triplicates and the Student t-test was used for statistics. $^{*}P < 0.05$; $^{***}P < 0.001$.

accordance with the principles expressed in the Declaration of Helsinki.

DNA methylation analysis. Aberrant promoter methylation of the RASSF genes was determined using MethyLight and COBRA. The methylation analysis of *RASSF1A*, *RASSF2,* and *RASSF6* genes in the ES tumors and the cell lines were performed using MethyLight as previously described.[22] The ALU gene was used as an internal reference and a fully methylated genomic DNA (Qiagen) was used as a reference sample to generate standard curves. MethyLight data are presented as the percentage methylated reference (PMR), which is defined by the *GENE*:*ALU* ratio of a sample. Occasionally, a PMR value of more than 100 can be observed due to incomplete methylation of the reference DNA at a particular site. The primer and probe sequences for *RASSF1A* and the ALU gene were from.[22] The primer and probe sequences for *RASSF2* and *RASSF6* are presented in **Table 1**. COBRA and MSP primers and conditions for the RASSF genes were previously described.[19]

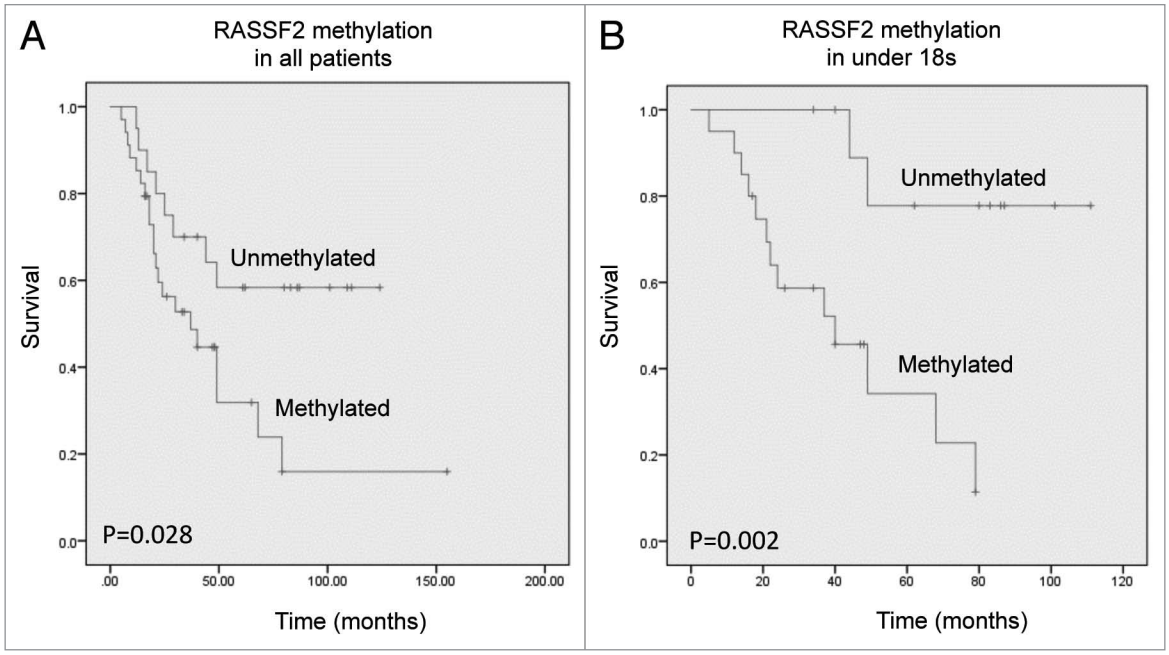

Figure 3. Kaplan-Meier survival curves for ES patients. (**A**) *RASSF2* methylation in the whole cohort of 55 patients; 34 methylated and 21 unmethylated measured by MethyLight. (**B**) *RASSF2* methylation in patients under the age of 18 y in a cohort of 31 patients; 20 methylated and 11 unmethylated measured by MethyLight. A percentage of fully methylated reference (PMR) >10 was considered methylated and the Log rank statistical test was performed.

Table 1. The primer and probe sequences for *RASSF2* and *RASSF6*

Gene Name	Forward 5'→3'	Reverse 5'→3'	Probe (FAM-BHQ)
RASSF2	GTTCGTCGTC GTTTTTTAGG C	ACCCTACGCC CCTCTAAAAC	TAGGTTTTAG TTTTCGGCGC G
RASSF6	GAAAAGGAGA AATAATTAAT AGTTTTTGG	CCCAAAACAT AACTCAACTA AAC	TTAGGATCGT TGATCGCGTC GGGGGTATT

Expression of *RASSF1A, RASSF2, RASSF6,* and *RASSF10* in Ewing sarcomas. One microgram RNA was used to create cDNA using the superscript III cDNA synthesis kit (Invitrogen). Expression primers used in RT-PCR were previously described by reference 19. The real time RT-PCR primers for RASSF2 were described in Schagdarsurengin U et al.[11] and human β actin primers were described in.[21] Power SYBR Green mix (Applied Biosystems) was used and the plates were read using BIO-RAD IQ5 machine. Relative expression was calculated as in Gharanei S et al.[21]

Plasmid constructs and colony formation assay. The RASSF1A and RASSF2 constructs have been described previously[3,8] and the transfections were performed accordingly. Briefly; 2 μg of empty vector and an equal molar amount of expression vectors were transfected using Fugene (Roche) following the manufacturer's instructions into 5×10^5 target cells. Forty eight hours after transfection, cells were seeded in a serial dilution and maintained in DMEM and 10% fetal bovine serum supplemented with 1 mg/ml G418 (Life Technologies). Surviving colonies were stained with 0.4% crystal violet (Sigma) in 50% methanol, 21 d after initial seeding, and counted. Each transfection was performed in triplicate. The expression was confirmed by western blotting.

Statistical analysis. The following statistical tests were performed: student t-test and log-rank test, as indicated, $P < 0.05$ was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/25617

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