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Hypermethylation-modulated Downregulation of *RASSF1A* Expression Is Associated with the Progression of Esophageal Cancer

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Background and Aims. Chromosome 3p21 is an important locus harboring critical tumor suppressor genes (TSGs) implicated in the pathogenesis of multiple tumors including esophageal carcinoma (EC). Aberrant promoter methylation is a fundamental mechanism of inactivation of TSGs in cancer. *RASSF1A*, a candidate tumor suppressor gene, recently cloned from the lung tumor locus at 3p21.3, is frequently inactivated by hypermethylation of its promoter region in a number of malignancies. We undertook this study to investigate the methylation status of *RASSF1A* and its significance in esophageal squamous cell carcinoma (ESCC).

Methods. Real-time RT-PCR and real-time methylation-specific PCR (real-time MSP) were used to detect *RASSF1A* expression and the methylation status of the *RASSF1A* promoter, respectively, in 124 primary ESCC tissues.

Results. Hypermethylation, partial methylation and unmethylation of the promoter region of *RASSF1A* were detected in 56 (45.2%), 23 (18.6%) and 45 (36.2%) of 124 ESCC samples, respectively. Unmethylation of the promoter region of *RASSF1A* was detected in 119 (96%) of the 124 corresponding noncancerous tissues. Five (4.0%) of 124 noncancerous tissues showed partial methylation. The presence of hypermethylation was statistically associated with loss of *RASSF1A* mRNA expression in primary ESCC ($p < 0.05$). There were statistically significant correlations between the presence of hypermethylation and regional lymph node involvement ($p = 0.000$), histological differentiation ($p = 0.009$) and tumor stage ($p = 0.000$).

Conclusions. Our results suggest that *RASSF1A* may be one of the ESCC-related TSGs located at 3p21, and hypermethylation of the CpG island promoter of the *RASSF1A* is associated with the progression of ESCC.

Key Words: Esophageal squamous cell carcinoma, *RASSF1A*, CpG islands, Methylation, Tumor suppressor genes, Prognosis.

Introduction

Esophageal cancer (EC) is one of the most serious tumor diseases worldwide, owing to its rapid development and a fatal prognosis in most cases (1). EC is characterized by striking geographic variations throughout the world. For example, the so-called Asian EC belt covers the Taihang Mountain region in northern China (2–4). So far, the basis for these variations is unknown. There are two major histological subtypes of EC: esophageal squamous cell carcinoma (ESCC), which is the predominant subtype in Asian populations, and esophageal adenocarcinoma (EADC), which is more common in Caucasians. Recently, despite advances in early diagnosis and multimodality therapy, in

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general the prognosis of EC is quite poor. Overall 5-year survival rate is $<20\%$, with most patients dying within the first year of diagnosis. Both ESCC and EADC remain a significant problem with very low 5-year survival rate (5).

Although multiple genetic and epigenetic alterations have been detected in ESCC (6−8), the precise molecular mechanisms of carcinogenesis and progression of ESCC remain unknown. Recently, loss of heterozygosity (LOH) analyses revealed that 3p21.3 is another region commonly deleted in several cancers, including EC (9). The downregulated expression of genes in the 3p21.3 has been observed in many kinds of cancers, implying their positive or negative regulation of different tumors (10−12). Transcriptional silencing by hypermethylation of CpG islands containing the promoter regions of tumor suppressor genes (TSGs) is becoming recognized as a common mechanism (13,14). *RASSF1A* has been proposed as a candidate TSG, isolated within the minimal homozygous deletion at 3p21.3 in lung cancer (15). The most frequent mechanism of inactivation of *RASSF1A* is hypermethylation of a CpG island in its promoter sequence, as reported in several types of human cancer cell lines as well as primary human cancer tissue samples such as lung, breast, prostate, and other solid tumors (16−19). Hypermethylation of *RASSF1A* has been highly correlated with a loss of gene expression, and demethylation by 5-aza-2-deoxycytidine (5-Aza-dC) treatment results in a restoration of gene expression, suggesting that hypermethylation of *RASSF1A* is one of the leading causes of silencing of the gene (15−21). Inactivation of *RASSF1A* stimulates anchor-dependent cell growth in lung cancer cell lines, and tumor growth is inhibited by transfection of wild-type *RASSF1A* into hypermethylated lung cancers, suggesting that *RASSF1A* functions as a TSG (22,23).

In this study we analyzed the expression of *RASSF1A* in ESCC cell lines and primary ESCC. To investigate whether hypermethylation of the *RASSF1A* promoter plays a role in the carcinogenesis and progression of ESCC, we determined the frequency and potential clinical implications of the aberrant hypermethylation of the *RASSF1A* promoter in primary ESCC by real-time MSP.

Materials and Methods

Cell Lines

Six ESCC cell lines (KYSE180, KYSE410, KYSE1170, EC1, EC18 and EC109) were used in this study and were maintained in a 1:1 mixture of RPMI 1640 (Invitrogen, Carlsbad, CA) and Ham's F12 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Gibco BRL Life Technologies, Rockville, MD) in humidified 5% CO_2-air at 37°C. To avoid possible effects on gene expression, antibiotic and antimycotic drugs were not used in the cell culture.

5-Aza-dC Treatments

Twenty four hours after seeding of 5×10^5 cells in a 25 cm^2 flask, the cells were exposed to 5-aza-dC (Sigma, St. Louis, MO) for 72 h at a final concentration of 1 μmol/L, which was prepared from 10 mmol/L stock solution dissolved in 50% acetic acid, or they were mock-treated with the same volume of phosphate-buffered saline containing 50% acetic acid. Culture media with and without treatment were changed every 24 h.

Patient Materials

The Institutional Review Board on Medical Ethics of the Zhejiang Province Cancer Hospital approved the method of tissue collection including informed consent. The present study was based on 124 patients who underwent esophagectomy for sporadic ESCC without preoperative radio- or chemotherapy. One hundred nine primary ESCC specimens were collected prospectively from patients in Zhejiang Cancer Hospital (Hangzhou, People's Republic of China) between July 2008 and Jan 2010, and 15 biopsies were obtained from primary ESCC patients in the Department of Surgery, Affiliated Hospital to the College of Medical Sciences, Zhengzhou University (24), Henan Province, China. Average age of the patients was 60.4 years (range: 39−76 years; median, 62 years). There were 101 males and 23 females. All tumor samples were identified histopathologically as ESCC by pathologists. The disease stage of the ESCC cases was classified according to the World Health Organization (WHO) and the tumor-node-metastasis (TNM) classification criteria of the International Union Against Cancer (UICC) (25): 11 were stage I, 46 were stage II, 63 were stage III, and four were stage IV (Table 1). Representative tissue samples from tumors and matching morphologically normal esophageal epithelium tissues (nontumor) at 6−10 cm away from the tumors were sampled during surgery in each patient. Thirteen esophageal dysplasia specimens were obtained from endoscopic biopsy. There were eight male cases and five female cases, aged from 43 to 65 years (median: 53 years). All samples were stored at −80°C until use. Sterile equipment was used for each sampling of tumor and nontumor specimens. Half of each sample was fixed in 10% formalin for histological assessment and for DNA extraction, and the other half was snap-frozen in liquid nitrogen and stored at −80°C for RNA extraction.

Cell Preparation by Laser Capture Microdissection (LCM) or Needle for DNA Extraction

We used 27-G needles to collect the target cells from 34 primary ESCC cases at the Zhejiang Cancer Research Institute. We used LCM to capture cells from 15 cases of primary ESCC in Zhengzhou University. After hematoxylin and eosin (HE) staining, tumor cells and the corresponding