Aberrant Methylation of the *CHFR* Gene is Frequently Detected in Non-invasive Colorectal Cancer

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Abstract. *Background: Aberrant methylation of the CHFR gene associated with gene silencing has been reported in several primary tumors. In order to define the role of CHFR in the tumorigenic pathway of the colorectum, the methylation of CHFR was examined in tumors from colorectal cancer patients. Materials and Methods: Ninety-eight colorectal cancer patients were examined using a methylation-specific PCR (MSP) for CHFR CpG island in primary tumors. Results: An aberrant methylation of the CHFR gene was detected in 25 out of 98 (26%) primary colorectal cancers. No methylation was detected in the corresponding normal tissue specimens. This finding suggested that an aberrant methylation of the CHFR gene occurs frequently in colorectal cancers. After a methylation analysis of all samples, the clinicopathological data were correlated with these results. A significant difference was found in the tumor (p=0.035), thus, indicating that in early colorectal cancer the CHFR gene was more frequently methylated than in advanced cases. Conclusion: These findings suggest that CHFR might act as a tumor suppressor in at least some colorectal cancers and that CHFR methylation might, therefore, be a particular phenomenon of early colorectal cancer.*

A series of genetic alterations in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human colorectal cancer. The activation of oncogenes, such as the *ras* gene, and the inactivation of tumor suppressor genes, such as the *APC* and *p53* genes, have been identified in colorectal cancer (1-3). In addition, several other genes have also been found to be related to the pathogenesis of colorectal cancer (4, 5). An investigation of the genetic changes is, therefore, important in clarifying the tumorigenic pathway of colorectal cancer (6).

Abbreviations: MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR.

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Key Words: CHFR, methylation-specific PCR, colorectal cancer.

In recent years, the *CHFR* (checkpoint with FHA and RING finger) gene has been reported as a new mitotic checkpoint gene. CHFR protein delayed chromosome condensation and entry into metaphase in response to the mitotic stress induced by a microtubule inhibitor (7). However, cancer cell lines lacking *CHFR* entered into metaphase without any delay after treatment with a microtubular inhibitor, thus, suggesting the function of *CHFR* as a mitotic checkpoint. Recently, the aberrant methylation of the *CHFR* gene associated with gene silencing has been reported in several primary tumors (8-12). These results prompted us to examine the methylation status of the *CHFR* gene in surgically removed colorectal cancers.

In the present study, the methylation status and gene expression of *CHFR* in several cancer cell lines were first examined using methylation-specific PCR (MSP) and reverse transcription-PCR (RT-PCR), respectively. The methylation status of the *CHFR* gene in primary tumors and corresponding normal tissues derived from 98 patients with colorectal cancer was then examined and the correlation between the methylation status and the clinicopathological findings was evaluated.

Materials and Methods

Sample collection and DNA preparation. An esophageal cancer cell line (NUEC3) and a gastric cancer cell line (NUGC3) were established in our laboratory. Three colorectal cancer cell lines (DLD1, SW480 and SW1116) were obtained from the American Type Culture Collection (Manassas, VA, USA). They were grown in RPMI 1640 supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37˚C.

Ninety-eight primary tumor and corresponding normal tissue specimens were collected consecutively at Nagoya University Hospital, Japan, from colorectal cancer patients during colorectal surgery. All tissue specimens were confirmed histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all patients. Collected samples were stored immediately at –80˚C until analysis. DNA was prepared as

Table I. *Clinicopathological features and CHFR promoter methylation in colorectal cancer.*

Clinicopathological features	Variable	No. of cases	*CHFR* methylation		*p*-value
			+	–	
Gender	male	58	14	44	0.708[1]
	female	40	11	29	
Age	<65	61	12	49	0.089[1]
	65≤	37	13	24	
Maximal size (cm)		98	5.1±1.4[3]	4.8±2.1	0.406[2]
Tumor extent	<mt[4]	75	23	52	0.035[1]
	mt≤	23	2	21	
TNM stage	I	18	2	16	0.121[1]
	II, III, IV	80	23	57	
Lymph node metastasis	+	36	7	29	0.294[1]
	–	62	18	44	
Total		98	25	73	

[1]Fisher's exact test; [2]Student's *t*-test; [3]mean±S.D; [4]mt, muscular tunic.

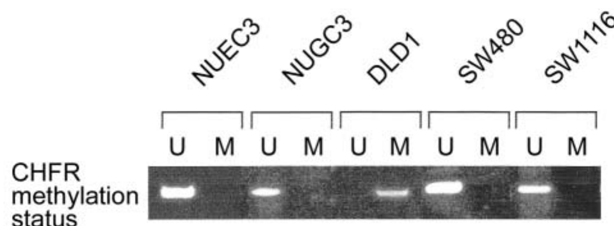

Figure 1. *Methylation-specific PCR analysis for the methylation status of CHFR promoter in human cancer cell lines. The presence of a visible PCR product in lane U indicates the existence of unmethylated genes; the presence of a product in lane M indicates the existence of methylated genes. The aberrant methylation of CHFR was detected in 1 colorectal cancer cell line (DLD1).*

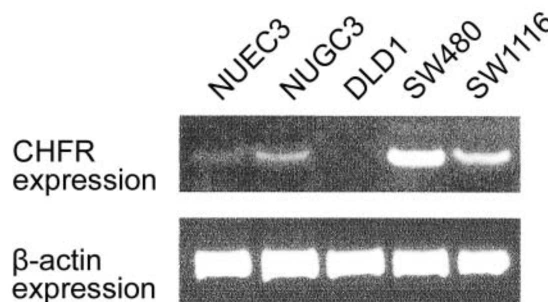

Figure 2. *An analysis of the CHFR expression by RT-PCR. DLD1, which showed a methylated allele in an MSP analysis, lacked any CHFR expression, whereas CHFR was expressed in all other cell lines with non-methylated CHFR promoter. The expression of β-actin was used as a control to confirm the success of the RT reaction.*

described elsewhere (13). The clinicopathological characteristics of the patients enrolled in the study are shown in Table I.

Sodium bisulfite modification. One μg of the genomic DNA extracted from the tumor and corresponding normal colorectal tissue specimens was subjected to bisulfite treatment, as described previously (14). Briefly, alkali-denatured DNA was modified by 2.1 M sodium bisulfite / 0.5 mM hydroquinone at pH 5.0. The bisulfite-reacted DNA was then treated by NaOH, purified using the Wizard DNA Clean-Up System (Promega, Madison, WI, USA), precipitated with ethanol and resuspended in distilled water.

MSP. The bisulfite-treated DNA was amplified with MSP. The primer sequences of *CHFR* for the unmethylated reaction were: *CHFR* UMS (sense), 5'-GTTTTAATATAATATGGTGTTGATT-3', and *CHFR* UMAS (antisense), 5'-AAAACAAC AACTAA AACAAAACCA-3', which amplify a 144-base pair product. Primer sequences of *CHFR* for the methylated reaction were: *CHFR* MS (sense), 5'-TTTTAATATAATATGGC GTCGATC-3', and *CHFR* MAS (antisense), 5'-AACGACAA CTAAAACGAAACCG-3', which amplify a 141-base pair product. The PCR amplification of modified DNA samples consisted of 1 cycle at 95˚C for 5 min, 1 cycle at 78˚C for 10 min, 37 cycles of denaturing at 95˚C for 30 sec, 1 min of annealing at 57˚C, 1 min of extension at 72˚C and a final extension step of 10 min at 72˚C. Modified DNAs obtained from NUEC3 and DLD1 were used as positive controls for unmethylated and methylated alleles, respectively. The controls without DNA were included in each assay. Ten μl of each PCR product was loaded directly onto non-denaturing 10% polyacrylamide gel, stained with ethidium bromide, and were then visualized under UV illumination. Each MSP was repeated at least twice.

RT-PCR. The expression of the *CHFR* gene was analyzed using RT-PCR. Total RNA was extracted from various cancer cell lines with ISOGEN® (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. First strand cDNA was generated from RNA, as described elsewhere (15). cDNA was then amplified using a primer set that was specific for the *CHFR* gene. The primer sequences were: *CHFR* S (sense), 5'-GGCGAGAGCGTTCCT CCAGTTG-3', and *CHFR* AS (antisense), 5'-GCATGTCAGCG TCTCCTCCATCTTG-3'. The PCR amplification consisted of 1 cycle at 94˚C for 2 min, 30 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, and 1 cycle at 72˚C for 5 min. The expression of β-actin was used as a control to confirm the success of the RT reaction. The PCR products were visualized on 1.3% agarose gel stained with ethidium bromide.

Statistical analysis. The associations between *CHFR* promoter methylation and clinicopathological parameters were analyzed using Fisher's exact tests or Student's *t*-tests. A *p*-value <0.05 indicated statistical significance.

Results

We first examined the methylation status of *CHFR* promoter in 1 esophageal, 1 gastric and 3 colorectal cancer cell lines using the MSP technique (Figure 1). The aberrant

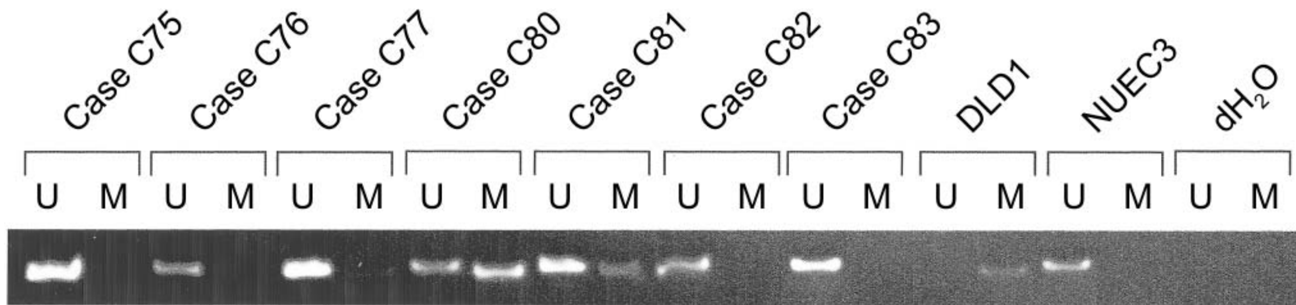

Figure 3. *The representative MSP of CHFR promoter in primary colorectal cancers. Modified DNAs obtained from DLD1 and NUEC3 were used as positive controls for the methylated and unmethylated alleles, respectively. Cases 80 and 81 exhibited methylation.*

methylation of *CHFR* was detected in 1 colorectal cancer cell line (DLD1). In order to confirm the status of the *CHFR* gene according to its methylation pattern, *CHFR* expression in these cell lines was examined using RT-PCR (Figure 2). As expected, DLD1 which was the only cell line to demonstrate methylation of the *CHFR* promoter, lacked *CHFR* expression, whereas *CHFR* was expressed in all other cell lines without methylation of the *CHFR* promoter.

The methylation status of the *CHFR* promoter was then examined in primary colorectal cancer samples (Figure 3). An aberrant methylation of the *CHFR* gene was detected in 25 out of 98 (26%) primary colon cancers. As a control, the corresponding normal tissues of these same patients was screened for *CHFR* methylation, and no methylation was detected. Our results suggested that the aberrant methylation of the *CHFR* gene was frequently observed in colorectal cancers.

After a methylation analysis of all samples, the clinicopathological data were correlated with these results. No significant correlations were observed between the presentations of abnormal methylation in colorectal cancers and patient gender, age or tumor maximal size, TNM stage and lymph node metastasis (Table I). A significant difference was observed in the tumor ($p=0.035$), indicating that early colorectal cancers were more frequently methylated than advanced ones.

Discussion

Colorectal cancer is one of the most aggressive cancers and occurs at a high incidence in most countries (16). In order to remove this fatal cancer from patients, surgical operations and subsequent chemotherapy and radiotherapy are performed. For this purpose, it is important to identify the occurrence of genetic alterations as a new parameter for the estimation of the malignancy of the cancer.

CHFR, a mitotic checkpoint gene, was recently cloned and localized to chromosome 12q24. It has been reported that the CHFR protein delays chromosome condensation and entry into metaphase in response to the mitotic stress induced by microtubule inhibitors, such as nucodasole or taxol (7). The CHFR protein contains three separate domains, a forkhead-associated (FHA), a ring finger (RF) and a cystein-rich (CR) domain. The FHA domain is conserved in several checkpoint genes, including *CHK2, RAD53* and *MDC1* (17-21). Based on a mutagenesis analysis, both the FHA and CR domains are required for CHFR's checkpoint function (7). The RF domain is also critical for the mitotic checkpoint activity, while also plays a role in the ubiquitination of substrates, such as polo-like kinase and *CHFR* itself (22).

In the present study, the frequent methylation of *CHFR* in colorectal cancer was observed, whereas the same methylation was not detected in corresponding normal tissue specimens. The methylation status of *CHFR* in colorectal cancer patients was also compared with their clinicopathological features and demonstrated that *CHFR* in early colorectal cancer patients was more frequently methylated than in advanced cases. Therefore, *CHFR* methylation could be used as a tumor marker in clinical samples, such as serum and stool, for the early detection of digestive tract cancers (23, 24).

Our findings suggest that *CHFR* may play a role in the carcinogenic pathway in some patients with colorectal cancers, and that tumor formation in the colorectum may be controlled by inducing the expression of silenced *CHFR* using demethylating reagents. This study provides solid evidence that can be used in further studies on the molecular mechanism of *CHFR* in colorectal cancers.

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Received July 31, 2006
Revised October 20, 2006
Accepted October 25, 2006