Expression of ECRG4, a novel esophageal cancer-related gene, downregulated by CpG island hypermethylation in human esophageal squamous cell carcinoma

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Abstract
AIM: To study the mechanisms responsible for inactivation of a novel esophageal cancer related gene 4 (ECRG4) in esophageal squamous cell carcinoma (ESCC).

METHODS: A pair of primers was designed to amplify a 220 bp fragment, which contains 16 CpG sites in the core promoter region of the ECRG 4 gene. PCR products of bisulfite-modified CpG islands were analyzed by denaturing high-performance liquid chromatography (DHPLC), which were confirmed by DNA sequencing. The methylation status of ECRG 4 promoter in 20 cases of esophageal cancer and the adjacent normal tissues, 5 human tumor cell lines (esophageal cancer cell line-NEC, EC109, EC9706; gastric cancer cell line- GLC; human embryo kidney cell line-Hek293) and 2 normal esophagus tissues were detected. The expression level of the ECRG 4 gene in these samples was examined by RT-PCR.

RESULTS: The expression level of ECRG 4 gene was varied. Of 20 esophageal cancer tissues, nine were unexpressed, six were lowly expressed and five were highly expressed compared with the adjacent tissues and the 2 normal esophageal epithelia. In addition, 4 out of the 5 human cell lines were also unexpressed. A high frequency of methylation was revealed in 12 (8 unexpressed and 4 lowly expressed) of the 15 (80 %) downregulated cancer tissues and 3 of the 4 unexpressed cell lines. No methylation peak was observed in the two highly expressed normal esophageal epithelia and the methylation frequency was low (3/20) among the 20 cases in the highly expressed adjacent tissues. The methylation status of the samples was consistent with the result of DNA sequencing.

CONCLUSION: These results indicate that the inactivation of ECRG 4 gene by hypermethylation is a frequent molecular event in ESCC and may be involved in the carcinogenesis of this cancer.

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INTRODUCTION
Esophageal cancer (EC) is one of the most common malignant tumors in the world. Previous studies have shown several genetic abnormalities including amplification of c-myc, int-2 and Hst, mutation and/or deletion of p53 and Rb in human EC and EC cell lines[12]. However, the genetic events leading to the development of EC are not clear yet. In recent years, many studies of EC focused on the clone and identification of novel EC-related genes, which might play an important role in the carcinogenesis and development of esophageal cancer[3-5].

Recently, we have cloned and identified a novel tumor candidate suppressor gene, ECRG 4 (Genbank Accession NO. AF 325503), from human normal esophageal epithelium[6, 7]. The ECRG 4 gene located in chromosome 2q14.1-14.3 contains 4 exons, spans about 13 kb and has a full-length cDNA of 772 bp. Analysis by bioinformatics has shown that the protein coded by ECRG 4 shows a 31 % homology with mouse IgG V region. The results of SAGE and RT-PCR detection have demonstrated the ECRG 4 gene is expressed in adult esophageal epithelium but is downregulated in esophageal squamous cell carcinoma (ESCC) and tumor cell lines. These findings suggest that the ECRG 4 gene might be involved in the development of ESCC, but the mechanism inactivating it remains to be determined.

According to the result of the sequence analysis in ECRG 4 gene, we found that there were CpG islands in the promoter region, exon 1 and part of intron 1 of the gene. Many tumor suppressor genes are downregulated by promoter methylation during the development and progression of cancer, and hypermethylation of gene-promoter regions is being revealed as one of the most frequent mechanisms in loss of gene function, thus detection of CpG methylation is important to understand the gene regulation of cancer[8-9]. It has been reported that the expression of some tumor suppressor genes, such as p16INK4a, p16INK4b, FHIT and E-cadherin are commonly downregulated by CpG island hypermethylation in ESCC[10-13]. However, the reason for reducing expression of ECRG 4 in ESCC is unknown.

In order to determine the mechanism involved in the downregulation of ECRG 4 in ESCC, we have examined the methylation status of the 5’ CpG island in promoter region of the ECRG 4 gene in 5 human cell lines, which include 3 esophageal cancer cell lines, 2 normal esophageal epithelia and 20 cases with ESCC and adjacent tissues. The methylation status of the cell lines and tissues were compared with the expression of the ECRG 4 gene in the same samples by RT-PCR respectively.

MATERIALS AND METHODS
Cell lines and tissue samples
Five cell lines, including 3 esophageal cancer cell line-NEC,
Thermal cycles were: at 94°C, 1.25 U LA Taq with 1× LA reaction buffer (TaKaRa).

CpG sites in shadow. The 5’ and 3’ primers are in the frames of the two ends of the fragment respectively. PCR containing 16 CpG sites and 4 cis-acting elements, and they were amplified as internal controls to normalize the amount of ECRG 4 specific products.

RESULTS

The promoter hypermethylation in ECRG 4 gene

Based on the flanking DNA sequences of the ECRG 4-core promoter region, PCR primers were designed to amplify a 220 bp fragment containing the 16 CpG sites (Figure 1).

EC109 and EC9706: 1 gastric cancer cell line- GLC and 1 human embryo kidney cell line-Hek293 were used in this study. All cell lines were routinely cultured in 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone) at 37°C with 5% CO2. 20 pairs of ESCC and corresponding tissues adjacent to the tumors were obtained from surgically removed specimens of individual patients who underwent an operation at the Cancer Hospital in Linxian County which has the highest age-adjusted mortality rate of this cancer. Two normal esophageal epithelia were collected from healthy individuals by biopsy. All the samples were frozen at -70°C before RNA and DNA were extracted with standard method as described previously[14].

**Bisulfite treatment of DNA**

Genomic DNA was treated with sodium bisulfite as described by Herman et al[15]. Briefly, 1 g DNA was denatured by adding freshly prepared NaOH with the final concentration 0.3 M for 15 min in a 37°C water bath. The denatured DNA was then diluted in 30 µl freshly prepared 10 mM hydroquinone (Sigma) and 520 µl freshly prepared 3 M sodium bisulfite (Sigma) at pH 5.0. The DNA was incubated at 50°C for 16 h and subsequently purified by the Wizard DNA Clean-Up System Kit (A7280; Promega).

20 µg of human placenta genomic DNA was incubated for 24 h with 20 units of SssI (New England Biolabs) as described in the instruction manual and the methylated DNA was treated with bisulfite and purified by the Wizard DNA Clean-Up System Kit (A7280; Promega) as described above.

**Design of primers and SsPCR condition**

Primers were designed according to the CpG island of the sense strand of the ECRG 4 gene. The strand-specific primers for the treated CpG island were used to amplify a 220 bp fragment containing 16 CpG sites and 4 cis-acting elements, and they were: 5’-AGT GGG GGG GGT AAG GAG ATAT T-3’ (forward), and 5’-CCC CTA AAC TCC AAA ACC AA-3’ (reverse). PCR was performed in a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, CT) with a 25 µl reaction mixture containing 100 ng DNA, 1.6 µmol each primer, 400 µmol each dNTPs, 1.25 U LA Taq with 1× LA reaction buffer (TaKaRa). Thermal cycles were: at 94°C for 2 min, then 40 cycles at 94°C for 30 sec, at 52°C for 30 sec, at 72°C for 1 min and 30 sec followed by extension at 72°C for 7 min. The β-actin transcripts in each sample were also amplified as internal controls to normalize the amount of ECRG 4 specific products.

**Analysis for methylation by DHPLC**

The SsPCR products of ECRG 4 were introduced into the mobile phase at an injection volume of 5 µl by the autosampler on a WAVE DNA Fragment Analysis System (Transgenomic) identical to that described by Deng et al[16]. Non-denaturing analysis was conducted at 48°C and partially denaturing analysis was conducted at 56°C, which was predicted by WAVEMaker.

The SsPCR product from the SsSI and bisulfite treated human placenta genomic DNA was the positive control of the experiment.

**DNA cloning and sequencing**

The PCR products amplified with primers specific either for the methylated or for the unmethylated DNA were purified and cloned into the pMD18-T Easy Vector (Promega) and sequenced on an ABI 377 automated sequencer (Applied Biosystems) by using M13 primers.

**RT-PCR detection**

Total RNA was isolated from cells and tissues using Trizol reagent (Invitrogen). Reverse transcription was carried out with the SuperScript TM First-Strand Synthesis System (Invitrogen). Approximately 3 µg total RNA was used in each reverse transcription reaction and the final volume was 20 µl. The ORF of ECRG 4 gene was amplified using the primers 5’-GGT TCT CCC TCG CAG CAC CT-3’ (forward), and 5’-CAG CGT GTG GCA AGT CAT GGT TAG T-3’ (reverse). PCR was performed in a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, CT) with a 25 µl reaction mixture containing 1 µl reverse transcription products, 200 pmol each primer, 200 µmol each dNTPs, 1.5 mM Mg2+, 2.0 U PLATINUM pfX DNA polymerase with 1x reaction buffer (Promega). Thermal cycles were: at 95°C for 2 min, then 30 cycles at 95°C for 30 sec, at 62°C for 30 sec, at 72°C for 1 min followed by extension at 72°C for 7 min. The β-actin transcripts in each sample were also amplified as internal controls to normalize the amount of ECRG 4 specific products.

**Figure 1**

The sequence of ECRG 4 fragment for bisulfite-DHPLC analysis. The fragment contains 4 cis-acting elements and 16 CpG sites in shadow. The 5’ and 3’ primers are in the frames of the two ends of the fragment respectively.
the ECRG4 promoter region, either the methylated or the unmethylated DNA was cloned and sequenced (Figure 5). The cytosines in the CpG sites of methylated ssPCR products remained unchanged, but the cytosines of unmethylated products were converted to thymines. The promoter methylation of ECRG4 gene in esophageal tissues is shown in Table 1. A high frequency of methylation was observed in 12 cancer tissues, 3 tumor adjacent tissues and 3 cell lines (EC 9706, EC 109 and GLC). No methylation peak was obtained in the two normal esophageal epithelia, the other tumor and adjacent tissues and the two cell lines (NEC and Hek293).

The expression level of ECRG4 gene was different, and a high frequency of methylation was revealed in 12 (8 unexpressed and 4 lowly expressed) of the 15 (80 %) cancer tissues and the 3 of the 4 unexpressed cell lines. No methylation peak was observed in the two highly expressed normal esophageal epithelia and the methylation frequency was low (3/20) among the 20 cases in the highly expressed adjacent tissues.

**Expression of ECRG4 gene related to methylation**

The expression level of the ECRG4 gene in the tissues and cell lines was examined by RT-PCR (Figure 4b). Out of 20 esophageal cancer tissues, nine were unexpressed, six were lowly expressed and five were highly expressed compared with the adjacent tissues and the 2 normal esophageal epithelia. In addition, 4 out of the 5 human cell lines were also unexpressed. The methylation was observed in 12 (8 unexpressed and 4 lowly expressed) of the 15 (80.0 %) cancer tissues and the 3 unexpressed cell lines (Table 1 and Table 2). Among the normal tissues corresponding to the 12-methylation cancer tissues, nine were highly expressed and unmethylated; three were lowly expressed or unexpressed and methylated (Table 1). No methylation peak was obtained in the highly expressed samples, including the two normal esophageal epithelia, the cell line Hek293 and the other tumor and adjacent tissues. The results demonstrated that the expression of ECRG4 was downregulated by CpG island hypermethylation in human esophageal squamous cell carcinoma.

### Table 1 The expression and methylation of ECRG4 in ESCC

| Cases | Gender | Pathological stage | Expression | Methylation |
|-------|--------|--------------------|------------|-------------|
| N1    | F      | Moderate           | ++         | +           |
| N2    | F      | ++                 | -          | -           |
| 1     | M      | Moderate           | ++         | +           |
| 2     | F      | Moderate           | +          | -           |
| 3     | M      | Moderate           | ++         | -           |
| 4     | M      | Moderate           | ++         | -           |
| 5     | F      | Moderate           | ++         | +           |
| 6     | F      | Moderate           | +          | +           |
| 7     | M      | Moderate           | ++         | -           |
| 8     | F      | Poor               | ++         | -           |
| 9     | M      | Moderate           | ++         | -           |
| 10    | M      | Poor               | ++         | -           |
| 11    | F      | Moderate           | +          | +           |
| 12    | F      | Moderate           | ++         | -           |
| 13    | F      | Moderate           | ++         | -           |
| 14    | M      | Moderate           | ++         | +           |
| 15    | M      | Moderate           | ++         | -           |
| 16    | F      | Moderate           | ++         | -           |
| 17    | M      | Moderate           | -          | +           |
| 18    | F      | Moderate           | ++         | -           |
| 19    | M      | Moderate           | ++         | -           |
| 20    | M      | Moderate           | ++         | -           |

### Table 2 The expression and methylation of ECRG4 in cell lines

| Cell lines | Expression | Methylation |
|------------|------------|-------------|
| NEC        | -          | -           |
| EC209      | -          | -           |
| EC9706     | -          | +           |
| GLC        | -          | +           |
| Hek293     | +          | -           |

a, Female; b, Male; ++, high expression; +, low expression; -, unexpression; *, unmethylation; †, methylation.
DISCUSSION

We used a high-throughput methylation assay, bisulfite-DHPLC assay to examine the methylation status of the ECRG4 gene promoter in ESCC. The results demonstrated for the first time that downregulated expression of ECRG4 in ESCC was associated with CpG island methylation in the core promoter region of the gene. These findings suggest that inactivation by the promoter hypermethylation of ECRG4 is a common molecular event in ESCC and it may be involved in the development of this cancer, since this epigenetic change of the ECRG4 gene was not found in the normal epithelium and immortalizing cell line Hek293. Eads et al reported that DNA hypermethylation was an early epigenetic alteration in the multistep progression of the esophageal adenocarcinoma, because they found that the premalignant tissue was significantly more methylated than the normal tissue[17]. Then, we can speculate that the inactivation by hypermethylation of ECRG4 might be an early event in the progression of ESCC carcinogenesis.

Because of the extent of methylation at various CpG sites of most genes, especially a newly identified gene is unknown, it is hard to design good MSP primers or MethyLight probes for methylated templates, which require full methylation at all CpG sites in their mating region[15,18]. However, the ssPCR for bisulfite-modified templates are not influenced by the extent of methylation of CpGs, because no CpG site exits in the primer sequence and the primer for modified DNA can amplify both methylated and unmethylated templates. Deng et al had compared the bisulfite-DHPLC with other methylation detection method, and demonstrated the bisulfite-DHPLC assay could be used to detect methylation in homoallelic and heteroallelic CpG islands in cell lines and tissues rapidly and reliably[16]. In the present study, we also confirmed the reliability of bisulfite-DHPLC assay by DNA sequencing.

Abnormal hypermethylation of CpG islands associated with tumor suppressor genes can lead to repression of gene expression and contribute significantly to tumorigenesis of many kinds of tumors, such as esophageal cancer, gastric cancer, lung cancer, breast cancer and cervical cancer[19-23]. Furthermore, each tumor type has a characteristic set of genes with an increased propensity to become methylated, and an individual tumor within a single patient has a unique epigenetic fingerprint[24]. Determining tumor-type specific and patient-specific fingerprints may provide biomarkers that can be used in diagnosis, such as cancer detection, cancer chemoprediction and prognostics[25, 26]. The recent study has been repleted with the examples of hypermethylation of CpG islands in the promoter region of more than 40 lung cancer related genes to analyze methylation patterns of multiple genes. They want to obtain complex DNA methylation signatures, which can provide a useful and highly specific tool for lung cancer diagnosis[27].

The promoter hypermethylation of the ESCC-related genes such as, p16INK4a, p15INK4b, hMLH1, E-cadherin, Chfr and HLA class I genes, has been shown to be a common epigenetic event in this cancer and the studies of these genes suggest that hypermethylation of key genes may be used in combination with other molecular changes, such as p53 mutation, in the development of biomarkers for predicting the risk for ESCC[28-30]. Our present study extended the findings of methylation signature in ESCC, and the methylation in more ESCC-related genes was studied, better understanding of the mechanisms underlying tumor progression in this cancer was

Figure 5 Sequencing of ssPCR products of the ECRG4 gene promoter region. All cytosines in CpG dinucleotides in the methylated ECRG4 remain as cytosines, indicating methylation (A), while all cytosines in unmethylated ECRG4 have been converted to thymidines, indicating unmethylation (B).
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