Promoter hypermethylation of the Chfr gene in neoplastic and non-neoplastic gastric epithelia

T Honda1,2, G Tamura*,1, T Waki1, S Kawata2, S Nishizuka3 and T Motoyama1

1Department of Pathology, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan; 2Internal Medicine, Yamagata University School of Medicine, 2-2-2 Iida-nishi, Yamagata 990-9585, Japan; 3Laboratory of Molecular Pharmacology, National Cancer Institute, National Institute of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

While chromosomal instability is a common feature of human solid tumours, no abnormalities in genes involved in the mitotic checkpoint have been identified. However, recently, Chfr (checkpoint with forkhead associated and ring finger), a mitotic stress checkpoint gene, has been reported to be inactivated due to promoter hypermethylation in several types of human malignancy. To clarify whether Chfr promoter hypermethylation is involved in gastric carcinogenesis, we investigated the promoter methylation status of the Chfr gene in gastric cancer cell lines and primary gastric cancers. Non-neoplastic gastric epithelia from cancer-bearing and noncancer-bearing stomachs were also examined for Chfr promoter hypermethylation to study its cancer specificity. Two of 10 gastric cancer cell lines (20%) showed Chfr promoter hypermethylation with resultant loss of expression, which could be restored by 5-aza-2’-deoxycytidine treatment. Chfr promoter hypermethylation was present in 35% (25 of 71) of primary tumours and occurred at similar frequencies in early and advanced stages. As for non-neoplastic gastric epithelia, 1% (one of 91) from noncancer-bearing and 5% (four of 71) from cancer-bearing stomachs exhibited Chfr promoter hypermethylation. Thus, Chfr promoter hypermethylation is mostly cancer specific and frequently leads to chromosome instability in gastric cancer.

Keywords: Chfr; hypermethylation; gastric cancer

Chromosomal instability (CIN) is commonly observed in human solid tumours, with the apparent gain or loss of large parts or whole chromosomes, leading to DNA aneuploidy (Lengauer et al, 1997; Duesberg et al, 1999). In previous studies, CIN has been associated in some cases with alterations in the cell-cycle checkpoint that monitors the integrity of the spindle apparatus, a structure critical for proper bipolar segregation of duplicated sister chromatids at mitosis (Chahil et al, 1999). A small fraction of CIN cancers are associated with dominant mutations in the human homologues of yeast spindle checkpoint genes BUB1 (Chahil et al, 1998; Imai et al, 1999; Gemma et al, 2000) and MAD2 (Li and Benezza, 1996; Cahil et al, 1999). However, BUB1 and MAD2 mutations are relatively rare, and gastric cancers frequently exhibit DNA aneuploidy (Abad et al, 1998; Esteban et al, 1999; Imai et al, 1999; Russo et al, 2000; Tanaka et al, 2001).

Recently, the Chfr (checkpoint with forkhead associated (FAH) and ring finger (RF)) gene, involved in the mitotic stress checkpoint, was cloned and located to chromosome 12q24.33. Its product, CHFR, mediates the delayed entry into metaphase characterised microscopically by delayed chromosomal condensation (Scolnick and Halazonetis, 2000). In addition, CHFR promotes cell survival in response to mitotic stress (Scolnick and Halazonetis, 2000). CHFR possesses an N-terminal FHA domain, a central RF domain and a C-terminal cysteine-rich (CR) region (Scolnick and Halazonetis, 2000). Based on functional analysis of Chfr deletion mutants, both the FHA and CR regions are required for its checkpoint function. CHFR also has ubiquitin ligase activity dependent on the RF domain (Chaturvedi et al, 2002). Northern blot analysis of Chfr using RNA from eight colon, osteosarcoma and neuroblastoma cancer cell lines revealed that Chfr expression was absent in three cell lines (Scolnick and Halazonetis, 2000). Loss of Chfr expression due to hypermethylation of a CpG island in the promoter region has been observed in tumour cell lines and primary cancers of the lung, oesophagus and colon (Mizuno et al, 2002; Shibata et al, 2002; Corn et al, 2003; Toyota et al, 2003). Thus, it is possible that Chfr promoter hypermethylation is also involved in gastric carcinogenesis.

As promoter hypermethylation of tumour suppressor or tumour-related genes are not always cancer specific, the significance of promoter methylation status can vary among different genes (Waki et al, 2003a, b). In the present study, we investigated Chfr promoter methylation status in gastric cancer cell lines, primary gastric cancers and corresponding non-neoplastic gastric epithelia, as well as in non-neoplastic gastric epithelia of noncancer-bearing stomachs to clarify both the significance and cancer specificity of Chfr promoter hypermethylation in gastric carcinogenesis.

MATERIALS AND METHODS

Gastric cancer cell lines

In all, 10 gastric cancer cell lines with variable histologies were used in our study and were cultured under appropriate conditions...
in our laboratory: MKN1, an adenosquamous cell carcinoma; MKN7, a well-differentiated adenocarcinoma; MKN28 and MKN74, moderately differentiated adenocarcinomas; MKN45 and KWS-I, poorly differentiated adenocarcinomas; KATO-III, a signet-ring cell carcinoma; TSG11, a hepatoid carcinoma; and ECC10 and ECC12, endocrine cell carcinomas.

Primary gastric cancers
In all, 71 pairs of gastric cancers (40 differentiated and 31 undifferentiated carcinomas; 15 early stage and 56 advanced stage) and corresponding non-neoplastic gastric mucosa were surgically obtained from 71 patients. Tissue samples were immediately frozen and stored at −80°C until analysis. All patients received a median of 36.7 months of follow-up care (range, 1–77 months).

Autopsy samples
Non-neoplastic gastric mucosa samples from noncancer-bearing stomachs were obtained from 34 autopsies. The autopsies consisted of 21 males and 13 females, ranging in age from 0.7 to 87 years (mean, 56 years). For most autopsies, tissue samples were obtained from the upper, middle and lower portions of the stomach. A total of 91 specimens were obtained, frozen and stored at −80°C until analysis.

DNA and RNA extraction
DNA was extracted from the 10 gastric carcinoma cell lines, 71 primary gastric cancers and their corresponding non-neoplastic gastric mucosa, and 91 non-neoplastic gastric mucosa from autopsies using SepaGene (Sanko-Junyaku, Tokyo, Japan). Total RNA was isolated from the 10 gastric carcinoma cell lines using TRIzol reagent (Gibco BRL, Life Technologies, Gaithersburg, MD, USA).

Bisulfite modification and methylation-specific polymerase chain reaction (MSP)
Sodium bisulfite treatment of DNA converts all unmethylated cytosines to uracils, but leaves methylated cytosines unaffected. Briefly, 2 µg aliquots of genomic DNA were denatured with sodium hydroxide and modified by sodium bisulfite. Samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, USA), treated with NaOH, recovered in ethanol and resuspended in 30 µl distilled water. Amplification was carried out in a 20 µl reaction volume containing 2 µl GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA, USA), 1.0 mm MgCl₂, 1 µl each primer, 0.2 µM dNTPs and 1 U Taq polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems). After heating at 94°C for 10 min, PCR was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 60 s and extension at 72°C for 60 s, followed by a final 7-min extension at 72°C. A positive control (Sss-I methylase-treated DNA) and negative control (distilled water without DNA) were included in each amplification. The PCR products were separated on 6% non-denaturing polyacrylamide gels. The following primer sets were used: Chfr M forward (5’-GTA ATG TTT TGT AGC GCC-3’) and Chfr M reverse (5’-AAT CCC CCT TCG CGG-3’) for methylated Chfr sequences; Chfr U forward (5’-GTT TGT TAT GAT GGT TGT TTA TAG TGG TGC T-3’) and Chfr U reverse (5’-CAA ATC CCC CTT CAC CA-3’) for unmethylated Chfr sequences (Corn et al., 2003).

Reverse transcription – PCR (RT – PCR)
Isolated RNA was reverse transcribed and amplified using the One-Step RT – PCR System (Gibco BRL). Primer sequences used were: Chfr forward (5’-TTG AAC ATG TAT TAA CAA GC-3’) and Chfr reverse (5’-AGG TAT CTT TGG TCC CAT GG-3’) for Chfr; and β-actin forward (5’-AAA TCT GGC ACC ACA CCT T-3’) and β-actin reverse (5’-AGC ACT GTG TGT GCG TAC AG-3’) for β-actin. RT – PCR products were separated on 3% agarose gels.

5-aza-2’-deoxycytidine (5-aza-dC) treatment
To examine the restoration of Chfr expression, two cell lines (MKN1 and KATO-III) were incubated for 96 h with 0.2 or 1 µM 5-aza-dC (Sigma), and then harvested for RNA extraction and RT-PCR.

Preparation of MSP-positve control
Sss-I methylase (New England BioLabs, Inc., Beverly, MA, USA) was used to methylate 100 µg peripheral blood DNA, which was modified by sodium bisulphite as described above.

Statistical analysis
Statistical comparisons were performed using Fisher’s exact test, with P<0.05 considered statistically significant. Survival analysis was performed using a Kaplan-Meier curve with a log-rank test.

RESULTS
Hypermethylation and expression of Chfr in gastric cancer cell lines
Chfr promoter hypermethylation was observed in two (MKN1 and KATO-III) of the 10 cell lines tested (Figure 1). The remaining cell lines (MKN7, MKN28, MKN45, MKN74, KWS-I, TSG11, ECC10 and ECC12) contained unmethylated Chfr alleles and expressed abundant Chfr mRNA. MKN1 and KATO-III exhibited loss of Chfr expression (Figure 1), which was restored after treatment with 5-aza-dC (Figure 1). Thus, promoter methylation status of Chfr directly correlated with expression.

Hypermethylation of Chfr in primary gastric cancers, corresponding non-neoplastic gastric mucosa and autopsy samples
Hypermethylation of Chfr was detected in 35% (25 of 71) of primary gastric cancers but only in 5% (four of 71) of the corresponding non-neoplastic gastric mucosa (Figure 2). Chfr hypermethylation was observed in only one (1%) of the 91 autopsy samples. This single sample showing Chfr hypermethylation was obtained from the lower portion of the stomach from an 82-year-old-male patient with Parkinson’s disease.

Correlation between Chfr promoter hypermethylation and clinicopathological parameters
Chfr hypermethylation occurred at a similar frequency in early and advanced gastric cancers, and no significant correlations between Chfr promoter methylation status and clinicopathological factors were observed (Table 1). Methylation status did not significantly influence event-free survival rate, as analysed by Kaplan-Meier curve with log-rank test and the Breslow-Gehan–Wilcoxon test (data not shown).

DISCUSSION
Although CIN is one of the most frequently recognised phenomenon in gastric cancer (Abad et al., 1998; Esteban et al., 1999; Russo et al., 2000), the mitotic checkpoint genes hsMAD2 and hBUB1 are
rarely mutated in gastric and other types of human malignancy (Imai et al., 1999; Tanaka et al., 2001). Checkpoints upstream of the spindle checkpoint that delays chromosome condensation in response to mitotic stress are regulated by CHFR. Normal primary cells and cancer cell lines that express CHFR exhibit delayed entry into metaphase after treatment with microtubule inhibitors (Scolnick and Halazonetis, 2000). In contrast, cancer cell lines that lack CHFR enter metaphase without delay, with ectopic expression of CHFR necessary and sufficient to restore cell-cycle delay (Scolnick and Halazonetis, 2000). Recent studies of human tumours have shown that Chfr inactivation can be due to hypermethylation of CpGs in the promoter region (Mizuno et al., 2002; Shibata et al., 2002). However, whether Chfr promoter hypermethylation is involved in gastric cancer has not yet been determined.

In the present study, we showed that Chfr promoter hypermethylation was present in two of 10 (20%) gastric cancer cell lines and in 25 of 71 (35%) primary gastric cancers. As for non-neoplastic gastric epithelia, 5% (four of 71) of samples from cancer-bearing and 1% (one of 91) from noncancer-bearing stomachs exhibited Chfr promoter hypermethylation. We have shown that many tumour suppressor and tumour-related genes, such as APC, DAP-kinase, DCC, E-cadherin, hMLH1, p16, RASSF1A and RUNX3, exhibit promoter hypermethylation in both neoplastic and non-neoplastic epithelia at variable frequencies (Tamura, 2004). While GSTP1 and PTEN promoters remained unmethylated in both neoplastic and non-neoplastic epithelia

| Table 1 Correlation between Chfr promoter methylation status and clinicopathological characteristics in gastric cancer patients |
|----------------------------------|-------------------|
| **Promoter methylation status**  | **Methylated** | **Unmethylated** |
| Number of patients               | 25               | 46               |
| Age (years) (mean)              | 72               | 66               |
| Gender                           |                  |
| Male                             | 20               | 31               |
| Female                           | 5                | 15               |
| Stage                            |                  |
| Early                            | 6                | 9                |
| Advanced                         | 19               | 37               |
| Histological differentiation     |                  |
| Differentiated                   | 13               | 27               |
| Undifferentiated                 | 12               | 19               |
| Location                         |                  |
| Lower                            | 12               | 17               |
| Middle                           | 10               | 15               |
| Upper                            | 2                | 12               |
| Unknown                          | 1                | 2                |
| Lymph node metastasis           |                  |
| Present                          | 20               | 38               |
| Absent                           | 5                | 8                |

NS = not significant by Fisher’s exact probability test. Chfr = checkpoint with forkhead associated and ring finger.
gastrointestinal epithelia (Sato et al., 2002; Tamura, 2004), TSLC1 promoter hypermethylation is highly cancer specific, but is observed at only a low frequency in gastric cancer (Honda et al., 2002).

Methylation generally increases with age in tissue-specific manner for different genes (Waki et al., 2003b). In the present study, the only sample of non-neoplastic gastric mucosa that exhibited Chfr hypermethylation was obtained from the non-cancer-bearing stomach of an 82-year-old male patient. In contrast, Chfr hypermethylation was present in cancer-bearing stomachs from patients at 66 years of age. Based on these observations, it appears that age-related Chfr hypermethylation may constitute a general defect where individuals may become predisposed to the development of gastric cancer. The cancer specificity of hypermethylation of a particular promoter can depend on the CpG site examined (Satoh et al., 2002). Our present study revealed that Chfr promoter hypermethylation appears to be one of the most cancer-specific alterations among the various examples of tumour suppressor and tumour-related gene hypermethylation reported to date (Tamura, 2004).

While Chfr promoter hypermethylation is a relatively infrequent non-neoplastic gastric epithelia, it occurs at similar frequencies in early and advanced gastric cancers. This suggests that Chfr promoter hypermethylation may be an early event in gastric carcinogenesis. DNA aneuploidy has been observed in 50–71% of gastric cancers and correlates with poor prognosis (Abad et al., 1998; Esteban et al., 1999; Russo et al., 2000). In the present study, we failed to find a statistically significant correlation between Chfr hypermethylation and gastric cancer patient survival. Nonetheless, our results did display a tendency towards a worse prognosis in patients with tumours that displayed Chfr hypermethylation. Owing to the lack of a significant correlation between Chfr methylation status and prognosis, and the relatively low frequency of Chfr hypermethylation compared to that of DNA aneuploidy, other gene(s) and/or mechanism(s) are likely to also contribute to CIN in gastric cancer.

In conclusion, Chfr promoter hypermethylation frequently occurs as an early event of gastric carcinogenesis. Owing to its cancer specificity, detection of Chfr promoter methylation could be useful as a molecular diagnostic marker for gastric cancer.

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