RASSF2 hypermethylation is present and related to shorter survival in squamous cervical cancer

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Ras association (RalGDS/AF-6) domain family member 2 (***RASSF2***) **is a gene involved in the progression of several human cancers, including breast, colorectal and lung cancer. The aims of this study were to determine the hypermethylation of the gene in squamous cervical cancer and precursor lesions, along with that of *RASSF1* and the recently described *EPB41L3*, and to analyze the potential prognostic role of these genes. Methylation-specific PCR and bisulfite sequencing were used to analyze the methylation status of *RASSF2* and *EPB41L3* gene in 60 squamous cervical cancer, 76 cervical intraepithelial neoplasias grade III, 16 grade II, 14 grade I and 13 cases of normal tissue adjacent to cervical intraepithelial neoplasia. RASSF2 expression was evaluated by immunohistochemistry and the re-expression of *RASSF2* and *EPB41L3* was analyzed by quantitative reverse-transcription PCR in HeLa, SiHa, C33A and A431 cell lines treated with 5-aza-2′-deoxycytidine and/or trichostatin. *RASSF1* hypermethylation and human papillomavirus type were also analyzed in all the cases by methylation-specific PCR and reverse line blot, respectively. *RASSF2* hypermethylation was predominant in squamous cervical cancer (60.9%) compared with cervical intraepithelial neoplasias (4.2%) and was associated with a lower level of RASSF2 expression and vascular invasion in squamous cervical cancer. *EPB41L3* and *RASSF1* hypermethylations were also more frequent in cancer than in precursor lesions. Patients with *RASSF2* hypermethylation had shorter survival time, independent of tumor stage (hazard ratio: 6.0; 95% confidence interval: 1.5–24.5). Finally, the expressions of *RASSF2* and *EPB41L3* were restored in several cell lines treated with 5-aza-2′-deoxycytidine. Taken together, our results suggest that *RASSF2* potentially functions as a new tumor-suppressor gene that is inactivated through hypermethylation in cervical cancer and is related to the bad prognosis of these patients.**

Cervical cancer is the third most common cause of cancer deaths in women worldwide;[1] the major etiological factor is the presence of human papillomavirus (HPV), with HPV 16, 18, 31, 33, 35, 45, 52 and 58 being the most prevalent types.[2] Infection with high-risk HPV is the main risk factor for developing high-grade cervical intraepithelial neoplasia grade II (moderate dysplasia) and grade III (severe dysplasia), which are precursor lesions of squamous cervical carcinomas.[3] The risk of developing squamous cervical carcinomas depends not only on HPV infection and persistence[4] but also on the presence of molecular alterations, including the hypermethylation of the CpG islands of tumor-suppressor genes.[5] These epigenetic abnormalities in cancer can affect a plethora of genes involved in key cellular pathways, including cell cycle control and cell adhesion.[5]

Ras association (RalGDS/AF-6) domain family member 2 (*RASSF2*) is a gene located at *20p13*, with RASSF2A as the most important putative transcript.[6] It belongs to the RAS association domain family of proteins, which has important functions in cell-cycle

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control, microtubule stabilization and motility.[7] The RASSF2A protein acts as a negative effector of K-RAS protein via the Ras effector domain,[8] and it relocates to the nucleus, where it performs important apoptotic functions.[9] *RASSF2* is inactivated by hypermethylation in breast, colorectal, endometrial, gastric, lung and thyroid cancers,[9–13] but the presence of this alteration in cervical cancer and precursor lesions and its prognostic role has not yet been analyzed. In this study, apart from *RASSF2*, we have also included the analysis of the hypermethylation in premalignant lesions and cervical cancer of *RASSF1* from the same family as *RASSF2*, and *EPB41L3*, a gene recently found to be hypermethylated in cervical cancer.

The *RASSF1* gene shares important functions with *RASSF2* in Ras signaling and microtubule stabilization,[14] and is located at *3p21*, where it is frequently deleted or hypermethylated in cervical cancer[11] and cervical intraepithelial neoplasia.[15] Its hypermethylation is of prognostic significance in association with *RASSF2* hypermethylation in oral squamous cell carcinoma.[16] On the other hand, *EPB41L3* hypermethylation has recently been described in cervical cancer,[17] but its prognostic role and involvement in precursor lesions are not known. This gene codes for a crucial protein in cell adhesion and is inactivated in several tumor types, such as breast tumors and renal cell carcinomas,[18,19] showing a bad prognosis role in non-small-cell lung cancer.[20]

Our objectives in this study were to analyze the methylation status of *RASSF2*, *RASSF1* and *EPB41L3* in cervical intraepithelial neoplasia and squamous cervical carcinomas, and to analyze their prognostic role. The treatment of HPV-positive and HPV-negative cell lines with the demethylating agent 5-aza-2′-deoxycytidine (dC) and/or the histone deacetylase inhibitor trichostatin A was also studied to examine the promoter demethylation and the restoration of the mRNA expression of the less studied *EPB41L3* and *RASSF2* genes.

Materials and methods

Cell Lines

HPV-positive cervical cell lines HeLa (HPV 18) and SiHa (HPV 16) and HPV-negative cervical cell line C33A and vulvar cell line A431 were included in this study. These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies, Carlsbad, CA, USA), and 1% penicillin/streptomycin (Invitrogen, Life Technologies) in a humidified atmosphere (5% CO_2, 37 °C) until 80% confluence was attained.

Patients

The group of cases studied consisted of patients diagnosed with squamous cervical carcinomas

(60 patients) and cervical intraepithelial neoplasia III lesions (76 patients) at Hospital Virgen del Camino (Navarra Hospital Complex, Navarra Health Service, Pamplona, Spain) between January 1995 and December 2006 (Table 1 and Figures 1a and b). In all, 14 and 16 cervical intraepithelial neoplasia I and II, respectively, and 13 cases of normal tissue adjacent to cervical intraepithelial neoplasia I, all randomly selected from the same period, were also analyzed. None of the patients had received radiation or chemotherapy before surgery. The study was approved by the Clinical Research Ethics Committee of Navarra.

All the cervical intraepithelial neoplasia lesions had been resected by loop electrosurgical excision procedure; 48 of 60 patients with squamous cervical carcinomas underwent primary surgical resection (total hysterectomy with bilateral salpingo-oophorectomy and lymphadenectomy), and 12 patients were diagnosed by biopsy on the basis of the patient's age and health status, and staged by radiology and lymphadenectomy. The diagnosis of cervical intraepithelial neoplasia lesions and squamous cervical carcinomas was confirmed following microscopic inspection by a gynecological pathologist.

The pathological staging of resected tumors according to the Federation Internationale de Gynécologie et d'Obstétrique (FIGO) was known for 44 out of 48 cases with primary surgical resection[3] (Table 1). Out of 19 stage I squamous cervical carcinomas, 6 were microinvasive carcinomas (early invasive squamous cell carcinoma), characterized by early stromal invasion according to previously

Table 1 Age of cervical intraepithelial neoplasia and squamous cervical carcinoma patients, and pathological data of resected squamous cervical tumors

Clinical data	
Mean age (s.d.)	
Cervical intraepithelial neoplasia	
I	34 (9.0) years
II	35 (8.5) years
III	39 (10) years
Squamous cervical carcinoma	55 (13) years
Pathological data	
Mean tumor size (s.d.) (cm)	2.7 (1.8)
FIGO stage	44 (100%)
I	19 (43%)
II	8 (18%)
III	12 (27%)
IV	5 (11%)
Grade of differentiation[a]	48 (100%)
Low	15 (31%)
Intermediate	25 (52%)
High	8 (17%)
Vascular invasion	41 (100%)
Yes	25 (61%)
No	16 (39%)
Histological type	48 (100%)
Microinvasive	6 (12.5%)
Squamous	42 (87.5%)

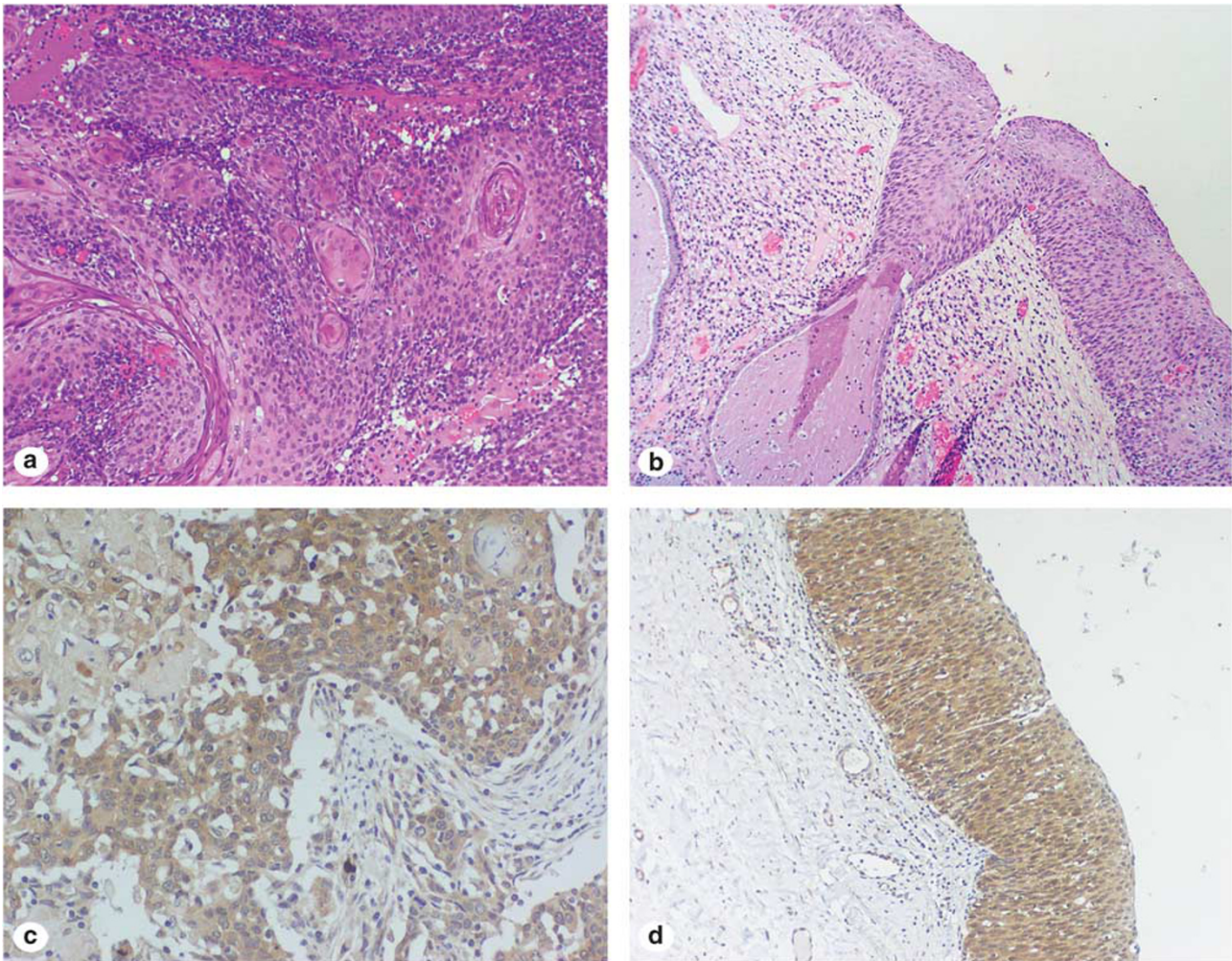[a]According to the World Health Organization grading system.

Figure 1 (**a**) Detail of a squamous cervical carcinoma (hematoxylin and eosin (H&E), ×400). (**b**) Panoramic view of cervical intraepithelial neoplasia grade III lesion with the loss of maturation of the epithelium (H&E, ×100). (**c**) Immunohistochemistry staining for a tumor positive (including positive endothelial cells as internal control) for RASSF2 expression, corresponding to an unmethylated case (×400). (**d**) Immunohistochemistry staining for a cervical intraepithelial neoplasia III, positive for RASSF2 (×100).

reported criteria.[3] Additional pathological data available for these cases are shown in Table 1.

The oncological treatment of the patients with advanced stages (II, III and IV) (56.9%) was complemented by radiotherapy, according to standard protocols.[21] The follow-up included physical and clinical examination, and a pelvic computed tomography scan and radiography every 3 months for the first 2 years, and every 6 months thereafter.

DNA Extraction from Cases

The pathologist selected a representative area of the lesions (cervical intraepithelial neoplasia, tumors) and normal tissue on formalin-fixed, paraffin-embedded sections stained with hematoxylin and eosin. The selected tissue was scraped from 5-μm-thick tissue sections under microscopic guidance, then deparaffinized in xylene and digested overnight at 56 °C in 50–400 μl buffer containing proteinase K (10 mg/ml) (Roche, Indianapolis, IN, USA), depending on the quantity of tissue. After inactivation of proteinase K at 96 °C for 10 min, DNA concentration and quality was measured for each sample using the NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). To assess DNA quality, the β-globin gene was amplified by PCR using primers BGPCO3 and BGPCO5, which generated a 209-bp product.[22]

Detection and Genotyping of HPV in Cases

We used PCR linked to a non-radioactive reverse-line blot procedure.[23] This technique amplifies a 150-bp fragment of viral DNA by adding GP5 + /GP6 + biotinylated primers. HPV genotypes were determined by hybridizing the GP5 + /6 + PCR products with specific oligoprobes containing a

5′-amino group on a carboxyl-coated nylon membrane (Biodyne C; Pall Corp., East Hills, NY, USA) in a miniblotter (MN45; Immunetics, Boston, MA, USA). The membrane was then incubated with streptavidin–POD conjugate (Roche, Indianapolis, IN, USA) subjected to enhanced chemiluminescence (ECL; GE Healthcare, Munich, Germany) and scanned in a densitometer (BioRad Laboratories, Hercules, CA, USA). A total of 36 types were identified using oligoprobes against high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82/MM4 and 82/IS39), probable high-risk types (HPV 26, 53 and 66) and low-risk types (HPV 6, 11, 34, 40, 42, 43, 44, 54, 55, 57, 61, 70, 71/ CP8061, 72, 81/CP83104, 83/MM7, 84/MM8 and CP6108). A case positive for HPV 16 and distilled water were included as positive and negative controls, respectively.

Methylation-Specific PCR in Cases

The methylation status of *RASSF2*, *RASSF1* and *EPB41L3* genes was determined in all the cases after sodium bisulfite modification of 1 μg of genomic DNA.[24] This treatment modifies unmethylated, but not methylated, cytosine to uracil and the modified DNA can be amplified with specific primers capable of annealing specifically methylated and unmethylated sequences. The primer sequences and the annealing temperatures (Sigma, St Louis, MO, USA) used to study *RASSF1* and *RASSF2* genes (Supplementary Table 1) have been described previously.[25] Primers used to amplify a region located −141 to −30 bp from the transcription start point of *EPB41L3* were designed using Methyl Primer Express v.1.0 (Applied Biosystems, Foster City, CA, USA) (Supplementary Table 1). Positive and negative controls were included in each bisulfite treatment and methylation-specific PCR to confirm the presence or absence of the band of expected size for each control, respectively. DNA from lymphocytes treated *in vitro* with *Sss*I methylase (New England Biolabs, Ipswich, MA, USA) was used as the positive control for methylated alleles of these genes. DNA from normal lymphocytes was used as the negative control. The presence of PCR products was verified by 2% agarose gel electrophoresis, stained with ethidium bromide and examined under ultraviolet illumination. Gene hypermethylation was defined as the presence of the band of expected size in the methylation reaction. The case was considered not analyzable if the gel band was not present in any of the two reactions (methylated and unmethylated).

5-AzadC and Trichostatin Treatment in Cell Lines

To determine the effects of DNA demethylation and acetylation on the restoration of mRNA expression of the two less-studied genes, *RASSF2* and *EPB41L3*, cell lines were cultured and treated with 5-azadC and/or trichostatin. A total of 10^6 cells were seeded and allowed to grow for 24 h before the treatment. Cells were treated separately with 10 μg/ml 5-azadC (Sigma), diluted in phosphate-buffered saline (PBS) or PBS alone for 96 h, and with 200 mg/ml trichostatin (Sigma) diluted in PBS or PBS alone for 24 h, or with both treatments (5-azadC for 96 h and trichostatin for the final 24 h). The medium containing the drug was replaced every 24 h. Cells receiving 5-azadC and combination treatment were trypsinized and harvested after 96 h, and cells in trichostatin treatment were collected after 24 h of treatment.

DNA and RNA were obtained for controls and for 5-azadC and/or trichostatin-treated cells using the EZNA Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA) and Ribopure Kit including a DNAse I treatment (Ambion, Life Technologies, Carlsbad, CA, USA), respectively, according to the manufacturer's instructions.

Quantitative Reverse Transcription-PCR for RASSF2 and EPB41L3 Expression in Cell Lines

Quantitative reverse-transcription PCR was performed to analyze the level of mRNA expression of *RASSF2* and *EPB41L3* genes in control and treated cell lines. cDNA was synthesized from 1 μg of RNA in a total volume of 50 μl with 250 U of reverse transcriptase, 2 × reverse transcriptase buffer, 1 μM random primers and 8 mM dNTPs, using a reverse transcription kit (Life Technologies).

Real-time PCR reactions were performed in triplicate from 100 ng of cDNA, for each control and treated cell line, together with positive and negative controls, using the Applied Biosystems 7300 Sequence Detection System (Life Technologies). Each real-time PCR reaction consisted of 50 μl of reaction mixture containing 25 μl PCR Master Mix (TaqMan Universal PCR Master Mix; Applied Biosystems) and 1 × TaqMan gene expression assays for target (*RASSF2*; Assay Id: Hs00248129_m1 (Applied Biosystems); *EPB41L3*; Assay Id: Hs00202360_m1) and endogenous control (*GAPDH*; Applied Biosystems) genes. The reaction consisted of 50 °C for 2 min (AmpErase UNG activation), followed by 95 °C for 10 min (AmpliTaq Gold activation) and 40 cycles of PCR amplification (95 °C for 15 s and 60 °C for 1 min). The cycle threshold value (Ct value) of each gene was normalized to that of housekeeping GAPDH obtained from the same sample. The delta-delta Ct method was used to calculate the relative fold-change in the expression of genes of each treatment relative to the control value.

Bisulfite Sequencing of *RASSF2* and *EPB41L3* Genes in Cases and Cell Lines

Bisulfite sequencing was performed to analyze *RASSF2* hypermethylation, which has not been described previously in cervical lesions, and

EPB41L3 hypermethylation, which has only recently been reported in cervical cancer.[17] This analysis was performed in cervical intrapithelial lesions, tumors and the HPV-positive (HeLa) and HPV-negative (A431) cell lines treated with 5-azadC or trichostatin, alone or in combination.

The analysis involved the use of 10 clones obtained from one case of normal tissue, cervical intraepithelial neoplasia I–III and tumor, following bisulfite conversion and amplification of a fragment containing CpG islands by PCR covering the same region as BS. Specific primers were designed using Methyl Primer Express v.1.0 (Applied Biosystems); sequences and conditions are described in Supplementary Table 1. PCR products were gel-purified and cloned into the pGEMT Easy Vector System (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol. In all, 10 colonies were randomly chosen, and DNA was extracted and sequenced using the 3100 Genetic Analyzer (Applied Biosystems). The results were transformed into percentages of CpGs showing methylation.

Immunohistochemistry

The 4-μm-thick sections from 20 cervical intraepithelial neoplasia and 20 tumors randomly selected (methylated and unmethylated cases) were mounted on glass slides, deparaffinized and rehydrated. Briefly, antigen retrieval was performed using Bond Epitope Retrieval Solution 2 (pH 9.0) (cat. no. AR9640; A Menarini Diagnostics Ltd, Berkshire, UK) at 95 °C for 30 min. Slides were then incubated with rabbit monoclonal RASSF2 antibody (clone EPR6621; Epitomics, San Diego, CA, USA) diluted 1:100 for 60 min at room temperature. The primary antibody was developed using Bond Polymer Refine Detection (cat. no. DS9800; Menarini Diagnostics Ltd) and visualized with 3, 3'-diaminobenzidine. Finally, slides were counterstained with hematoxylin. RASSF2 staining was expressed diffusely in the cytoplasm and, in some cases, in the nucleus. The extent and intensity of immunopositivity were both considered, and evaluation of immunohistochemistry was independently evaluated by two pathologists, who scored the cases as positive or weakly negative, on the basis of the previous criteria.[26]

Statistical Analysis

Associations between gene hypermethylation, pathological and clinical variables of this retrospective study were assessed with the χ^2 test or Fisher's exact test. Disease-free survival was studied in patients with squamous cervical carcinomas undergoing primary surgical resection ($n = 48$) and overall survival in all patients with squamous cervical cancer ($n = 60$).

Survival curves were calculated using the Kaplan–Meier method and statistically compared by the log-rank test. Cox proportional hazard regression models were used for univariate analysis and the proportional hazard ratio and the 95% confidence intervals (95% CI) were calculated for each factor. Hazard risk was adjusted for tumor stage and patient age. Statistical significance was concluded for values of $P < 0.05$ in all cases.

Results

HPV Typing

HPV was present in 93.4% and 94.9% of cervical intraepithelial neoplasia III and squamous cervical carcinomas, respectively (Supplementary Figure 1). The percentages were lower in the case of cervical intraepithelial neoplasia I (64.3%) and II (75%) lesions. All the HPV-positive lesions contained high-risk HPV types, with HPV 16 and HPV 18 being the most prevalent, alone or in combination with other high-risk types (Supplementary Table 2).

Methylation-Specific PCR Results

There were different numbers of analyzable cases for the *RASSF2*, *RASSF1* and *EPB41L3* genes (Table 2, Supplementary Table 3 and Figure 2). The percentages of methylation were higher for all the genes in tumors (65.5%, 46.3%, 66.1% for *RASSF2*, *RASSF1* and *EPB41L3*, respectively) than in cervical intrapithelial neoplasia III (4.2%, 11.8% and 32.4%, respectively), the differences being statistically significant for all the genes (Table 2). On the other hand, cervical intraepithelial neoplasia I and II cases were slightly methylated for all the genes except for *RASSF2*, which was not methylated in any cervical intraepithelial neoplasia I–II case (Supplementary Table 3). Normal tissue was not methylated for any of the genes.

It is notable that the proportion of *RASSF2* hypermethylation was much greater among squamous cervical carcinomas (38 of 58 analyzable cases) than among cervical intraepithelial neoplasia III lesions (3 of 71 analyzable cases) ($P < 0.001$) (Table 2). In addition, there was a clear relationship between *RASSF2* gene hypermethylation and vascular invasion ($P = 0.033$) (Table 2). All the patients who showed metastasis at the time of diagnosis exhibited hypermethylation of the *RASSF2* gene (five patients), but there was no association between this alteration and lymph node involvement.

In univariate analyses, *RASSF2* and *EPB41L3* hypermethylations were both significantly associated with HPV presence ($P = 0.004$ and 0.034, respectively). Nevertheless, these associations were not significant when adjusting for disease category in logistic models (Supplementary Table 4). Finally,

Table 2 Association of gene hypermethylation with pathological diagnosis, vascular invasion and histological type

Gene meth	Diagnosis			Vascular invasion			Histological type		
	Cervical intraepithelial neoplasia III, n (%)[a]	Squamous cervical carcinoma, n (%)	P-value[b]	Yes, n (%)	No, n (%)	P-value[b]	Squamous, n (%)	Microinv n (%)	P-value[b]
EPB41L3									
Yes	24 (32.4)	39 (66.1)	<**0.001**	17 (68.0)	11 (73.3)	1.000	29 (72.5)	2 (33.3)	0.078
No	50 (67.6)	20 (33.9)		8 (32.0)	4 (26.7)		11 (27.5)	4 (66.7)	
RASSF1									
Yes	9 (11.8)	25 (46.3)	<**0.001**	12 (50.0)	6 (40.0)	0.74	16 (41.0)	6 (100.0)	**0.009**
No	67 (88.2)	29 (53.7)		12 (50.0)	9 (60.0)		23 (59.0)	0 (0.0)	
RASSF2									
Yes	3 (4.2)	38 (65.5)	<**0.001**	17 (68)	5 (33.3)	**0.033**	22 (55.0)	5 (83.3)	0.370
No	68 (95.8)	20 (34.5)		8 (32)	10 (66.7)		18 (45.0)	1 (16.7)	

Abbreviations: meth, methylation; microinv, microinvasive type.
[a]Number and percentage of cases with available data.
[b]χ^2-test *P*-values (statistically significant *P*-values in bold).

RASSF2 and *RASSF1* hypermethylations were not associated with one another.

BS Results for *RASSF2* and *EPB41L3* Genes

In the case of *RASSF2*, promoter hypermethylation was confirmed to be preferentially present in tumors compared with cervical intraepithelial neoplasia III (Figure 3, up). On the other hand, cervical intra-epithelial neoplasia II had a low level of *RASSF2* hypermethylation (methylation in 16.7% of the CpGs analyzed) and normal cervical tissue and cervical intraepithelial neoplasia I revealed un-methylated *RASSF2* for all the clones analyzed.

In the case of *EPB41L3*, bisulfite sequencing also enabled us to confirm the increasing hypermethyla-tion of this gene from cervical intraepithelial neoplasia to tumor, with CpG methylation in 15.9%, 26.3% and 68.8% of the clones, respectively (Figure 3, down).

Quantitative Reverse Transcription-PCR Results for the *RASSF2* and *EPB41L3* Genes

In the case of *RASSF2*, quantitative reverse-tran-scription PCR confirmed that treatment with tri-chostatin led to partial re-expression of *RASSF2* in HeLa cells ($P = 0.045$) (Figure 4, up). In C33A cells, the treatment with 5-azadC alone led to higher levels of *RASSF2*, especially when combined with trichostatin ($P = 0.040$). The effect of these agents was also analyzed in A431 cells, which showed prominent, approximately 11-fold ($P = 0.001$) and 15-fold ($P = 0.001$), *RASSF2* re-expression, respectively.

In the case of *EPB41L3*, 5-azadC treatment was sufficient to boost its expression only in the SiHa cell line, treated with this agent alone or in combination with trichostatin ($P = 0.040$). Conver-

sely, gene re-expression was absent from HeLa, C33A and A431 cell lines (Figure 4, down).

RASSF2 Immunohistochemistry Results

In squamous cervical carcinomas, expression was diffuse in the cytoplasm and present in some isolated nuclei in all the cases with unmethylated promoter (Figure 1c). Conversely, RASSF2 staining was characterized as being reduced or absent in the majority of cases with methylated promoter ($P = 0.001$). Endothelial cells were clearly stained and were used as the positive internal control, in cases that showed weak or no expression. On the other hand, all the cervical intraepithelial neoplasia cases and normal tissue stained intensely positive for this protein (Figure 1d).

Follow-Up Results

Disease-free survival and overall survival were shorter for patients with more advanced stages of squamous cervical carcinomas and vascular inva-sion at the time of diagnosis (Table 3). It is notable that overall survival was significantly shorter for patients with *RASSF2* hypermethylation ($P = 0.019$; Table 3 and Figure 5). After adjusting for age and tumor stage, the risk of recurrence remained higher in patients with *RASSF2* hypermethylation (hazard ratio = 6.2; 95% CI: 1.2–32.4) (Table 4). Risk estimates related to mortality in all the patients were similar, with a hazard ratio for *RASSF2* hypermethylation of 6.0 (CI: 1.5–24.5).

Discussion

The analysis of markers and their prognostic role in cervical cancer, such as the epigenetic analysis of
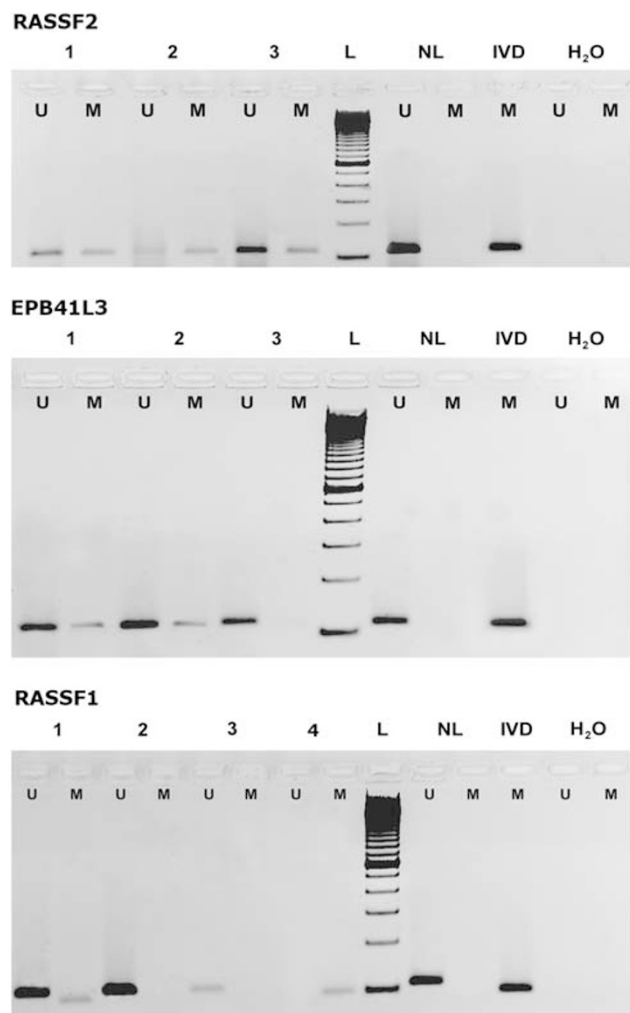
Figure 2 Methylation-specific *RASSF2*, *EPB41L3* and *RASSF1* genes. Both the unmethylated (U) and methylated (M) PCR products are shown for each case. DNA from normal lymphocytes (NL) and *in vitro*-methylated DNA (IVD) are used as a positive PCR control for the U and M reactions, respectively; H_2O: negative control.

tumor-suppressor genes, is crucial for detecting more aggressive squamous cervical carcinomas that are characterized by their lethality.[27] Gene hypermethylation has been described in various stages of HPV 16/18-transfected keratinocytes,[28] and is preferentially present in repetitive DNA sequences[29] and specific genes.[30] These epigenetic alterations are involved in the pathogenesis and progression of cervical carcinoma from cervical intraepithelial neoplasia to invasive tumors, as was reported for *MGMT* and *p16* genes, among others.[15,31]

This association between the methylation changes and malignancy has been found in our squamous tumors, in which all the genes were more methylated than in cervical intraepithelial neoplasia III cases. This difference is remarkable in the case of *RASSF2*, as this gene is almost exclusively hypermethylated in tumors (65.5%); it is found in a very

small proportion of cervical intraepithelial neoplasia III (4.2%). The value of *RASSF2* hypermethylation as a tumor marker has already been noted in patients with gastric,[9] colorectal,[32] non-small-cell lung cancer and breast cancer.[10]

In our study, the upregulation of RASSF2 mRNA expression was much higher in HPV-negative cell lines treated by 5-azadC treatment and in HPV-positive cells treated by trichostatin. These results suggest that histone deacetylation contributes to this silencing in HPV-positive cell lines probably caused by the virus, as was previously suggested for the re-expression of the cystatin gene after trichostatin treatment in SiHa cells.[33] The involvement of the *RASSF2* gene in RAS signaling,[34] and the promotion of apoptosis and ras-dependent cell growth inhibition demonstrated in pancreatic cancer cells and breast cancer cells transfected with the *RASSF2* gene[10,35] highlight the need for further studies of the role of this gene in cervical cancer cell lines.

We note that the presence of *RASSF2* hypermethylation in gynecological cancer in comparison with benign lesions was first reported by our group in vulvar squamous cell carcinoma.[36] To our knowledge, this is the first description of the same alteration in a high proportion of squamous cervical carcinomas, correlated with the loss of expression of the RASSF2 protein detected by immunohistochemistry. *RASSF2* hypermethylation is equally common in tumor stages I–IV of squamous cervical carcinomas, suggesting that it is an early event in cancer progression, as was described for this gene in colorectal cancer, and is equally hypermethylated in stages I and II.[32] In addition, its possible oncogenic role is reflected in its association with vascular invasion, which is a bad prognostic factor for our patients.[37] It should also be stressed that *RASSF2* hypermethylation is a bad prognostic factor, with respect to overall survival, in our patients. The bad prognostic role of this gene is also characterized by radioresistance and shorter survival in oral squamous carcinoma, and pancreatic and gastric cancers, in which it is more frequent in cases with peritoneal recurrence.[16,38–40]

On the other hand, in our study, *RASSF1* hypermethylation was also more frequent in squamous cervical carcinomas than in cervical intraepithelial neoplasia III and was not found to be of prognostic significance, as reported previously.[41,42] This epigenetic alteration was also present in the six microinvasive cancers examined here, suggesting that this gene is involved in early tumor invasion, as was described for *CCNA1* and *C13ORF18* hypermethylations in high-grade cervical lesions and microinvasive cervical cancer.[43] In fact, a similar finding was described for the clear association between *RASSF1* hypermethylation and microinvasion in ovarian borderline tumors.[44] More studies with a wide range of microinvasive populations in cervical cancer are needed to confirm this finding.

Figure 3 Bisulfite sequencing of *RASSF2* (up) and *EPB41L3* (down) genes in clones derived from normal, cervical intraepithelial neoplasia I–III lesions and tumors. Each vertical bar of circles represents a CpG site in the promoter region and the methylation status of the corresponding site is indicated in the circle below. Open and filled circles represent unmethylated and methylated CpG sites, respectively, and each row represents a single clone.

Finally, the predominance of this alteration in squamous cervical carcinomas compared with precursor lesions has also been found for the *EPB41L3* gene, which is hypermethylated in cervical cancer.[17,45] In non-cancerous cells, EPB41L3 protein anchors to the actin cytoskeleton at the cell–cell attachment site, crucial to the adhesion of adjacent cells.[45] Additional functions of *EPB41L3* in relation

RASSF2 hypermethylation in cervical cancer
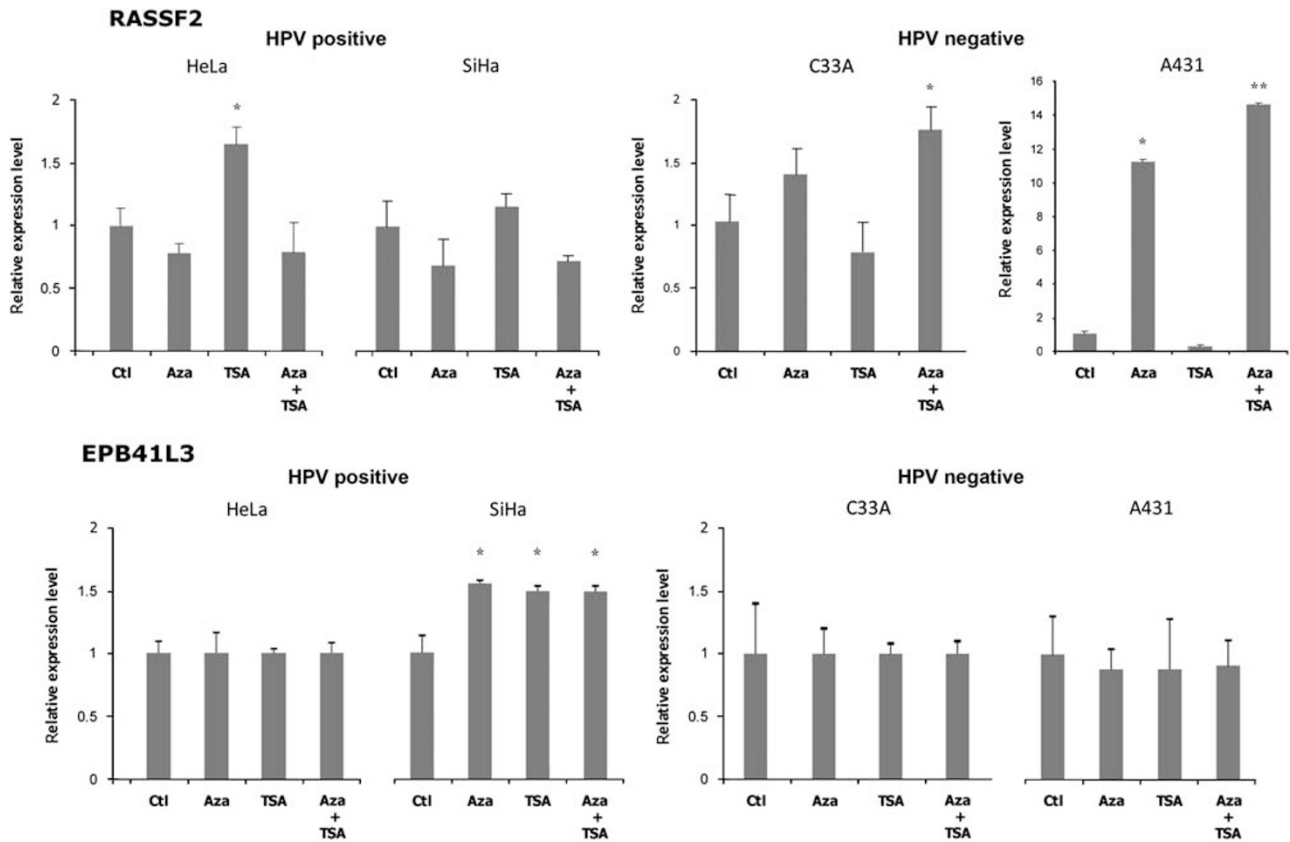D Guerrero-Setas *et al*
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Figure 4 Expression level of *RASSF2* (up) and *EPB41L3* (down) in human papillomavirus (HPV)-positive and HPV-negative cells treated with 5-aza-2'-deoxycytidine and/or trichostatin. Relative level of expression after quantitative real-time PCR (qRT-PCR) was compared with the untreated cells (control, Ctl), whose value was taken as 100%. Bars represent mean ± s.d. of the relative expression level of three experiments. Statistically significant (*$P < 0.05$) and very significant (**$P < 0.01$) change in gene expression in treated compared with untreated cells.

Table 3 Univariate Kaplan-Meier analysis of the risk of recurrence or death related to pathological and molecular variables in patients with squamous cervical cancer

Variables	Disease-free survival[a]				Overall survival[b]			
	n[c]	Recurrence		HR (95% CI)	P-value[d]	n[c]	Death		HR (95% CI)	P-value[d]
		Yes	No				Yes	No		
Stage										
I	19	4	15	Ref.		19	3	16	Ref.	
II	8	3	5	2.0 (0.5–9.1)	**0.004**	8	4	4	7.5 (1.4–38.7)	**0.001**
III	12	5	7	2.1 (0.5–8.6)		12	6	6	6.8 (1.3–36.2)	
IV	5	4	1	10.0 (2.3–43.2)		5	4	1	18.7 (3.4–104.2)	
Vascular invasion										
No	13	3	10	Ref.	0.07	16	5	11	Ref.	**0.015**
Yes	20	11	9	3.1 (0.9–11.2)		25	15	10	3.4 (1.2–9.6)	
RASSF2 meth										
No	18	3	15	Ref.	0.07	20	5	15	Ref.	**0.019**
Yes	28	12	16	2.9 (0.8–10.3)		38	24	14	3.0 (1.1–7.9)	

Abbreviations: CI, confidence interval; HR, hazard ratio; meth, methylation; Ref., reference value.
[a]Patients with squamous cervical cancer and primary surgical resection.
[b]All the patients with squamous cervical cancer.
[c]Case number (with available data).
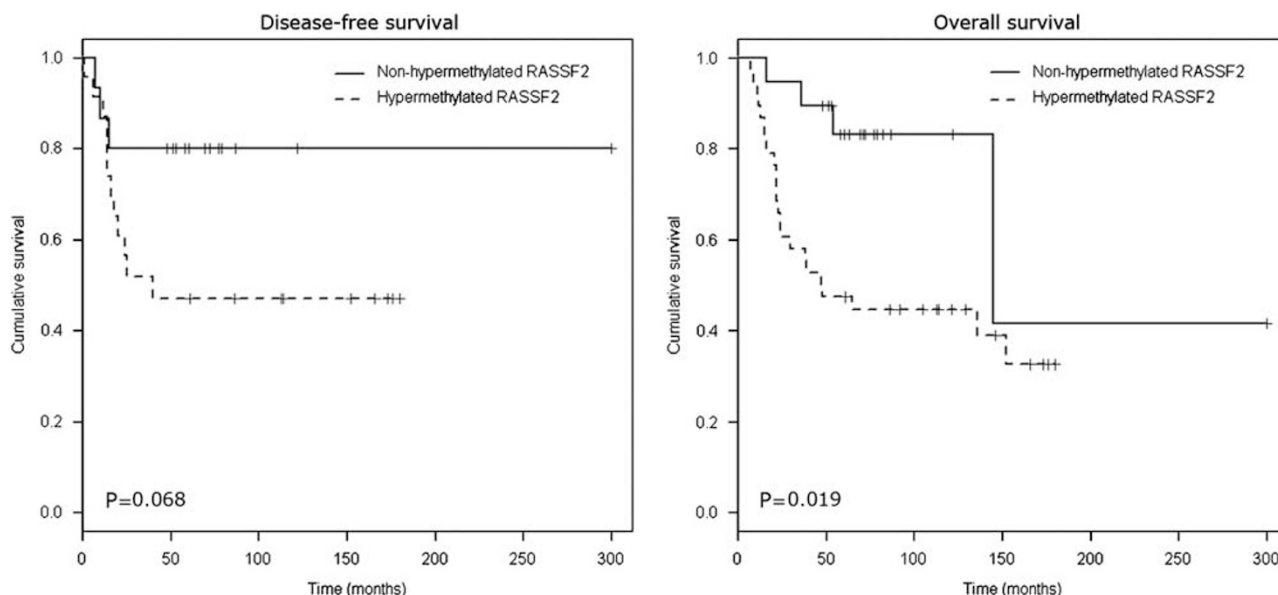[d]Log-rank test value (statistically significant *P*-values in bold).

Figure 5 Kaplan–Meier plots stratified for *RASSF2* promoter hypermethylation. (**a**) Curves for disease-free survival in patients with invasive carcinoma undergoing primary surgery. (**b**) Curves for overall survival in all squamous cervical carcinoma patients (primary surgical resection and biopsy).

Table 4 Cox proportional analysis of the risk of recurrence or death related to pathological and molecular variables in patients with squamous cervical cancer

Variable	Disease-free survival[a]		Overall survival[b]	
	HR (95% CI)	P-value[c]	HR (95% CI)	P-value[c]
RASSF2A meth				
No	Ref.	**0.03**	Ref.	**0.012**
Yes	6.2 (1.2–32.4)		6.0 (1.5–24.5)	
Stage				
I	Ref.		Ref.	
II	2.6 (0.46–14.7)	0.06	5.8 (1–32.6)	**0.005**
III	5.6 (1–31.4)		21.3 (3.8–118.6)	
IV	9.9 (1.8–53.9)		20.6 (3.2–133.9)	
Age (years)	1.039 (0.98–1.1)	0.17	1.038 (0.99–1.1)	0.13

Abbreviations: CI, confidence interval; HR, hazard ratio; meth, methylation; Ref., reference value.
[a]Patients with squamous cervical cancer and primary surgical resection.
[b]All the patients with squamous cervical cancer.
[c]*P*-value (statistically significant values in bold).

to growth suppression and induction of apoptosis are also crucial for the progression of ovarian and lung cancers.[45,46] In this study, we also found re-expression of this gene in SiHa cells after adding demethylating agents, demonstrating the role of hypermethylation in silencing this gene in cervical cancer. However, it was not associated with the clinicopathological variables included here.

In conclusion, we describe for the first time the preferential hypermethylation of the *RASSF2* gene in squamous cervical carcinomas relative to precursor cervical intraepithelial neoplasia and also the association of this alteration with vascular invasion.

The hypermethylation of *RASSF1* and *EPB41L3* genes involved in cell signaling and cell adhesion, respectively, was also more frequent in squamous cervical cancer, although they were not of prognostic significance. Conversely, *RASSF2* hypermethylation had a bad prognostic role in our study, which could be important to consider a stricter follow-up and treatment of these patients.

Acknowledgements

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)