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• ESOPHAGEAL CANCER •

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[1,2]. 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 $p16^{INK4a}$, *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,

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 ℃ with 5 % CO_2. 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 ℃ 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 ℃ 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 ℃ 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 by 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 GGA GTT AAG GAG ATA TT -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 about 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 ℃ for 2 min, then 40 cycles at 94 ℃ for 30 sec, at 52 ℃ for 30 sec, at 72 ℃ for 1 min and 30 sec followed by extension at 72 ℃ for 7 min. The PCR products were detected in 1.5 % agarose gels.

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 ℃ and partially denaturing analysis was conducted at 56 ℃, 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 Mg^{2+}, 2.0 U PLATINUM pfx DNA polymerase with 1×reaction buffer (Promega). Thermal cycles were: at 95 ℃ for 2 min, then 30 cycles at 95 ℃ for 30 sec, at 62 ℃ for 30 sec, at 72 ℃ for 1 min followed by extension at 72 ℃ 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.

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). Using the ssPCR-specific primers, a 220 bp product was successfully obtained from each bisulfite-treated sample, which was detected by 1.5 % agarose gels and DHPLC size analysis, and the specific band (Figure 2) and the single chromatogram peak (Figure 3) were obtained respectively. The agarose gel detection and the size analysis on DHPLC all indicating the quality and quantity of the ssPCR products were high and the products could be used in the methylation analysis on DHPLC. Figure 4a shows the detection of methylated and unmethylated CpG islands in ssPCR products by DHPLC. Compared with the peak of positive control from the *SssI* treated human fetal DNA, the methylated and unmethylated samples could be easily discerned. The different proportion of methylated peak represented the different methylation levels in different samples. To confirm the reliability of the ssPCR products of

agtgg**gggag c**caaggagac accccag**cg**c tgggatc**cg**g caagtcctcc ctc**tgagtgg**
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box
gcttgt**ccta accgctttcg ct**g**cg**ggcag **cg**ctggcca**c gcg**gcccc**cg** c**cg**c**cg**g**cg**g
BARBIE box
ttctc**cgtgg ccaa**gcatcc ttggccttgg agcccagggg
CAAT box

Figure 1 The sequence of *ECRG 4* fregment 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).

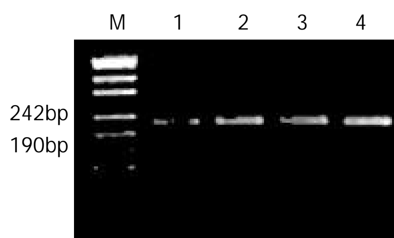

Figure 2 The 1.5 % agarose gel detection of the ssPCR products of *ECRG 4*. M; pUC19 DNA/ *Msp I Hap II)* Marker. 1, 2, 3, 4; four tissue samples.

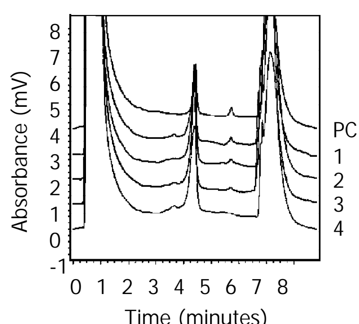

Figure 3 The sizing analysis of ssPCR products of *ECRG 4* on DHPLC at 48 ℃. PC was the product from the *Sss I* treated human placenta genomic DNA. 1, 2, 3, 4; the same samples as in Figure 2.

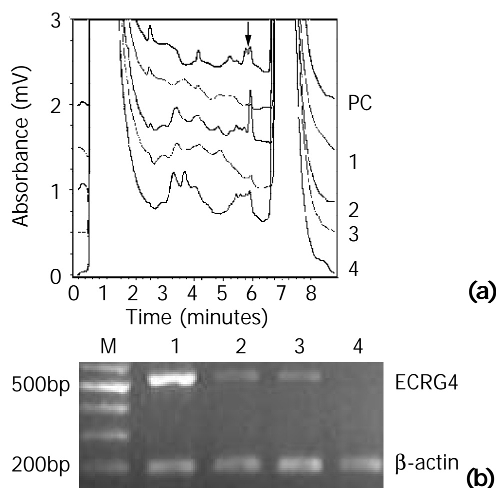

Figure 4 **(a)** The methylation detection of ssPCR products at 56 ℃ on DHPLC. The methylation peak was emphasized by the arrow. PC was the product from the *SssI* treated human placenta genomic DNA. 1, 2, 3, 4; the same samples as Figure 2. **(b)** The expression level of *ECRG 4* by RT-PCR using the primer set flanking the ORF of the gene in the same samples was detected on DHPLC. The β-Actin gene was amplified as internal control. M; 1 kb DNA Ladder Marker. 1, 2, 3, 4; the same samples as Figure 2.

The expression level of *ECRG 4* 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 *ECRG 4* 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 *ECRG 4* in ESCC

Cases	Gender	Pathological stage	Expression		Methylation	
			Normal	Cancer	Normal	Cancer
N1	F[a]		++[c]		-	
N2	F		++		-	
1	M[b]	Moderate	++	+[d]	-[f]	+[g]
2	F	Moderate	+	-	-	+
3	M	Moderate	++	++	-	-
4	M	Moderate	++	++	-	-
5	F	Moderate	++	-[e]	-	+
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	++	-	-	+

[a], Female; [b], Male; [c]++, high expression; [d]+, low expression; [e]-, unexpression; [f]-, unmethylation; [g]+, methylation.

Table 2 The expression and methylation of *ECRG 4* in cell lines

Cell lines	Expression	Methylation
NEC	-[a]	-[c]
EC109	-	+[d]
EC9706	-	+
GLC	-	+
Hek293	+[b]	-

[a]-, unexpression; [b]+, expression; [c]-, unmethylation; [d]+,methylation.

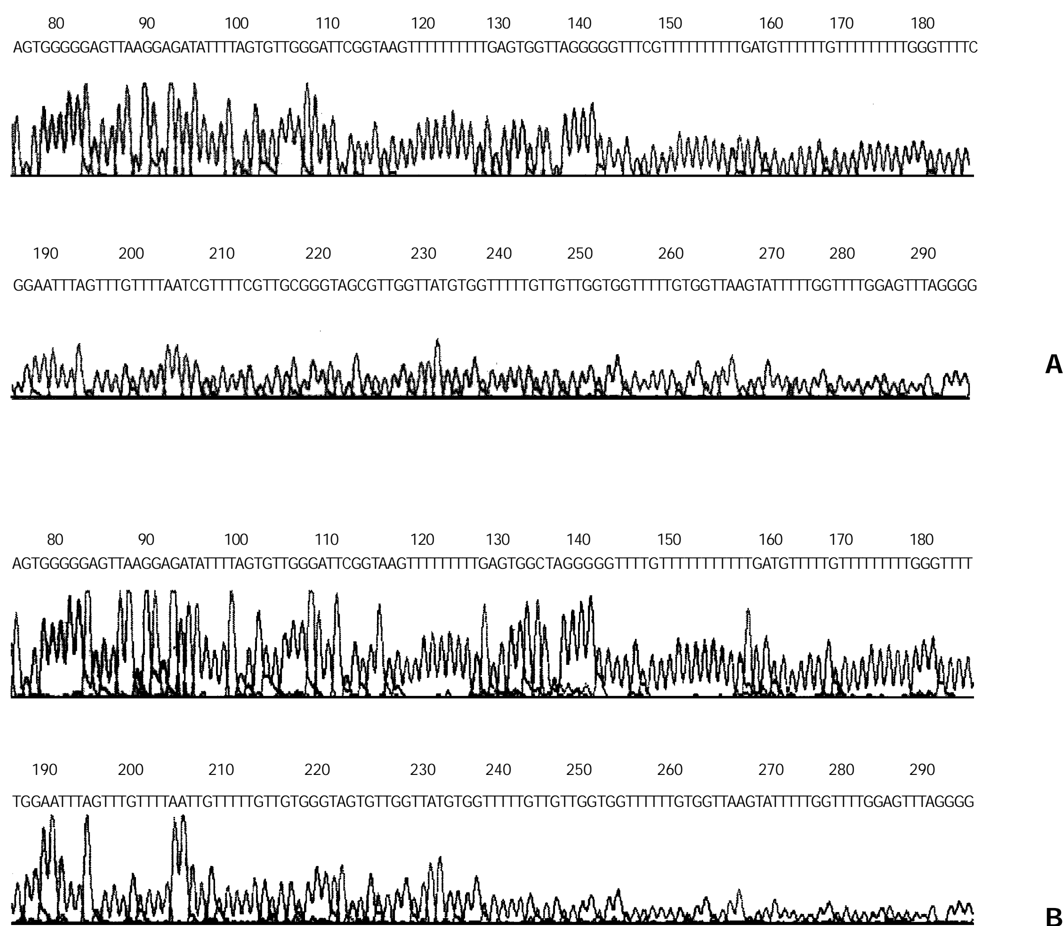

Figure 5 Sequencing of ssPCR products of the ECRG4 gene promoter region. All cytosines in CpG dinuleotides in the methylated ECRG4 remain as cytosines, indicating methylation (**A**), while all cytosines in unmethylated ECRG4 have been converted to thymidines, indicating unmethylation (**B**).

DISCUSSION

We used a high-throughout methylation assay, bisulfite-DHPLC assay to examine the methylation status of the *ECRG 4* gene promoter in ESCC. The results demonstrated for the first time that downregulated expression of *ECRG 4* 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 *ECRG 4* is a common molecular event in ESCC and it may be involved in the development of this cancer, since this epigenetic change of the *ECRG 4* gene was not found in the normal epithelium and immortalling 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 *ECRG 4* 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 novelly 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 analyse 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 hypermethlation of the ESCC-related genes such as, *p16*^{INK4a}, *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 understaning of the mechanisms underlying tumor progression in this cancer was

obtained, so that improved diagnosis and therapy can be facilitated.

In summary, our study demonstrated that aberrant methylation of CpG islands in the core promoter of the *ECRG 4* gene was a frequent molecular event in ESCC and proved for the first time that loss or lower expression of *ECRG 4* was associated with *ECRG 4* CpG island methylation. These results indicate that the inactivation of *ECRG 4* gene by hypermethylation in ESCC may be involved in the carcinogenesis of the cancer.

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