The RASSF1A Tumor Suppressor Gene Is Commonly Inactivated in Adenocarcinoma of the Uterine Cervix

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ABSTRACT

Purpose: Development of adenocarcinoma (AC) of the uterine cervix, as well as squamous cell carcinoma (SCC), is strongly linked to infection by high-risk human papillomavirus (HPV) types. Human HPV E6 and E7 proteins inactivate the tumor suppressor genes p53 and retinoblastoma, respectively. However, additional genetic alterations may be required to maintain a malignant phenotype. Allelic loss at the short arm of chromosome 3 is one of the most frequent genetic changes found in cervical cancer and various other types of human cancer, including lung, breast, and ovarian cancer. This implies that a resident tumor-suppressor gene in this region is involved in the genesis of these tumors. RASSF1A, which is located at 3p21.3, is rarely inactivated by mutations but has been suggested as a target tumor suppressor gene on the basis of its frequent inactivation through promoter hypermethylation and loss of heterozygosity in a variety of primary human cancers. In the present study, we sought to determine whether epigenetic silencing of RASSF1A caused by hypermethylation of the promoter region plays a role in the development of uterine cervical cancer.

Experimental Design: We studied 51 uterine cervical carcinoma samples. These 51 cases were comprised of 31 SCCs and 20 ACs. Real-time methylation-specific PCR system was used for the detection and quantification of the bisulfite-converted methylated version of the RASSF1A promoter region. The 20 cases of cervical AC were also analyzed for the presence of oncogenic HPV 16 DNA using a PCR-based method.

Results: We found complete methylation of the RASSF1A promoter in 45% (9 of 20 samples) of AC cases. There was no promoter methylation observed in any of the 31 cases of SCC. We also correlated RASSF1A promoter hypermethylation to oncogenic HPV 16 infection. HPV 16 DNA was found in 3 of 9 (33%) AC tumors with RASSF1A promoter hypermethylation and 5 of 11 (45%) AC tumors without RASSF1A promoter hypermethylation. We could not demonstrate an inverse correlation between RASSF1A methylation and HPV 16 infection in AC of the uterine cervix.

Conclusions: Hypermethylation of the RASSF1A promoter region is common in AC of the uterine cervix and rare in squamous carcinoma of uterine cervix. HPV infection does not correlate with RASSF1A methylation status in AC of the uterine cervix, but the absence of RASSF1A methylation in SCC of the uterine cervix coupled with the high incidence of HPV 16 infection in this subtype is in accord with previous reports. Our results suggest that epigenetic silencing of RASSF1A may play a role in the development of AC of the uterine cervix.

INTRODUCTION

Cervical cancer is the leading cause of death from cancer among women in most developing countries (1). The development of cervical cancer is strongly linked to infection by high-risk HPV (2). HPV-16 is the most prevalent type detected in cervical cancer and, along with types 18, 31, 33, and 45, has been classified as a class I carcinogen (3). The HPV virus E6 and E7 proteins inactivate the products of tumor suppressor genes p53 and Rb, respectively (4). This inactivation is important in the process of maintaining abnormal cellular proliferation (5). Because the majority of patients with HPV infection do not develop invasive lesions, HPV infection alone is probably insufficient for the complete neoplastic transformation of cervical cells, suggesting the involvement of other important genetic and epigenetic events in cervical carcinogenesis.

Classical genetic mechanisms, including deletions caused by LOH and inactivating mutations, as well as gain of function mutations, are the hallmark of cancer cells. In addition, growth regulatory genes can be inactivated epigenetically (6). Epigenetic gene silencing through promoter hypermethylation of normally unmethylated CpG islands occurs in major tumor suppressor genes, such as VHL, hMLH1, E-cadherin, APC, and p16 (7–9). LOH on chromosome 3p is a common and early event in the pathogenesis of cervical cancer (10) and many other solid tumors, (11), suggesting the presence of a resident tumor sup-

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2 The abbreviations used are: HPV, human papilloma virus; LOH, loss of heterozygosity; AC, adenocarcinoma; Rb, retinoblastoma; SCC, squamous cell carcinoma.
pressor gene on this chromosome arm. Recently, Dammann et al. (12) identified and cloned a gene from the common homozygous deletion area at 3p21.3. This gene, named \textit{RASSF1A}, has homology to mammalian RAS effectors (12). Although little is known about the function of \textit{RASSF1A}, it has been shown to dramatically reduce tumorigenicity \textit{in vivo} (12, 13). \textit{RASSF1A} is frequently inactivated in a variety of primary human cancers, but inactivating mutations of this gene are very rare (14–17). Epigenetic silencing of \textit{RASSF1A} leads to absence of the protein and appears functionally analogous to a homozygous deletion or an inactivating mutation (12).

On the basis of these observations, we tested whether epigenetic silencing of \textit{RASSF1A} plays a role in the development of cervical cancer. We found common \textit{RASSF1A} methylation in a specific subset of these tumors.

**MATERIALS AND METHODS**

**Cervical Cancer Samples.** Fifty-one samples of invasive cervical carcinoma, including 31 SCCs and 20 ACs, were collected from the Samsung Cheil Hospital (Seoul, Korea) with institutional review board approval. The histological type and grade of the tumors were classified according to the World Health Organization criteria. H&E-stained, 10-μm-section slides were prepared from paraffin-embedded tumor tissue. Areas with >75% of tumor cells were used for microdissection.

**Bisulfite Modification.** One μg of genomic DNA was denatured with NaOH. The bisulfite treatment (during which methylated DNA is protected and unmethylated cytosine is converted to uracil) was carried out for 16 h at 50°C on the denatured genomic DNA as described by Herman et al. (19). DNA samples were then purified using the Wizard DNA Clean-Up System (Promega, Madison, WI), treated again with NaOH, ethanol-precipitated, and resuspended in water.

**Real-time Methylation-specific PCR.** After sodium bisulfite conversion, genomic DNA was analyzed by ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously (20, 21). Briefly, two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: (a) a methylated set for \textit{RASSF1A} gene and (b) a reference set for β-actin gene (\textit{ACTB}) to normalize for input DNA. The primers and probe sequences used are the following: (a) \textit{RASSF1A}-5’ GCG TTG AAG TCG GGG TTC 3’ (sense); (b) 5’ CCC GTA CTT CGC TAA CTT TAA ACG 3’ (antisense); (c) 5’ FAM-ACA AAC GCC AAC AGA AAA CCA-TAMRA 3’ (probe); (d) β-actin (\textit{ACTB})-5’ TGG TGA TGG AGG AGG TTT AGT AAG 3’ (sense); (e) 5’ AAC CAA TAA AAC CTA CTC CTC CCT TAA 3’ (antisense); and (f) 5’ FAM-ACC ACC ACC AAC ATA ACA AAC ACA-TAMRA 3’ (probe). Specificity of the reactions for methylated DNA was confirmed separately using human sperm DNA (unmethylated) and SssI (New England Biolabs)-treated sperm DNA (methylated). The reference primers and probe were designed in a region of the \textit{ACTB} gene that lacks any CpG dinucleotides to allow for equal amplification, regardless of methylation levels. The relative degree of methylation of each DNA sample (as a percentage of total alleles) was calculated using the method described by Monney et al. (22).

**HPV Type-specific PCR.** Purified genomic DNA was amplified by PCR for the HPV-16 E7 gene, as well as for an internal reference gene, β-globin. Oligonucleotide primers used were as follows: (a) HPV-16 E7, 5’-ATTAATGACAGCTCA-GAGGA-3’ (sense) and (b) 5’-GTTTTGTACGACAACAGCAG-3’ (antisense). Primers for β-globin were 5’-GAGGAGCCAAAGGACAGGTAC-3’ (sense) and 5’-CAACTTGATCCAGGTAC-3’ (antisense). DNA made from a con-

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**Fig. 1** Representative negative (A) and positive (B) \textit{RASSF1A} amplification curves. Samples were run in duplicates. Amplification curve of \textit{ACTB} represents input DNA, whereas amplification of \textit{RASSF1A} indicates the presence of aberrant promoter methylation.
fluent culture of the human cervical carcinoma cell lines SiHa (HPV-16; American Type Culture Collection, Manassas, VA) was used as a positive control for each HPV subtype.

Statistical Analysis. Comparisons were made by two-tailed Fisher’s exact test.

RESULTS

We tested 51 primary cervical cancers for aberrant RASSF1A promoter methylation using real-time methylation-specific PCR; aberrant promoter hypermethylation was detected in 17.5% (9 of 51) of the cancers (Fig. 1). Remarkably, aberrant promoter hypermethylation was detected in 45% (9 of 20) of the AC cases but in none (0 of 31) of the SCC cases (Fisher’s exact test; P < 0.0005; Table 1). All 10 normal cervical glandular tissues used as control were negative for RASSF1A promoter hypermethylation. Oncogenic HPV-16 E7 DNA was detected in 25 of 31 (81%) of the SCC tumors and in 8 of 20 (40%) of the AC tumors. In this small sample size study, there was no statistical association between methylation status and HPV infection (Fisher’s exact test).

DISCUSSION

RASSF1A is frequently inactivated in a variety of primary human tumors, including lung, breast, ovarian, prostate, and bladder (12, 16, 17, 23, 24). A previous study on RASSF1A inactivation in cervical cancer (17) failed to detect RASSF1A methylation in 22 cases of cervical cancer. In this study, we demonstrate RASSF1A frequent inactivation by promoter hypermethylation in AC but not in SCC of the uterine cervix. Our observation that RASSF1A methylation is absent in SCC (the most common subtype of cervical cancer) may explain the findings in the previous study (17).

Understanding the relationship between HPV infection and RASSF1A inactivation is important in cervical cancer, as well as in other HPV-related tumors. RASSF1A blocks cell cycle progression by engaging the Rb family cell cycle checkpoint and inhibits cyclin D1 accumulation (25). By bypassing the Rb family, dependent cell cycle checkpoint E7 papillomavirus protein can overcome RASSF1A-induced cell cycle arrest (25). The observed inverse correlation between HPV 16 infection and RASSF1A methylation in primary SCC of the head and neck cancers (26) could reflect a functional interaction between the cellular RASSF1A and viral E6/E7 proteins. This interaction could play an important role in both neoplastic transformation and immortalization of cervical epithelial cells. Although we could not demonstrate an inverse correlation between RASSF1A methylation and HPV 16 infection in ACs of the uterine cervix, the absence of RASSF1A methylation in SCCs of the uterine cervix coupled with the high incidence of HPV 16 infection in this subtype do mirror the observed inverse correlation in head and neck SCC (26). Larger studies are needed to formally establish the relationship between RASSF1A methylation and HPV 16 infection in ACs of the uterine cervix and in all HPV-related SCCs.

RASSF1A has been shown to dramatically reduce tumorigenicity in vivo and in vitro (12, 13), and inactivation of both RASSF1A alleles is a critical event in small cell carcinoma of the lung (13, 17). LOH in chromosome 3p is also an early and common event in AC cervical cancer (10). Our data support the notion that epigenetic inactivation of one RASSF1A allele followed by LOH at 3p is important for the development of cervical AC. Moreover, we found that RASSF1A promoter methylation segregates with AC and is distinctly uncommon in SCC of the uterine cervix. These observations suggest distinct patterns of tumor progression in these major histological subtypes of cervical cancer.

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