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Inactivation of *RAS Association Domain Family 1A* Gene in Cervical Carcinomas and the Role of Human Papillomavirus Infection[1]

Igor Kuzmin,[2,3] Limin Liu,[3] Reinhard Dammann, Laura Geil, Eric J. Stanbridge, Sharon P. Wilczynski, Michael I. Lerman, and Gerd P. Pfeifer[2]

Basic Research Program, Science Applications International Corporation-Frederick, Inc. [I. K., L. G.], and Laboratory of Immunobiology [M. I. L.], NCI Frederick, Frederick, Maryland, 21702; Department of Biology, Beckman Research Institute [L. L., G. P. P.] and Department of Anatomic Pathology, City of Hope Medical Center [S. P. W.], Duarte, California, 91010; Institut für Humangenetik und Medizinische Biologie, Martin-Luther-Universität Halle-Wittenberg, D-06097 Halle/Saale, Germany [R. D.]; and Department of Microbiology and Molecular Genetics, University of California, Irvine, California, 92697 [E. J. S.]

ABSTRACT

Recently, we have identified a new putative tumor suppressor gene, *RASSF1A* (Ras association domain family 1A gene), located at human chromosome 3p21.3, the segment that is often lost in many types of human cancers. The *RASSF1A* promoter was shown to be frequently hypermethylated in various epithelial tumors, including small cell lung, breast, bladder, prostate, gastric, and renal cell carcinomas. In this study, we have analyzed the methylation status of the *RASSF1A* gene in primary human cervical cancers and in eight cervical cancer cell lines. The *RASSF1A* promoter is hypermethylated in 4 of 42 (= 10%) of squamous cell carcinomas, in 4 of 19 (= 21%) of adenosquamous carcinomas, and in 8 of 34 (= 24%) of cervical adenocarcinomas. Although in adenocarcinomas, methylation of *RASSF1A* and presence of human papillomavirus (HPV) type 16 or 18 sometimes coexisted, not a single case of HPV-16/18-positive squamous cell carcinomas had *RASSF1A* methylation. Similarly, in all eight analyzed cervical cell lines, *RASSF1A* inactivation and HPV infection were mutually exclusive (Fisher's exact test; $P = 0.0357$): two HPV-negative cervical cancer cell lines had a methylated and silenced *RASSF1A* promoter (C-33A and HT-3), whereas the other six HPV-positive lines expressed *RASSF1A* mRNA (ME 180, MS751, SiHa, C-4I, HeLa, and CaSki). For cervical tumors and cell lines combined, the Pearson's χ^2 test ($\chi^2 = 3.99$; $P \leq 0.05$) indicates a borderline-significant reverse correlation between inactivation of *RASSF1A* and the presence of high-risk HPVs. Our data imply that the presence of HPVs in cervical carcinomas alleviates the requirement for *RASSF1A* inactivation and suggests that these two events may engage the same tumorigenic pathway.

INTRODUCTION

Cervical cancer is the leading gynecological malignancy worldwide and is one of the most common cancers diagnosed in women in the United States (1). HPV[4] infection is frequently detected in cervical intraepithelial neoplasia and invasive cervical carcinoma (2). It is estimated that >90% of cervical SCCs and >50% of A/Cs contain HPV DNA (3). Certain subtypes of HPV such as HPV-16 and HPV-18 are most commonly associated with cervical carcinoma. Oncogenic HPVs carry the transforming *E6* and *E7* genes. These genes are necessary and sufficient for malignant transformation and immortalization of cervical epithelial cells (2, 4). The *E6* gene product promotes degradation of the p53 tumor suppressor protein (5), whereas the E7 protein inactivates the Rb protein and related pocket proteins (6, 7). However, the tumorigenic properties of the E6 and E7 proteins may not necessarily be limited only to the Rb and p53-related pathways (8, 9).

During malignant progression, tumor suppressor genes can be inactivated by different means. It is becoming increasingly clear that intragenic point mutations or small deletions are not the most frequent events leading to gene inactivation in cancer. Larger chromosomal deletions and promoter methylation are much more common. In cervical cancers, loss of heterozygosity has been reported for different chromosomal regions (10, 11). One of the frequently deleted chromosomal arms is the short arm of chromosome 3 (11).

Epigenetic inactivation of tumor suppressor genes through DNA methylation of promoter proximal CpG islands is an important event in tumorigenesis (12–14). In cervical cancer, aberrant methylation of the CpG islands of the *p16*, *death-associated protein kinase*, *HIC-1*, *APC*, *FHIT*, *retinoic acid receptor β*, and *E-cadherin* genes has been observed in 25–50% of the tumors (15–18). Other genes that are hypermethylated in their promoter regions less frequently in cervical carcinomas include the *GSTP1* gene and the *MLH1* gene (17).

Recently, we and others (19–21) have cloned and characterized the *RASSF1* gene. This gene is localized at 3p21.3, a chromosomal site where loss of genetic material is one of the most frequently observed events in many types of human solid tumors (20, 22–24). One of the two major isoforms transcribed from this locus, *RASSF1A*, was absent in human lung and breast tumors because of promoter methylation (19, 21, 25–27). Promoter hypermethylation, loss of heterozygosity, and even homozygous deletions are the major mechanisms inactivating the *RASSF1A* gene, whereas mutations are infrequent. Hypermethylation of the *RASSF1A* promoter region has been found in several types of primary human tumors (28), and *RASSF1A* is probably the most commonly inactivated gene thus far reported in human cancer. Ectopic expression of *RASSF1A* in cancer cells reduced colony formation, suppressed anchorage-independent growth, and inhibited tumor formation in nude mice (19, 21, 29, 30).

The precise function of the RASSF1A protein is not yet known. The homology of the *RASSF1A* gene with the mammalian Ras effector *NORE1* suggests that the *RASSF1A* gene product may function in signal transduction pathways involving Ras-like proteins. It was shown that RASSF1 binds Ras in a GTP-dependent manner and that its overexpression induces apoptosis (31). However, our recent data indicate that the proapoptotic effect of RASSF1 may require heterodimerization with NORE1 and that RASSF1 itself binds to Ras only weakly (32). In addition, there is evidence for an association of both NORE1 and RASSF1A with the proapoptotic kinase MST1 and that this interaction is involved in apoptosis induced by activated Ras (33). Other investigations have uncovered a role of RASSF1A in suppression of cyclin D accumulation and cell cycle progression (34). Available data suggest that inactivation of *RASSF1A* may be involved in the malignant processes leading to many types of human cancers.

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[2] To whom requests for reprints should be addressed, at Basic Research Program, Building 560, Room 12-34, SAIC-Frederick, Inc., NCI Frederick, Frederick, MD 21702. Phone: (301) 846-7320; Fax: (301) 846-6145; E-mail: kuzmin@mail.ncifcrf.gov (to I. K.). Department of Biology, Beckman Research Institute of the City of Hope, Duarte, CA 91010. Phone: (626) 301-8853; Fax: (626) 358-7703; E-mail: gpfeifer@coh.org (to G. P. P.).

[3] I. K. and L. L. contributed equally to this work.

[4] The abbreviations used are: HPV, human papillomavirus; Rb, retinoblastoma; A/C, adenocarcinoma; A/S, adenosquamous carcinoma; COBRA, combined bisulfite restriction analysis; *RASSF1*, RAS association domain family 1 gene; RT-PCR, reverse transcription-PCR; SCC, squamous cell carcinoma.

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The frequency of *RASSF1A* inactivation in human tumors is generally quite high. For example, *RASSF1A* is shown to be methylated in >70% of small cell lung cancers (21, 26), 91% of renal cell carcinomas (29), 62% of bladder cancers (35), 71% of thyroid carcinomas (36), 84% of nasopharyngeal cancers (37), and in >70% of prostate cancers (30, 38). In a previous study of prostate cancer, it was observed that prostate cell lines immortalized by infection with HPV expressed *RASSF1A* mRNA, whereas spontaneously or telomerase-immortalized prostate cell lines methylated and silenced the *RASSF1A* gene (30). This data suggested that *RASSF1A* inactivation and HPV infection might be mutually exclusive.

Because HPV infection is common in cervical carcinomas, a detailed investigation of *RASSF1A* methylation in this type of tumor is warranted. In this study, we investigated the methylation status of *RASSF1A* in primary human cervical cancers and in cervical cancer cell lines to explore a possible involvement of *RASSF1A* as a tumor suppressor gene in cervical carcinoma and to determine its relationship with HPV infection.

MATERIALS AND METHODS

Tissue Samples. All primary frozen cervical carcinoma tissues, matching adjacent tissues, and the relevant pathological data were obtained from the tumor tissue bank of the Anatomical Pathology Department of the City of Hope National Medical Center (Duarte, CA). All tissue was collected with Institutional Review Board approval. The tissue used for the DNA extraction was examined histologically and the presence of tumor in the sample confirmed. Details of DNA extraction and estimation of tumor in the sample extracted were described previously (39). Cervical carcinoma cell lines C-33A, HT-3, ME 180, MS751, SiHa, C-4I, HeLa, and CaSki were purchased from American Type Culture Collection (Manassas, VA).

Analysis of HPV Status. The presence of HPV in the tissue was determined by PCR using M09/MY11 consensus primers and type specific primers as reported earlier (39). It is possible that our HPV detection method underestimated the frequency of HPV-positive cases. The HPV testing was done using the MY09/MY11 consensus primers without microdissection of tumors as reported previously (39). MY09/MY11 are located in the *L1* gene, which is frequently lost in some cervical cancers during integration. Also, these primers do not detect all of the HPV types. Some tumors only retain the *E6/E7* genes for which no efficient consensus primers could be designed. The presence and expression of the papillomaviral sequences in human cervical carcinoma cell lines were extensively analyzed elsewhere (40–47).

Expression and Methylation Analysis of RASSF1A. The methylation status of the *RASSF1A* promoter region was determined by a bisulfite modification method (19, 48). For COBRA (49), 100 ng of bisulfite-treated DNA was PCR-amplified with primers MU379 (5′-GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT-3′) and ML730 (5′-ACCCTCTTCCTCTAACACAATAAAACTAACC-3′) in a 25-μl reaction volume containing 200 μM of each deoxynucleotide triphosphate and Taq polymerase (Roche; Indianapolis, IN). The PCR conditions consisted of an initial incubation for 3 min at 95°C, followed by 25 cycles of 20 s at 95°C, 20 s at 56°C, 40 s at 73°C, and finally a 4-min extension at 74°C. One-fifth of the PCR product was used as templates for a second PCR reaction with an internal primer ML561 (5′-CCCCACAATCCCTACACCCAAAT-3′) and primer MU379 with similar conditions as described for the preceding PCR but for 30 cycles. The PCR products were purified using a QIAquick PCR purification kit (Qiagen). Twenty to 50 ng purified PCR products were digested with 10 units of TaqI (New England Biolabs). The digested products were resolved on 2% Tris-acetate-EDTA agarose gels.

RASSF1A promoter bisulfite sequencing, COBRA, and RT-PCR quantification of the cervical cell lines were performed as described elsewhere (30). Evaluation of the *RASSF1A* mRNA by real-time PCR in cell lines was conducted according to standard protocols for TaqMan Gold RT-PCR kit using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The following *RASSF1A* oligonucleotides were used for amplification: RASSF1A-D (5′-ACGCACACGTGGTGC-3′); RASSF1A-R (5′-AGTGGCAGGTGAACTTGCA-3′); and for the probe RASSF1A-HY (5′FAM-TCGTGCGCAAAGGCCTGCAGTG-TAMRA-3′). RNA normalization and estimation were performed with standard ABI TaqMan glyceraldehyde-3-phosphate dehydrogenase control reagents and *in vitro* transcribed *RASSF1A* RNA.

RESULTS

HPV and *RASSF1A* Methylation Analysis of the Primary Cervical Tumors. We analyzed the methylation status of the *RASSF1A* gene's 5′ CpG island and promoter region in human cervical cancer samples. We treated genomic DNA with sodium bisulfite, followed by a COBRA (49) to analyze the methylation status of this gene in 95 primary cervical cancer samples and 58 matching adjacent normal tissues. After bisulfite treatment, all unmethylated cytosines are converted to uracils and then to thymines during subsequent PCR steps. All cytosines after subsequent PCR derive solely from 5-methylcytosines, which are nonresponsive to bisulfite. With COBRA, our target sequence was a 205 bp PCR fragment, which contains 16 CpG dinucleotides (19). TaqI (5′-TCGA-3′) can digest this fragment at two CpG sites (6 and 16), only when the genomic DNA is methylated at these sites.

Genomic HeLa cell line DNA and A549 lung cancer cell line DNA have been shown to be unmethylated and methylated, respectively, in the *RASSF1A* promoter region in our previous work (19). These DNAs were used as negative and positive controls for methylation in our methylation analysis. We analyzed 34 cervical A/Cs, 19 A/Ss, and 42 SCCs (Fig. 1). Four of 42 (= 10%) of SCCs and 4 of 19 (= 21%) of A/Ss were methylated in the *RASSF1A* promoter (Fig. 1; Table 1). For cervical A/Cs, methylation frequencies were somewhat higher (8 of 34 tumors were methylated = 24%; Table 1). We analyzed 58 normal tissues in parallel with the matching tumors (Fig. 1*B* and data not shown). Four normal samples were partially methylated but to a much lower extent than the corresponding tumor tissue. This result might be attributable to tumor cell contamination of the normal tissue or to preexisting methylation. *RASSF1A* was methylated only in those normal tissues in which the cancerous counterparts also showed methylation (Fig. 1*B* and data not shown). The presence of a methylated *RASSF1A* promoter did not correlate with tumor stage or grading (data not shown). Thus, tumors with high or low grades or stage could be methylated.

We next investigated the relationship of *RASSF1A* methylation and presence of HPV DNA in the cervical tumor samples. Five of 24 (= 21%) of HPV-positive A/Cs carried methylated *RASSF1A* alleles. There were four cases of HPV-16 and one case of HPV-18 in the *RASSF1A*-methylated samples. Three of 10 HPV-negative A/Cs had methylated *RASSF1A*. Seventeen of 19 adenosquamous cancers were HPV positive, and the four methylated samples were also HPV positive (all were HPV-16). Among the SCCs, 34 of 42 (= 81%) were HPV positive. Two of four tumors with *RASSF1A* methylation were HPV negative, and two others carried HPV45 sequences. Thus, none of the SCCs that carried high-risk HPV-16 or HPV-18 genomes contained a methylated *RASSF1A* gene (Table 1). In total, 25.0% (5 of 20) of HPV-negative primary tumor samples and 14.7% (11 of 75) of HPV-positive tumors were methylated in the promoter region of *RASSF1A*.

***RASSF1A* Expression and Methylation Analysis of Cervical Carcinoma Cell Lines.** To further investigate the connection between HPV infection and *RASSF1A* methylation, we analyzed eight cervical carcinoma cell lines. In earlier studies, the cell lines were evaluated in detail for the presence and expression of HPV sequences. In C-33A and HT-3 cells, no HPV viral DNA or RNA were found (41, 43). SiHa and CaSki cells contain HPV-16 DNA, whereas C-4I and HeLa have HPV-18 DNA (40, 41, 43). HPV-39-related virus was

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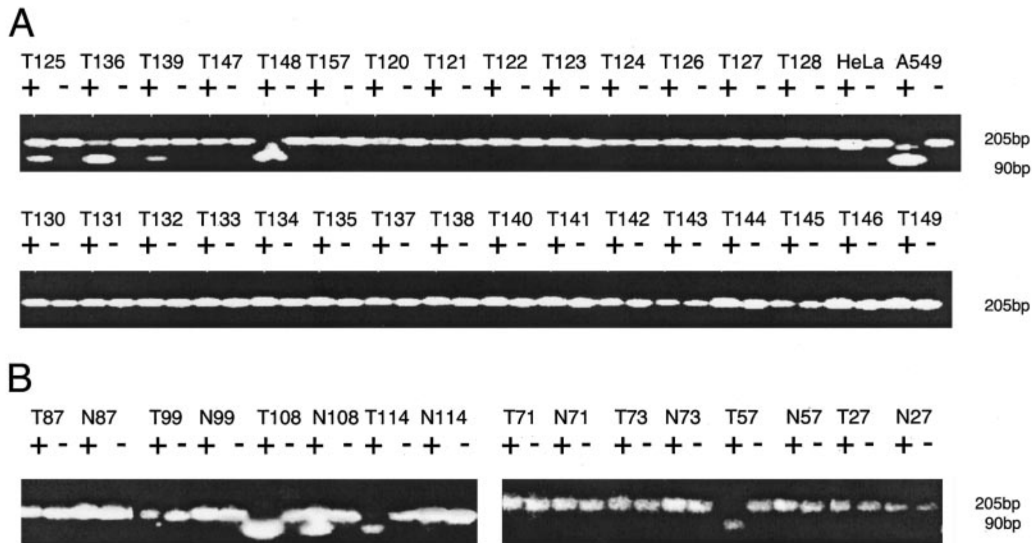

Fig. 1. Methylation analysis of primary cervical tumors. COBRA of primary cervical cancer samples. PCR products from bisulfite-treated DNA obtained from primary tumors (T) were digested (+) or mock digested (−) with TaqI. The digested products were resolved on 2% Tris-acetate-EDTA agarose gels and visualized by ethidium bromide staining. Unmethylated HeLa DNA and methylated A549 cell DNA were used as controls. *A*, tumor samples; *B*, matching tumor and adjacent normal tissues. We analyzed the PCR fragments obtained from bisulfite-modified DNA by digestion with a restriction enzyme that has a CpG recognition sequence (49). TaqI (5′-TCGA-3′) will cut only previously methylated DNA after bisulfite treatment and PCR. The consensus sequence will be lost by cytosine deamination in unmethylated samples. The analyzed 205-bp fragment of *RASSF1A* contains two TaqI restriction sites after bisulfite conversion of CpG methylated DNA. Restriction digestion of PCR products obtained from DNA methylated at both TaqI sites results in three bands (90, 81, and 34 bp; the two larger ones migrating together and the smaller band not visible). The criteria for scoring a sample as methylation positive was the presence of an additional band migrating ~90 bp upon TaqI digestion. The data obtained with the bisulfite restriction assay were confirmed by methylation-specific PCR using primers specific for unmethylated and methylated CpGs, respectively, in the *RASSF1A* CpG island (data not shown).

found in ME-180 (46), and HPV-45 was reported in MS751 (47). All HPV-positive cell lines express viral RNA transcribed from the E6-E7 region (41–45).

We analyzed the methylation status of the *RASSF1A* promoter by COBRA. C-33A and HT-3 showed complete methylation, whereas ME-180, MS751, SiHa, C-4I, HeLa, and CaSki were not methylated (Table 2). In addition, we performed bisulfite sequencing of the *RASSF1A* promoter in C-33A and HT-3 cells and found complete methylation of the promoter region (data not shown).

We next quantified expression of *RASSF1A* mRNA in these cells. *RASSF1A*-specific RT-PCR was performed in the presence of different amounts of *in vitro* transcribed *RASSF1A* mRNA, producing a shorter PCR fragment (Fig. 2). *RASSF1A* mRNA content was calculated by comparing the intensity of both bands in each lane. RT-PCR quantification and the data on HPV status are presented in Table 2. In both HPV-negative cell lines (C-33A and HT-3), the *RASSF1A* transcript was not detectable (<0.0001% of total RNA). In contrast, six other cell lines, which contained HPV DNA and RNA (SiHa, HeLa, ME-180, MS751, CaSki, and C-4I), expressed *RASSF1A* mRNA at levels 0.01–0.05% of total RNA. These levels of expression are comparable with those found in prostate cell lines transfected with E6-E7-expressing plasmids (30). In agreement with the data obtained from primary cervical tumors, we found a somewhat lower content of *RASSF1A* mRNA in the cell lines infected with HPV-39 (0.02% of total RNA in ME 180) and especially HPV-45 (0.01% in MS751) when compared with four HPV-16/-18-infected cell lines (0.05% in SiHa, HeLa, CaSki, and C-4I; Table 2).

Similar results were obtained using a real time PCR procedure. The percentages of *RASSF1A* mRNA were normalized to the total RNA concentration (Table 2). In addition, *RASSF1A* mRNA was normalized to *glyceraldehyde-3-phosphate dehydrogenase* mRNA (data not shown), but this did not considerably alter the established ratios of *RASSF1A* expression between the cell lines.

To assess the mechanisms of *RASSF1A* transcriptional inactivation, we treated C-33A cells with 5-aza-2′deoxycytidine for 1 week. RT-PCR titration of the *RASSF1A* indicated that its expression was not fully restored, even in the presence of high concentrations (15 μM) of the drug (data not shown). Sequencing of the *RASSF1A* promoter in C-33A cells did not reveal any nucleotide changes, therefore, such inactivation most likely occurs because of a lack of *trans*-acting factors and the promoter methylation may represent a secondary event.

DISCUSSION

HPV infection is recognized as a major contributor to the development of cervical cancer. Interestingly, *RASSF1A* methylation was unexpectedly low in primary cervical tumors. An earlier study had found no *RASSF1A* methylation in 22 cervical cancers analyzed (27). However, HPV presence was not assessed in that particular study. Our simultaneous assessment of *RASSF1A* inactivation and HPV presence in cervical carcinomas has revealed several interesting (albeit not statistically significant) correlations. We found that *RASSF1A* methylation was more common in cervi-

Table 1 *Summary of methylation analysis and HPV status of primary human cervical cancer samples*

	HPV16+	HPV18+	HPV45+	Other HPVs	HPV−	Total
SCC	0/26 (0)[a]	0/3 (0)	2/3 (66)	0/2 (0)	2/8 (25)	4/42 (10)
A/C	4/15 (26)	1/9 (11)	0/0 (0)	0/0 (0)	3/10 (30)	8/34 (24)
A/S	4/8 (50)	0/6 (0)	0/1 (0)	0/2 (0)	0/2 (0)	4/19 (21)
Total	8/49 (16)	1/18 (5)	2/4 (50)	0/4 (0)	5/20 (25)	16/95 (17)

[a] Each value indicates the number of RASSF1A-methylated tumors (in numerator) *versus* the total number of tumors (in denominator). The percentage is in parentheses.

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Table 2 *HPV status, RASSF1A expression, and promoter methylation in cervical cell lines*

Cell line, origin	*RASSF1A* expression, % of total RNA		*RASSF1A* methylation	HPV DNA, E6/E7 expression and references
	Visual estimate from Fig. 2	Real-time PCR		
C-33 A, carcinoma	<0.0001	not detectable	+	Negative for HPV DNA and RNA (41, 43)
HT-3, carcinoma	<0.0001	not detectable	+	Negative for HPV DNA, and RNA (41, 43)
SiHa, SCC	0.05	0.110 ± 0.018	−	HPV-16 DNA (41, 43), E6/E7 RNA (45)
HeLa, A/C	0.05	0.081 ± 0.003	−	HPV-18 DNA (40, 41, 43), E6/E7 RNA (42, 44)
ME-180, epidermoid carcinoma	0.02	0.018 ± 0.003	−	HPV-39 DNA (46), E6/E7 RNA (46)
MS751, epidermoid carcinoma	0.01	0.009 ± 0.001	−	HPV-45 DNA (47), E6/E7 RNA (47)
CaSki, epidermoid carcinoma	0.05	0.041 ± 0.007	−	HPV-16 DNA (41, 43), E6/E7 RNA (45)
C-4I, carcinoma	0.05	0.028 ± 0.003	−	HPV-18 DNA (40, 41, 43), E6/E7 RNA (42, 44)

cal A/Cs than in SCCs (24 *versus* 10%, respectively). In A/Cs, methylation of the *RASSF1A* promoter could coexist with the presence of HPV-16 or HPV-18. However, this was not the case with cervical SCC in which none of the samples with HPV-16 or HPV-18 genomes had methylated *RASSF1A* alleles. Concordantly, lower levels of *RASSF1A* mRNA were found in HPV-39/-45-infected cell lines compared with HPV-16/-18-infected cell lines. The presence of both *RASSF1A* methylation and HPV infection in some primary tumor samples may indicate that the methylation assay we used may not always precisely reflect the expression status of the *RASSF1A* gene. Indeed, when *RASSF1A* mRNA was titrated in cell lines, we never found *RASSF1A* silencing and HPV infections in the same samples. Unfortunately, *RASSF1A* mRNA is difficult to quantify in primary tumors because of the presence of normal cells and lower quality of the extracted RNA. In addition, our HPV detection method could underestimate the number of HPV-positive cases in primary tumors (see "Materials and Methods"). These limitations may contribute to the reason why the HPV/*RASSF1A* correlation does not reach statistical significance in primary cervical tumors alone (Fisher's exact test; $P = 0.24$), even after these difficult-to-score HPV-45-positive samples were excluded ($P = 0.11$). However, when only SCC tumors are considered (excluding three HPV-45-infected samples), this correlation was quite significant (Fisher's test; $P = 0.038$). For all cervical tumors and cell lines combined, both Pearson's ($\chi^2 = 3.99$; $P \leq 0.05$) and Fisher's ($P = 0.05$) tests indicate a borderline-significant reverse correlation between inactivation of *RASSF1A* and the presence of high-risk papillomaviruses.

Moreover, the *RASSF1A* gene was expressed in all six HPV-positive cervical lines and completely silenced in two HPV-negative lines, indicating statistically significant reverse correlation (Fisher's exact test; $P = 0.0357$).

In the same panel of eight cervical cell lines, a similar correlation was established earlier in connection with the *Rb* and *p53* mutations. Only HPV-negative cells contained mutated *Rb* and *p53* genes, whereas the wild-type genes were retained in all six HPV-positive cell lines (7), implying that the wild-type Rb and p53 proteins were inactivated by the viral oncoproteins E6 and E7.

Recently, the absence of *RASSF1A* methylation in HPV-immortalized prostate cell lines has been reported (30). The expression of the *RASSF1A* gene was tested in four prostate carcinoma cell lines, adapted to grow in cell culture by transfection with DNA, which encodes E6/E7-transforming proteins of HPV serotype 16 or 18. All four tested HPV-transformed cell lines expressed normal levels of *RASSF1A* mRNA. In total, using the same semiquantitative RT-PCR analysis, we measured *RASSF1A* mRNA expression in 17 cervical and prostate carcinoma cell lines with known HPV status. In all 7 HPV-negative lines, the *RASSF1A* gene was silenced, whereas it was expressed in the other 10 HPV-positive lines. These data also points to a possible correlation between HPV infection and *RASSF1A* expression (Fisher's exact test $P = 0.00005$). This correlation could reflect a functional interaction between the cellular RASSF1A and the viral E6/E7 proteins, which may play important roles in both neoplastic transformation and immortalization of certain epithelial cells.

Evidence for a causal association between HPV and a subset of head and neck cancers has also been reported (50). Therefore, a similar reverse correlation between HPV infection and *RASSF1A* methylation may exist for this type of cancer but this has not yet been investigated. It is of note, however, that the methylation frequency of *RASSF1A* in primary head and neck tumors is quite low (5–15%; Refs. 28, 51). However, *RASSF1A* is frequently methylated in nasopharyngeal tumors, which may not be related to HPV infection (37).

The reverse correlation between *RASSF1A* inactivation and presence of HPV-transforming gene products is intriguing but is presently unexplained. The presence of HPV-16 or HPV-18 in cervical carcinomas may alleviate the requirement for *RASSF1A* inactivation. There is not enough information available regarding the exact biological function of the RASSF1 proteins. One piece of evidence points to an inhibitory effect of RASSF1A on cyclin D protein levels (34). Cyclin D-dependent phosphorylation negatively regulates Rb. Because the

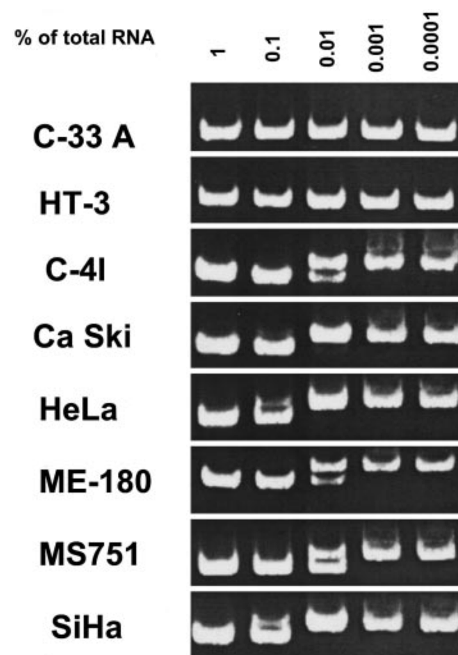

Fig. 2. Quantification of *RASSF1A* mRNA in cervical carcinoma cell lines. *RASSF1A*-specific RT-PCR was performed in presence of different amounts of artificial *RASSF1A* mRNA, producing a shorter PCR fragment. The amount of artificial mRNA (as percentage of total RNA) is marked on the top.

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E7 protein of high-risk papillomaviruses can inactivate the Rb protein, both RASSF1A and E7 may interact in the same pathway. However, other evidence suggests that it is unlikely that RASSF1A functions in the Rb pathway. The Rb pathway appears to be inactivated in the majority of human cancers by ways that do not seem to depend on *RASSF1*. For example, the Rb protein is inactivated in the majority of small cell lung cancers (52), and yet *RASSF1A* is inactivated in ~80% of these tumors (19, 21, 26, 27). Cyclin D1 overexpression and Rb inactivation can coexist in small cell lung cancer (53). Other evidence also argues against a placement of *RASSF1A* into the known p16/Rb pathway. Ninety percent of thyroid tumors with p16 inactivation were also silenced for *RASSF1A* expression (36). Thus, the precise mechanistic connection between HPV infection and *RASSF1A* inactivation remains to be determined. Our data suggest that E6 and E7 proteins may target not only the Rb- and p53-related tumor suppressing mechanisms but also some other essential pathways, most likely involving Ras signaling.

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