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Chfr expression is downregulated by CpG island hypermethylation in esophageal cancer

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Cell cycle progression is monitored by checkpoint mechanisms to ensure the integrity of the genome and the fidelity of sister chromatid separation. Failure of such checkpoint functions results in genomic instability, a condition that predisposes cells to neoplastic transformation and tumor progression. Recently, Scolnick and Halazonetis defined a new mitotic checkpoint that acts at prophase and delays chromosome condensation in response to mitotic stress, and identified a gene, named checkpoint with FHA and ring finger (*Chfr*), that seems to be required for delaying prophase in human cells. In the present study, we examined human *Chfr* mRNA expression in 15 human esophageal cancer cell lines and 43 primary esophageal cancers to investigate the potential involvement of *Chfr* in the pathogenesis of esophageal cancers. We report here that a significant proportion of human esophageal cancer has loss of expression of *Chfr* gene. Furthermore, we found aberrant hypermethylation of the promoter region of this checkpoint gene in four of 15 (26.7%) esophageal cancer cell lines and in seven of 43 (16.3%) primary cancers.

Introduction

Esophageal squamous cell carcinoma is one of the most common cancers worldwide. Patients with this cancer generally have a poor prognosis, because of a virulent malignancy that often is diagnosed in its late stage. Although treatment such as pre-operative chemotherapy and chemoradiation therapy are currently used for the patients with advanced esophageal cancer, their results are not satisfactory (1,2). Even in early-stage disease, we have experienced many patients who developed local recurrence of tumor or distant metastasis within a short period after operation. Accumulating evidence in the field of molecular carcinogenesis for this fatal disease is expected to yield new medical treatment strategy.

Recent molecular biological studies have clearly indicated that many cancers including esophageal cancer are diseases caused by the accumulation of multiple genetic defects in dominant oncogenes and tumor suppressor genes, which are involved in various cellular processes such as cell cycle regulation, growth signal transduction and induction of apoptotic cell death (3). A number of surveillance mechanisms exist in cells to ensure maintenance of genomic stability against various types of damage to the genome. The G_1 checkpoint prevents replication of damaged DNA, while genomic integrity before mitosis is monitored by the G_2 checkpoint, which promotes G_2 arrest on detection of DNA damage. Failure of such checkpoint functions results in genomic instability, a mutagenic condition that predisposes cells to neoplastic transformation and tumor progression (4,5). We have reported previously *in vivo CHK2* inactivation, which suggests the possible involvement of G_2 checkpoint impairment in the pathogenesis of human lung cancer (6).

During mitosis the replicated genetic material is segregated, with one copy of each chromosome going to each daughter cell with fidelity. Mitosis is monitored by several checkpoint systems (7,8). Recently, Scolnick and Halazonetis (9) defined a new mitotic checkpoint which delays chromosome condensation in response to mitotic stress induced by taxol or nocodazole, and also identified a gene, named checkpoint with FHA and ring finger (*Chfr*), that seems to be required for delaying prophase in human cells. They investigated eight different human tumor cell lines, and found that three of these cell lines do not express the *Chfr* gene (9). Although the prophase checkpoint genes are potential targets for genetic alteration in human cancers, no information is available whether such defect in human esophageal cancer exists.

In the present study, we examined human *Chfr* expression in 15 human esophageal cancer cell lines and 43 primary esophageal cancers to investigate the potential involvement of *Chfr* in the pathogenesis of esophageal cancers. We report here a significant proportion of human esophageal cancer has loss of expression of *Chfr* gene. Furthermore, we searched for epigenetic alterations in this prophase checkpoint gene and found aberrant hypermethylation of its promoter region in four of 15 esophageal cancer cell lines and in seven of 43 primary cancers.

Materials and methods

Esophageal cancer cell lines and specimens

Fifteen (14 squamous cell carcinomas, one adenocarcinoma) esophageal cancer cell lines were analyzed in this study. One colon cancer cell line DLD1 used by Scolnick and Halazonetis was also included as control (9). Forty-three patients with primary esophageal squamous cell carcinomas underwent resection at Nagoya City University Hospital from January 1996 to December 2000. All patients had a single tumor and no distant metastasis. None of the patients died of postoperative complications within 30 days. There were 32 males and 11 females, and the mean age was 62.7 years (47–80 years). There were one stage 0, three stage I, six stage II, 17 stage III and 16 stage IV tumors. Samples of the tumors and paired normal esophageal tissues were collected at resection and immediately frozen in liquid nitrogen.

Northern blot analysis

Northern blot analysis was performed using 10 μg of total RNA according to the standard protocol. A 761 bp cDNA probe for the *Chfr* gene was generated by PCR amplification with F1 (sense; 5′-GCATACCTCATCCAGCATCC) and R (antisense; 5′-TAGGTCAGCTCACGGAAGCT) oligonucleotide primers.

Abbreviations: GAPDH, glyceraldehydes-3-phosphate dehydrogenase; MSP, methylation-specific PCR.

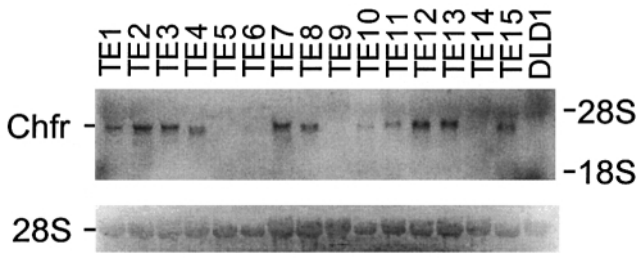

Fig. 1. Loss of Chfr expression in esophageal cancer cell lines. Northern blot analysis showed that Chfr expression was undetectable or negligible in TE5, TE6, TE9 and TE14. One colon cancer cell line, DLD1, used by Scolnick and Halazonetis was included as a negative control.

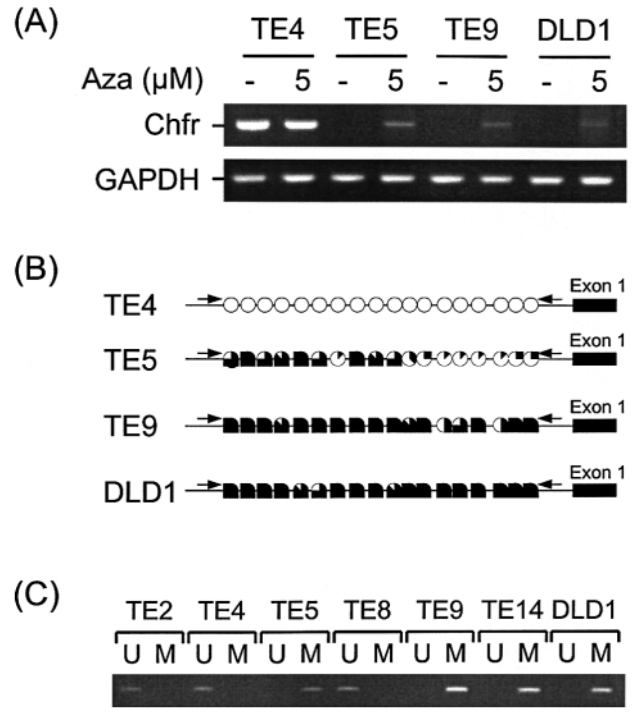

Fig. 2. (A) Treatment with 5-aza-2′-deoxycytidine in TE4, TE5, TE9 and DLD1. RT–PCR showed obvious restoration of *Chfr* mRNA expression in TE5, TE9, DLD1 supporting the possibility of aberrant hypermethylation. **(B)** Summarized sequence of genomic DNAs treated with sodium bisulfite. Filled circles, completely methylated; open circles, unmethylated; partially filled, partially methylated. Sequence of at least eight clones clarified dense aberrant hypermethylation in TE9 and DLD1, and heterogenous methylation in TE5; these cell lines showed decreased *Chfr* mRNA expression. Whereas in TE4, which showed normal *Chfr* mRNA expression, no CpG site was methylated. **(C)** MSP analysis of DNAs from cell lines. Four of 15 (26.7%) esophageal cancer cell lines indicated apparent hypermethylation of the *Chfr* promoter region. M, methylated DNA-specific amplification; U, unmethylated DNA-specific amplification.

RT–PCR

RNA extraction and RT reaction was performed as described previously (10). PCR using random primed cDNAs was performed using F (sense; 5′-AGCTCAACCTGGGTGACAAG) and R (antisense; see above) oligonucleotide primers, which generated a 229 bp PCR product. PCR amplification consisted of 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) was used as loading control, and the primers used were S (sense; 5′-AATCAAGTGGGG-CGATGCTG) and AS (antisense; 5′-GCAGAGATGATGACCCTTTTG), which generated a 118 bp PCR product. The same temperature profile was used except for the annealing step, which was 55°C for 30 s.

5-aza-2′-deoxycytidine treatment

Four cell lines (TE4, TE5, TE9 and DLD1) were seeded at a density of 2×10^5 cells/100 mm plate. Thirty-six hours later cells were treated with 0, 2, 5 or 10 μm 5-aza-2′-deoxycytidine (Sigma, St Louis, MO). Cells were grown in a medium containing 5-aza-2′-deoxycytidine for 4 days, the medium and drug were replaced every 48 h.

Methylation analysis

Sodium bisulfite conversion of genomic DNA of esophageal cancer cell lines and primary esophageal cancers was performed essentially as described by Ferguson *et al.* (11), followed by PCR amplification using MetF (sense; 5′-GGTTAGGATTAAAGATGGT) and MetR (antisense; 5′-ACTCCCTC-AACTAATCC) oligonucleotide primers, which generated a 155 bp PCR product. PCR amplification consisted of 35 cycles of 94°C for 25 s, 53°C for 25 s and 72°C for 25 s. Genomic organization of the *Chfr* gene was obtained through GenBank (accession no. AC023047). The resultant products were purified using QIA quick Gel Extraction Kit (Qiagen, Chatsworth, CA). The purified PCR product was cloned using TA Cloning Kit Dual Promoter (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's recommendations. At least eight clones were sequenced using M13 R primer with an ABI3100 DNA sequencer and a Big Dye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Bisulfite conversion was confirmed to be complete by presence of substituted thymine for all cytosine residues at non-CpG sites.

Methylation-specific PCR

Methylation-specific PCR (MSP) amplification was carried out as described by Herman *et al.* (12) with the following oligonucleotide primers, which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite conversion as described above. Methylated DNA-specific primers were MS (sense; 5′-TTTCGTGATTCGTAGGCGAC), MAS (antisense; 5′-GCGATTAACTAACGACGACG). Unmethylated DNA-specific primers were UMS (sense; 5′-TTTTGTGATTTGTAGGTGAT), UMAS (antisense; 5′-ACAATTAACTAACAACAACA). PCR amplification consisted of 35 cycles of 94°C for 25 s, 58°C for 25 s and 72°C for 25 s (MS and MAS), 94°C for 25 s, 53°C for 25 s and 72°C for 25 s (UMS and UMAS), which generated a 155 bp PCR product. The resultant PCR products were separated on 3% agarose gels stained with ethidium bromide and visualized under UV illumination. Each MSP was repeated at least three times.

Real-time RT–PCR with the LightCycler

Real-time RT–PCR was performed with a single-step method using *Chfr*-specific oligonucleotide primers F and R. PCR amplification using a LightCycler (FastStart DNA Master SYBR Green I) instrument (Roche, Mannheim, Germany) was carried out according to the manufacturer's instructions. Real-time PCR monitoring was achieved by measuring the fluorescent signal at the end of the annealing phase for each cycle. External standards for *Chfr* and *GAPDH* mRNA were prepared by the serial dilutions (1:1 to 1:128) of cDNA from TE8 esophageal carcinoma cell line. Each run consisted of eight external standards, a negative control without a template and patient samples with unknown mRNA concentrations. Quantification of mRNA in each sample was then performed automatically by reference to the standard curve constructed each time according to the LightCycler software.

Statistical analyses

The relative mRNA expression levels (*Chfr*/*GAPDH*) were calculated from quantified data. The statistical software package StatView 5.0 was used. Association between *Chfr* expression [tumor/normal tissue (T/N)] and MSP status was examined using Mann–Whitney's *U* test. A P value <0.05 indicated statistical significance.

Results

The expression of *Chfr* mRNA was examined in 15 esophageal cancer cell lines (14 squamous carcinoma cell lines and one adenocarcinoma cell line) with the aid of northern blot analysis. Four of 15 cell lines (TE5, TE6, TE9 and TE14) showed no or negligible expression. DLD1, a colon cancer cell line used as a control, did not show *Chfr* mRNA expression as reported by Scolnick and Halazonetis (9) (Figure 1).

The putative promoter region of the *Chfr* gene appeared to harbor a CpG island. To investigate the possibility that the reduced expression of *Chfr* was caused by its promoter hypermethylation, four cell lines including those with low or negative *Chfr* mRNA expression (TE4, TE5, TE9 and DLD1) were treated with 5-aza-2′-deoxycytidine. RT–PCR showed obvious restoration of *Chfr* mRNA expression in TE5, TE9 and DLD1 supporting the possibility of aberrant hypermethylation

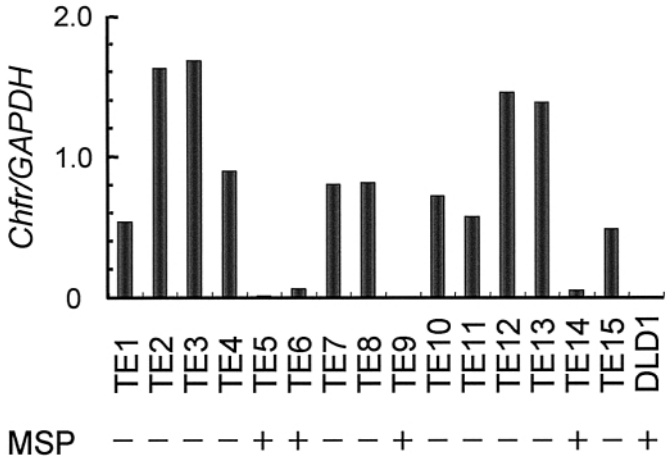

Fig. 3. Summary of Chfr mRNA quantification with LightCycler and MSP status in cell lines. The expression of northern blotting analysis is similar to that of LightCycler. Four cell lines with methylated CpG island showed no or negligible level of expression, whereas 11 cell lines that are not methylated expressed Chfr at various levels.

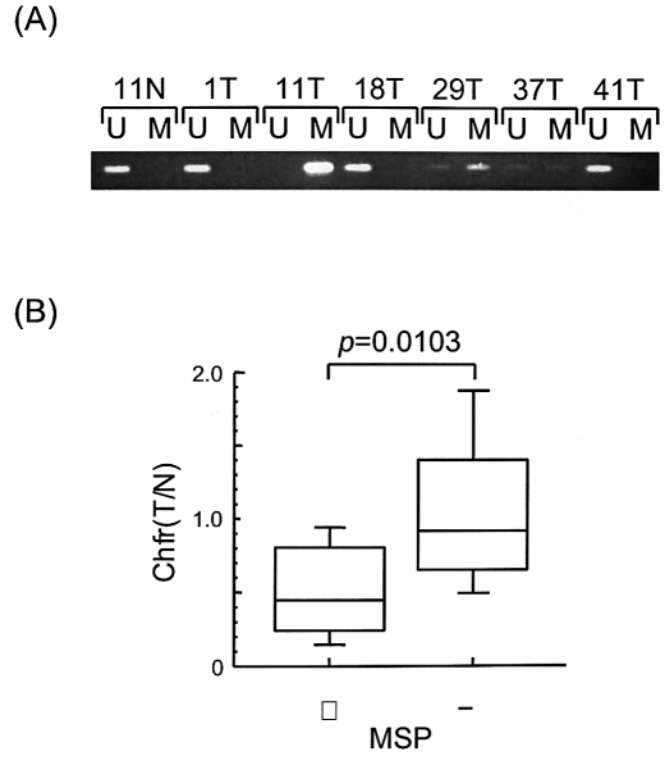

Fig. 4. (A) MSP analysis of DNAs from primary tumors. Seven of 43 (16.3%) primary cases showed apparent hypermethylation of the *Chfr* promoter region. M, methylated DNA-specific amplification; U, unmethylated DNA-specific amplification. **(B)** Summary of *Chfr* mRNA expression (T/N) and MSP status. *Chfr* mRNA was quantified using LightCycler and standardized with *GAPDH* mRNA expression. *Chfr* mRNA expression as a ratio of expression in the tumor to that in the normal esophageal mucosa is shown for MSP positive and negative samples. Box indicates 75 and 25 percentile, horizontal line indicates the mean; bars indicate 10 and 90 percentile. MSP status was significantly correlated with the *Chfr* mRNA expression ($P = 0.0103$, Mann–Whitney's *U* test).

(Figure 2A). Expression of *Chfr* mRNA was not changed by 5-aza-2′-deoxycytidine treatment in TE4, which expressed *Chfr* mRNA.

We investigated methylation status of 5′ region of *Chfr* gene in esophageal cancer cell line. Genomic DNA extracted from the four cell lines was treated with sodium bisulfite, followed by PCR amplification using MetF and MetR primers. Because unmethylated cytosine residues were converted to thymine, whereas methylated cytosine residues were resistant to bisulfite modification, different sequences should be created according to the methylation status. The PCR products were then cloned and sequenced. Cumulative data derived from sequence information from multiple clones revealed that the 5′ CpG island of the *Chfr* gene was densely methylated in TE9 and DLD1. In TE5, the CpG island of the *Chfr* gene was heterogeneously methylated in this region. On the contrary, no CpG sites were methylated in TE4, which showed normal *Chfr* mRNA expression (Figure 2B).

MSP was performed using DNA from 16 cell lines (TE1–TE15 and DLD1). The presence of a visible PCR product with methylated DNA-specific primers was shown in four of 15 (26.7%) esophageal cancer cell lines (Figure 2C).

Using real-time RT–PCR, we quantified the *Chfr* mRNA expression in these cell lines. The relative level of *Chfr* mRNA expression was shown as the ratio of the mRNA of *Chfr* to that of *GAPDH*. The data with real-time RT–PCR was similar to that obtained with Northern blotting analysis. As shown in Figure 3, the cell lines with methylated CpG island showed no or only a negligible level of expression. Eleven cell lines with unmethylated *Chfr* gene promoter expressed *Chfr* at various levels.

Next, we investigated the methylation status in 43 primary esophageal cancers using the MSP technique. One normal and six tumor samples are shown as an examples in Figure 4A. Seven of 43 primary samples showed the presence of a visible PCR product with the aid of methylated DNA-specific primers, indicating that these promoter regions were methylated. In the normal esophageal mucosa, no methylation of the *Chfr* gene was observed. In some cases, concurrent amplification by both methylated DNA-specific and unmethylated DNA-specific PCR primers was observed. It may be due to either contamination with normal tissue or the presence of hemizygous methylation. Subsequently, we investigated the expression level in 43 primary esophageal cancers using real-time RT–PCR. All tumor and corresponding normal samples showed a variety of *Chfr* mRNA expression. MSP status was significantly correlated with the *Chfr* mRNA expression level ($P = 0.0103$; Mann–Whitney's *U* test) (Figure 4B). Four cases out of seven cases with significantly decreased *Chfr* expression (<50% of that in corresponding normal mucosa) had aberrant methylation in their tumor DNA. MSP status and *Chfr* mRNA expression had no statistical relationship with the clinicopathological features, such as tumor size, lymphatic metastatic status, stage, histological subtype and survival after surgical treatment (Table I).

Discussion

The segregation of chromosome at mitosis involves a series of steps, including condensation of chromosome and separation of the centrosome, chromosomal alignment and sister-chromatid separation. Mitosis and cytokinesis are undoubtedly the most spectacular parts of the cell cycle. Error in the choreography of these processes could lead to aneuploidy or genetic instability, fostering cell death or disease. We and others found that the mitotic checkpoint function is impaired in a significant proportion of human cancer cell lines (13,14). Genetic alterations in this checkpoint gene were also reported

Table I. Correlation of Chfr expression in esophageal cancer

Characteristics	No. of patients	Chfr mRNA expression (T/N) relative to GAPDH (mean ± SD)	*P* value*
Age at surgery (year)			
<65	25	1.047 ± 0.917	0.844
>65	18	1.436 ± 1.775	
Sex			
Male	32	1.207 ± 1.235	0.373
Female	11	1.216 ± 1.675	
Pathological stage			
0, I, II	10	0.867 ± 0.667	0.288
III, IV	33	1.313 ± 1.477	
Tumor factor			
pT1, pT2, pT3	32	1.216 ± 1.228	0.231
pT4	11	1.191 ± 1.691	
Nodal factor			
pN0, pN1, pN2, pN3	34	1.200 ± 1.340	0.835
pN4	9	2.004 ± 1.415	
Histological subtype, squamous cell carcinoma			
Well, moderately	36	1.072 ± 1.178	0.065
Poorly	4	2.459 ± 2.513	
Vein invasion			
Negative	14	1.412 ± 1.188	0.770
Positive	25	1.273 ± 1.523	
Lymphatic invasion			
Negative	5	1.862 ± 1.790	0.425
Positive	34	1.159 ± 1.338	
MSP status			
Negative	36	344 ± 1.421	
Positive	7	0.519 ± 0.317	0.0103

*Mann–Whitney's *U* test.

as a target (13,15,16). These studies support a possible link between impaired mitotic checkpoint and oncogenesis.

In this study we investigated the expression of *Chfr*, a recently discovered gene involved in prophase checkpoint, in esophageal cancer cell lines and primary tumor samples. Our study demonstrated that four out of 15 esophageal cancer cell lines showed loss of expression of *Chfr* mRNA. Identification of frequent loss of expression led us to search the mechanism involved in this reduction. We showed that aberrant methylation of promoter region correlated well with loss of mRNA expression, and treatment with the methyltransferase inhibitor 5-aza-2′-deoxycytidine induced re-expression of the gene. Epigenetic changes, particularly DNA methylation, are susceptible to modulation by exogenous factors and may be a candidate mechanism to explain how certain environmental factors may increase the risk of cancer (17,18). Several tumor suppressor genes contain CpG islands in their promoters, suggesting a role of methylation in silencing these genes (18,19). Using primary esophageal cancer samples, we showed that aberrant hypermethylation of the 5′ CpG island of the *Chfr* gene is closely associated with transcriptional inactivation and might be involved in tumor development of the esophagus.

Many anticancer drugs work by disturbing microtubule function. The most investigated new agent in esophageal cancer is paclitaxel (20–22). Cells that lack Chfr may be more sensitive to microtubule poisons, resulting from a failure of checkpoint function. We did not examine the genetic alteration in the *Chfr* gene. Further study is warranted to study whether the *Chfr* gene is mutated in esophageal cancers as reported in U2OS neuroblastoma cell line (9).

In this study, we showed that ~20% of esophageal cancer had decreased the level of *Chfr* mRNA expression; this reduction was due to aberrant hypermethylation of the promoter region of the *Chfr* gene. We also reported previously *Chfr* promoter hypermethylation in lung cancer (23). In some other cancers, *Chfr* might be downregulated by promoter hypermethylation. Abrogation of these mechanisms may have an important permissive role in the development and progression of cancer by allowing cells to progress through abnormal mitosis that could generate genetically unstable progeny. It will be interesting to examine the relationship between the prophase checkpoint defect and chromosome instability. Further study will shed light on the mechanism of cancer development as well as more effective cancer therapies.

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