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Identification of genes epigenetically silenced by CpG methylation in human gastric carcinoma

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ABSTRACT

To identify novel methylation-silenced genes in gastric cancer, we carried out a genome-wide search for genes that are up-regulated after treatment with the demethylating agent, 5-aza-2′-deoxycytidine (5Aza-dC). When three gastric cancer cell lines (SNU-1,-601, and -719) were treated with 5Aza-dC, 143 genes were found to be upregulated by twofold or more using oligonucleotide microarrays. Six of these genes, i.e. *TFPI2*, *GPX3*, *GPX1*, *IGFBP6*, *IRF7* and *DMRT1*, showed promoter hypermethylation in one or more gastric cancer cell lines, but were unmethylated in normal gastric mucosa by bisulphite sequencing and methylation-specific PCR analysis. The following percentages of these genes were found to be aberrantly methylated in gastric cancer samples; *TFPI2* (80.9%), *GPX3* (30.1%), *DMRT1* (46.9%), *GPX1* (16.7%), *IGFBP6* (22.6%) and *IRF7* (32.1%). Interestingly, the survival of patients possessing methylated alleles of *TFPI2* (123/152, 80.9%) was poorer than that of patients with unmethylated alleles ($p = 0.023$). Multivariate analysis confirmed that *TFPI2* methylation is a significant and independent prognostic factor in gastric carcinoma. Furthermore, altered *TFPI2* expression, as demonstrated by immunohistochemistry in 566 consecutive gastric cancer tissues, was found to be significantly associated with sex ($p = 0.003$), WHO classification ($p < 0.001$), and a mixed subtype by Lauren's classification ($p < 0.001$). Thus, the present study identified several novel genes, which were methylated in gastric cancer and among them, methylation of *TFPI2* was an unfavourable prognostic marker.

1. Introduction

Globally, gastric cancer is the second most common cause of cancer death, [1] and although the incidence of gastric cancer is declining in developed countries, it remains a leading cause of cancer-related death in the majority of developing countries. Genetic and environmental factors, including *Helicobacter pylori* infection, are considered to contribute to the development of gastric cancer. Moreover, recent studies have shown that the silencing of tumour suppressor genes by epigenetic modification is a fundamental mechanism of the pathogenesis of human cancer.[2] Gastric carcinogenesis is thought to be a multistep process that involves multiple genetic and epigenetic events, but its underlying mechanisms have yet to be clarified, although it is known that promoter hypermethylation plays an essential role in tumour suppressor gene loss of function. In terms of the molecular basis of gastric cancer, it is believed that the aberrant DNA methylation of known tumour suppressor genes is more common that their mutation.[3] However, the roles played by many of the genes affected by

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methylation-silencing in gastric cancer remain un-revealed, and further studies are necessary to identify all the silenced genes.

The epigenetic alterations of CpG islands in the promoter region of genes by aberrant DNA methylation have been established to be a common mechanism for silencing tumour suppressor genes in cancer cells.[4,5] Aberrant DNA methylation affects important cellular pathways by silencing tumour-related genes.[6] Generally, tumour cells have promoter methylation patterns that differ from those of normal cells.[7] Furthermore, recently, DNA methylation has emerged as promising biomarker for cancer detection[8], and a number of genes, e.g. *APC, COX2, DAP-K, CDH1, p14, CDKN2A, THBS1, TIMP3, hMLH1, p15, MINT1, MINT2, MINT25, CHFR* and *CDH4*, have been found to be aberrantly methylated in gastric cancer.[9–14] Nevertheless, many of the specific molecular events involved in the pathogenesis and progression of gastric cancer remains to be elucidated. A global approach to the identification of epigenetically silenced genes in gastric cancer cells could provide methylation signatures for early detection and prognostic stratification, identify novel targets for therapy and lead to a deeper understanding of the biology of gastric cancer.

Epigenetic silencing is not an irreversible process, for example, drugs like 5-aza-2′-deoxycytidine (5Aza-dC) cause generalised demethylation. 5Aza-dC is known to act by being incorporated into new DNA strands during replication, where it forms a covalent complex with methyltransferase active sites, thus reducing methyltransferase activity.[4] On the other hand, chromatin is a DNA/histone complex, and histone deacetylation is also known to impair gene transcription.[15] Furthermore, DNA methylation has been reported to promote histone deacetylation.[16] Methyl-CpG binding protein 2 (MeCP2) appears to reside as a complex with histone deacetylase[17], whereas DNA methyltransferase binds to histone deacetylase 2 (HDAC2) and a transcriptional co-repressor, DMAP1.[18] Thus, densely methylated DNA associates with transcriptionally repressive chromatin, which is characterised by the presence of under-acetylated histones. Trichostatin A (TSA; a histone deacetylase inhibitor) has been reported to reverse the formation of transcriptionally repressive chromatin on methylated promoter templates.[19] Thus, epigenetic alterations are dynamically linked, and synergy between DNA demethylation and histone deacetylase inhibition using TSA has been shown to reactivate genes silenced in carcinoma more robustly than 5Aza-dC alone.[20,21]

A number of different approaches have been developed for the genome-wide identification of cancer-associated hypermethylated genes, such as, restriction landmark genome scanning, methylation-sensitive arbitrarily primed PCR and methylated CpG island amplification.[22] An alternative approach is to treat cells with epigenetic modifying drugs to facilitate the expressions of hypermethylated/silenced genes and then to compare gene expression profiles by microarray analysis to identify putatively hypermethylated genes. The methylation status of such genes can then be confirmed in untreated cells by bisulphite sequencing or methylation-specific PCR analyses. Global demethylation and microarray analysis have been successfully used to identify a number of novel genes hypermethylated in colorectal and pancreatic cancer.[21,23] In the present study, we examined the global reactivations of epigenetically silenced genes using an oligonucleotide microarray in three gastric cancer cell lines. Genes that were markedly upregulated (>twofold) by 5Aza-dC treatment in these cancer cell lines were considered genes of interest. Subsequently, bisulphite sequencing and methylation-specific PCR analyses were carried out on these genes to confirm the presence of aberrantly methylated CpG dinucleotides. In the present study, we show that the methods employed preferentially selected epigenetically silenced hypermethylated genes in primary gastric carcinomas. Finally, six genes found to be hypermethylated in at least one of the three cell lines were investigated to determine whether their methylation statuses were correlated with clinicopathological parameters.

2. Materials and methods

2.1. Cell lines and tissue samples

Three human gastric cancer cell lines, (SNU-1, -601 and -719) were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were grown in RPMI1640 supplemented with 10% foetal bovine serum (FBS; Hyclone, Lorgan, UT, USA) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37 °C in a humidified 5% CO_2 incubator.

Formalin-fixed, paraffin-embedded samples of 566 gastric cancer tissue specimens, resected at Seoul National University Hospital from January 1995 to December 1995, were used in this study.[24] Age, sex, histologic type (according to WHO and Lauren's classifications), lymphatic invasion and pTNM (pathologic tumour-node-metastasis) stage were evaluated by reviewing medical charts, pathology reports and glass slides. Patient clinical outcomes were followed from date of surgery to date of death or until 1st December, 2000. Follow-up periods ranged from 1 month to approximately 72 months (mean, 49 months). Cases lost to follow-up and deaths attributed to causes other than gastric cancer were censored during the survival analysis.

2.2. Treatments with 5-aza-2′-deoxycytidine and/or TSA

Three gastric cancer cell lines (SNU-1, -601 and -719) were treated with 5-aza-2′-deoxycytidine (5Aza-dC) and/or trichostatin A (TSA) (both Sigma Aldrich, St. Louis, MO, USA). Cells were exposed continuously to 5Aza-dC (5 μM) for 4 d or to TSA (0.3 μM) for 24 h. Because we observed previously that treatment of these cell lines with 5Aza-dC (5 μM) for 4 d results in the re-expression of genes silenced by aberrant methylation without evidence of excessive cell death, we used this time for the experiment.[25] For combined treatment, these cells were cultured in the presence of 5Aza-dC (5 μM) for 3 d and then treated for another 24 h with TSA (0.3 μM).

2.3. Oligonucleotide microarray analysis

Total RNA was isolated from treated or untreated cells using RNeasy Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Target preparation and microarray processing procedures were performed as

described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA, USA). First- and second-stranded cDNA were synthesised from 10 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and T7-$(dT)_{24}$ primer (Metabion, Germany). Labelled cRNA was synthesised from purified cDNA by *in vitro* transcription using BioArray High Yield RNA Transcript Labelling Kits (ENZO, Farmingdale, NY, USA) at 37 °C for 6 h. Fifteen microgram of cRNA was fragmented at 94 °C for 35 min in a fragmentation buffer containing 40 mM Tris–acetate (pH 8.1), 100 mM potassium acetate and 30 mM magnesium acetate. Fragmented cRNA was then hybridised to an oligonucleotide microarray (GeneChip Human Genome U133A 2.0) containing 22,277 transcripts at 45 °C for 16 h. Washing and staining (streptavidin–phycoerythrin) procedures were performed in an Affymetrix Fluidics Station, according to the manufacturer's instructions. Probes were then scanned using a laser scanner, and transcript signal intensities (background-subtracted and adjusted for noise) were calculated using Microarray Suite Software 5.0 (Affymetrix). All genes represented on the GeneChip were globally normalised and scaled to a signal intensity of 500. Fold changes were calculated by comparing the signal intensities of transcripts in untreated and treated cell lines. Microarray Analysis Suite software and the Wilcoxon's test were used to generate detected (present or absent) or changed (increased or decreased) calls, and these calls were used to statistically determine whether a transcript was expressed, and whether its expression had been altered by treatment. A *p*-value of 0.003 was used to identify increased expression.

2.4. Isolation and bisulphite modification of genomic DNA

Genomic DNA was isolated from cells and tissues by standard phenol–chloroform extraction. To denature DNA, 2 μg of genomic DNA was incubated with 1 μg of salmon sperm DNA (Sigma Aldrich, St. Louis, MO, USA) in 0.3 M NaOH for 20 min at 37 °C in a total volume of 50 μl, diluted with 550 μl of a 3.5 M sodium bisulphite (pH 5.0)/1 mM hydroquie solution (both Sigma Aldrich, St. Louis, MO, USA), and incubated at 55 °C for 16 h. The modified DNA was then purified using Wizard DNA Clean Up System (Promega, Madison, WI, USA). The purified DNA was incubated with 0.3 M NaOH for 10 min at 37 °C. DNA was precipitated with ethanol, dissolved in 20 μl Tris–EDTA (pH 8.0) and stored at –20 °C. The bisulphite modification of DNA converts unmethylated cytosines to uracils, whereas methylated cytosines are resistant to modification.

2.5. Bisulphite sequencing of gene promoter CpG islands

Bisulphite-treated genomic DNA was used as a template to amplify fragments of 220–351 bp with a high CpG content from around the transcription start site using the oligonucleotides listed in the Supplementary data (Table S1). Amplified PCR products were purified enzymatically using a pre-sequencing kit (Amersham Life Science, Cleveland, OH, USA), according to the manufacturer's instructions, and then directly sequenced using a BigDye terminator (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were run on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA) and collected data were analysed using DNA sequencing analysis 3.7 software (Applied Biosystems, Foster City, CA, USA). Bisulphite sequencing was performed in both directions using the primers used for PCR amplification.

2.6. Methylation-specific PCR

Bisulphite-treated DNA was amplified with either a methylation-specific or unmethylation-specific oligonucleotide set in a total volume of 20 μl using two units of Ex Taq-polymerase (TAKARA, Tokyo, Japan) per reaction and the oligonucleotide primers listed in Supplementary data (Table S2). After denaturation at 95 °C for 5 min, 35 PCR-cycles were done when template genomic DNA was obtained from cell lines or 39–40 cycles were performed when DNA was obtained from formalin-fixed, paraffin-embedded tissue blocks. After PCR, samples were run in 2% agarose gel with appropriate size markers, and the presence or absence of PCR products was analysed. DNA from normal gastric tissues recognised as unmethylated by bisulphite sequencing was used as negative controls for methylated alleles, whereas DNA from cell lines recognised as methylated and CpGenome Universal Methylated DNA (Chemicon, Temecula, CA, USA) was used as positive controls.

2.7. Reverse transcription-PCR analysis

Total RNA (2 μg) was reverse transcribed for 1 h at 37 °C in 20 μl using 4 μl of 5× reverse transcriptase buffer, 1 μl of 100 pmol oligo dT_{15} primer, 2 μl of 0.1 M 1,4-dithiothreitole, 1 μl of RNAsin (Invitrogen, Carlsbad, CA, USA) and 200 U of MMLV-reverse transcriptase (Bioneer, Korea). One μl of cDNA was used as a template for each subsequent 20 micro litre PCR-reaction; the sequence-specific oligonucleotide primers used are listed in Supplementary data (Table S3). Amplification was performed in a ABI 9700 DNA thermal cycler (Applied Biosystems, Foster City, CA, USA) for 35 cycles with a final extension at 72 °C for 10 min. PCR products were electrophoresed through in 2% agarose gel containing ethidium bromide and visualised using UV light; β-actin was used as an internal standard to confirm equal loadings.

2.8. Immunohistochemistry

Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumour tissue samples (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea).[26] The resulting tissue array blocks contained up to 60 specimens. A total of 10 tissue array blocks were constructed for the 566 gastric carcinoma tissue samples. An adequate case was defined as one with a tumour occupancy exceeding 10% of the core area. Each block contained non-neoplastic gastric mucosa samples from body and antrum, and an intestinal metaplasia sample as an internal control. Four-μm thick sections were cut from each tissue array block, deparaffinised in xylene and rehydrated using graded alcohols. Immunohistochemical staining against mouse anti-TFPI2 monoclonal antibody (1:50; R&D Systems,

Minneapolis, MN, USA) was performed using a streptavidin-peroxidase procedure after microwave antigen retrieval. Antigen-bound primary antibody was detected using a standard avidin–biotin immunoperoxidase complex method (Vectastatin Elite ABC peroxidase kit; Vector Laboratories, Burlingame, CA, USA). Immunostaining results were considered positive when more than 10% of tumour cells were cytoplasmically stained.

2.9. Statistical analyses

The Fisher's exact test (two-sided) and chi-square test were used to assess the significances of associations between *TFPI2* expression and clinicopathological parameters. Survival curves were plotted using the Kaplan–Meier product-limit method, and the log-rank test was used to compute differences between curves. Multivariate survival analysis was performed using the Cox proportional hazards model. A p-value of <0.05 was considered statistically significant. All statistical analyses were conducted using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Identification of candidate genes reactivated by 5Aza-dC in gastric cancer cell lines

Global changes in gene expression profiles induced by 5Aza-dC in the three gastric cancer cell lines (SNU-1, -601 and -719) were determined using Affymetrix HG U133 2.0 oligonucleotide microarrays containing 22,277 transcripts representing 14,500 well-characterised human genes. A comprehensive survey for hypermethylated gene candidates associated with gastric cancer was performed by comparing mock-treated and 5Aza-dC-treated gastric cancer cells. A summary of our differential and epigenetic gene expression profiling flowchart is shown Fig. 1. As compared with mock-treated counterparts, 5Aza-dC treatment substantially increased the signal intensities of 953 transcripts (4.3% of the transcripts analysed) in SNU-1, of 1566 transcripts (7.0%) in SNU-601 and of 748 transcripts (3.4%) in SNU-719. As complete silencing is a characteristic of methylated genes in cancer cells[27], we excluded transcripts present in cell lines before drug treatment. Accordingly, we were left with 148 (SNU-1), 242 (SNU-601) and 108 (SNU-719) transcripts, which in total amounted to 361 unique transcripts that were significantly up-regulated by more than twofold in response to 5Aza-dC in at least one of the gastric cancer cell lines. We further reduced the number of candidate genes by excluding unknown genes (e.g. expressed sequence tags, complementary DNA clones and hypothetical proteins). We excluded genes from the X chromosome and well-known imprinted genes, such as *H19* and *IGF2*, because their methylation is not cancer cell specific. In addition, we also excluded genes without CpG islands because these genes cannot be validated by bisulphite sequencing or by using methylation-specific PCR. After filtering genes using the above criteria, 143 candidate genes finally remained for further study, and of these 143 genes, we selected 18 genes with known tumour-associated functions, i.e. involvement in DNA repair, negative cell cycle regulation, apoptosis, detoxification, differentiation or transcription regulation (Table 1). Furthermore, because the bisulphite sequencing of seven genes was unsuccessful, 11 of the 18 genes were selected as candidates for methylation analysis. None of these 11 genes had been previously known to be associated with aberrant methylation in gastric cancer. They comprised; *RASSF4* (Ras association domain family 4), *LGALS1* (Lectin, galactoside-binding, soluble, 1), *GPX1* (Glutathione peroxidase 1), *TUSC3* (Tumour suppressor candidate 3), *IGFBP6* (Insulin-like growth factor binding protein 6), *IRF7* (IFN regulatory factor 7), *GPX3* (Glutathione peroxidase 3), *SLIT3* (Slit homologue 3), *TFPI2* (Tissue factor pathway inhibitor 2), *DMRT1* (Doublesex and mab-3 related transcription factor 1), and *SOCS3* (Suppressor of cytokine signalling 3). A recent study has shown that *TFPI2* is a novel placental-specific imprinted gene, but that the monoallelic expression is restricted to extra-embryonic tissues, and therefore this gene remains a valid candidate gene.

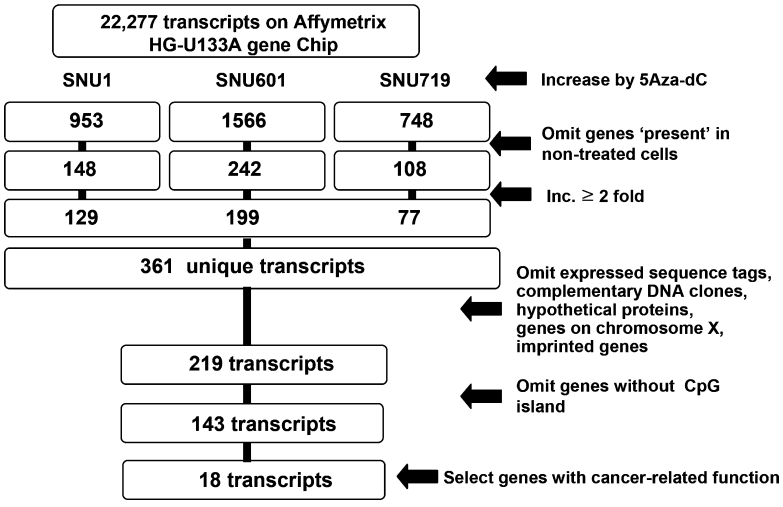

Fig. 1 – Overall flow-chart for the selection of candidate methylation-silenced targets.

Table 1 – Candidate epigenetically silenced genes as identified by microarray analysis.

Gene title	Symbol	Chr. location	Function
Hect domain and RLD 5	HERC5	4q22.1–q23	Protein kinase activity
Ras association domain family 4	RASSF4	10q11.21	Signal transduction
Lectin, galactoside-binding, soluble, 1	LGALS1	22q13.1	Signal transduction
Cut-like 2	CUTL2	12q24.11	Transcription factor
Dickkopf homologue 3	DKK3	11p15.2	Wnt signalling
Glutathione peroxidase 1	GPX1	3p21.3	Detoxification
Tumour suppressor candidate 3	TUSC3	8p22	Electron transporter
Human T-cell leukaemia virus enhancer factor	HTLF	2p22	Transcription factor
Insulin-like growth factor binding protein 6	IGFBP6	12q13	Growth factor
IFN regulatory factor 7	IRF7	11p15	Regulation of cell growth
Glutathione peroxidase 3	GPX3	5q23	Detoxification
Trefoil factor 2	TFF2	21q22.3	Digestion
Slit homologue 3	SLIT3	5q35	Differentiation
Tissue factor pathway inhibitor 2	TFPI2	7q22	Protease inhibitor activity
Doublesex and mab-3-related transcription factor 1	DMRT1	9p24.3	Transcription factor activity
Caveolin 1, caveolae protein	CAV1	7q31.1	Structural molecule activity
Claudin 3	CLDN3	7q11.23	Structural molecule activity
Suppressor of cytokine signalling 3	SOCS3	17q25.3	Regulation of cell growth

3.2. *Validation of the candidate genes in gastric cancer cell lines*

We analysed the methylation statuses of the promoter CpG islands proximal to the transcription start sites of the 11 genes in the three gastric cancer cell lines and in normal gastric tissues by bisulphite sequencing. Initial screening showed that 6 of the 11 genes tested (*GPX1*, *IGFBP6*, *IRF7*, *GPX3*, *TFPI2* and *DMRT1*) showed 5′ CpG island hypermethylation in gastric cancer cells, but not in normal gastric tissues (Fig. 2). Bisulphite treatment efficiency was determined by calculating the C to T conversion rate for all cytosine bases other than those in CpG dinucleotides. To calculate the conversion rate, the resulting samples from each bisulphite treatment were aligned and the converted cytosines were counted. The ratio of converted cytosine to the total of cytosines before the treatment was calculated in percent. The primer sequences and filtered reads were not included in this analysis. The bisulphite conversion rate in our experiment was estimated to be 99.2%. Three genes (*TFPI2*, *GPX3* and *DMRT1*) were densely methylated in all three untreated gastric cancer cell lines, indicating that these genes are likely to be epigenetically regulated. These CpG methylation patterns were then used to assign positions for methylation-specific PCR (MSP) primers (Table S2). MSP analysis of the three gastric cancer cell lines and of normal gastric tissues produced results that concurred with bisulphite sequencing analysis (Fig. 3a), which indicates that promoter hypermethylations of *GPX1*, *IGFBP6*, *IRF7*, *GPX3*, *TFPI2* and *DMRT1* are cancer cell specific because methylated CpG islands were only present in the gastric cancer cell lines.

In addition, RT-PCR was performed on five genes in untreated and treated cells to confirm the up-regulations of their expressions observed by microarray analysis (Fig. 3b). Using RT-PCR, the re-expressions of *TFPI2* and *GPX3* mRNA were detected in all three gastric cancer cell lines treated with 5Aza-dC. However, the upregulations of *TFPI2* and *GPX3* were not detected after treatment with TSA (0.3 μM) alone, though treatment with 5Aza-dC plus trichostatin A (TSA) markedly increased *TFPI2* expression in the gastric cancer cell lines, indicating the presence of a synergistic effect.[20] In addition, *GPX1*, *IGFBP6*, *GPX3* and *IRF7* were also strongly up-regulated in SNU-1 after treatment with 5Aza-dC and/or TSA (Fig. 3b).

In order to determine whether CpG methylation is related to the reduced or absent expression of respective genes, we analysed the expression levels of the six genes which showed CpG methylation in the gastric cancer cell lines. RT-PCR analysis of cDNAs obtained from four normal gastric tissues and the three cell lines revealed several distinct patterns of mRNA expression. The CpG methylation as determined using the MSP primers was found to be related to silenced gene expression for *TFPI2*, *GPX1*, *GPX3*, *IGFBP6* and *IRF7*, whereas the detected CpG methylation of *DMRT1* was not accompanied by decreased gene expression (Fig. 3c), which demonstrates that CpG methylation of a promoter should not be interpreted as proof of its transcriptional repression.

3.3. *Methylation analysis of candidate genes in clinical gastric cancer samples*

The methylation status of cancer cell lines does not always reflect those of primary tumours.[28] Therefore, the methylation status of these six genes was examined in 152 surgically resected gastric cancer cases using the MSP method. The PCR product in the unmethylated lane from tumour DNA probably arose from normal cells in the tumour specimen (Fig. 3d). Aberrant methylation was detected in 80.9% of primary gastric cancers for *TFPI2*, in 30.1% for *GPX3*, 46.9% for *DMRT1*, 16.7% for *GPX1*, 22.6% for *IGFBP6* and 32.1% for *IRF7*. These results confirm the abnormal methylation patterns of these six genes in primary gastric cancer tissues and in gastric cancer cell lines. We then investigated whether methylation of these six genes is associated with sex, age, tumour stage, histology and overall survival. No significant association was found between the methylation statuses of these genes and these clinicopathological variables. However, the survival of those with

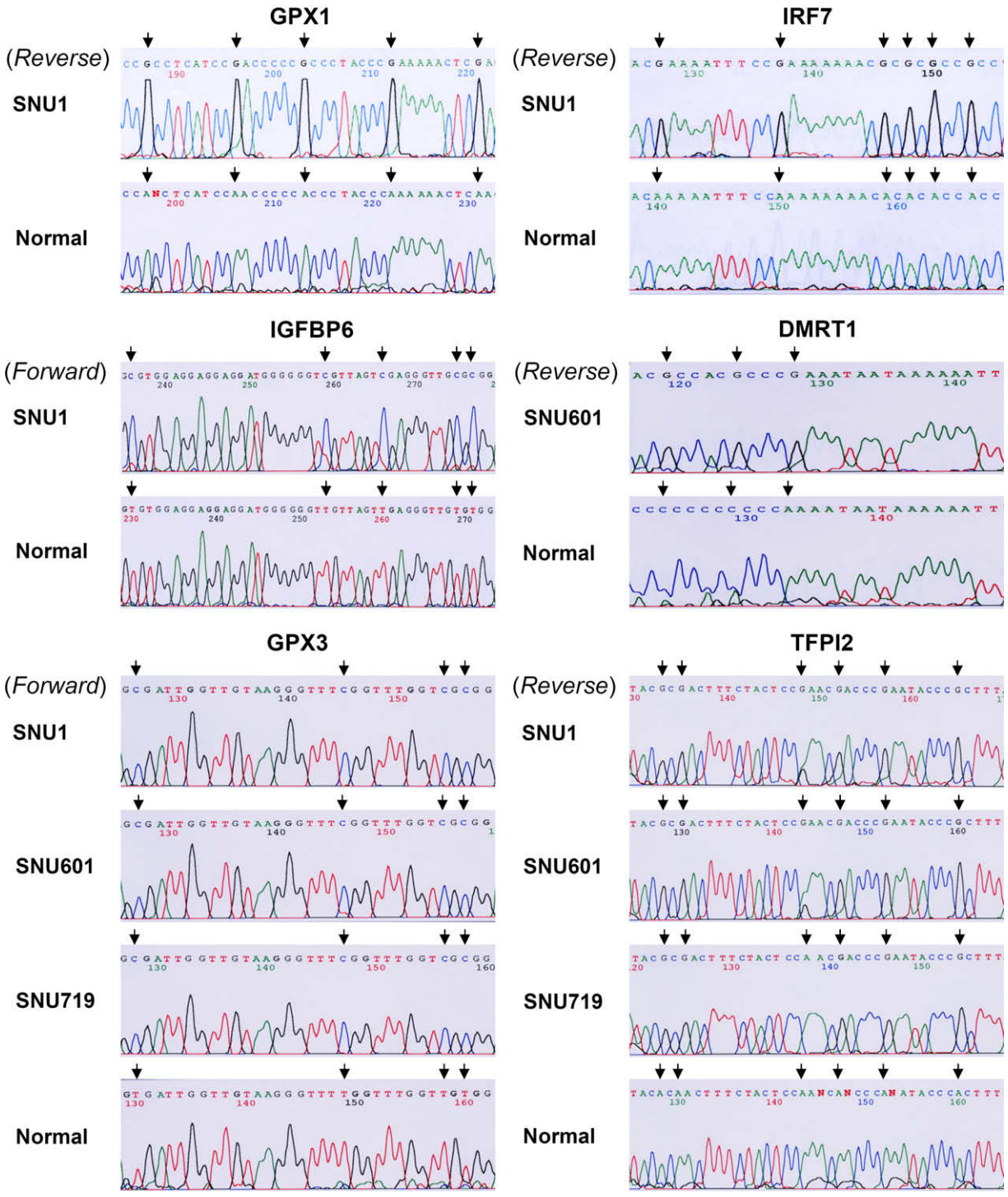

Fig. 2 – Bisulphite sequencing of gene promoter CpG islands in gastric cancer and normal tissue samples. Unmethylated cytosines (C) are converted to uracils (T). The presence of C preceding a G in the site indicated by arrows shows that these cytosines were methylated in gastric cancer cell lines.

a tumour containing methylated alleles of *TFPI2* (123/152, 80.9%) was significantly poorer than that of those with a tumour possessing unmethylated alleles ($p = 0.023$) (Fig. 4). Furthermore, multivariate analysis confirmed that *TFPI2* methylation is a significant and independent prognostic indicator in gastric cancer (Table 2).

3.4. *Immunohistochemical expression and clinicopathologic correlations*

The expression pattern of TFPI2 protein was determined in primary gastric cancer tissues. Immunohistochemical labelling was performed using a monoclonal antibody to TFPI2

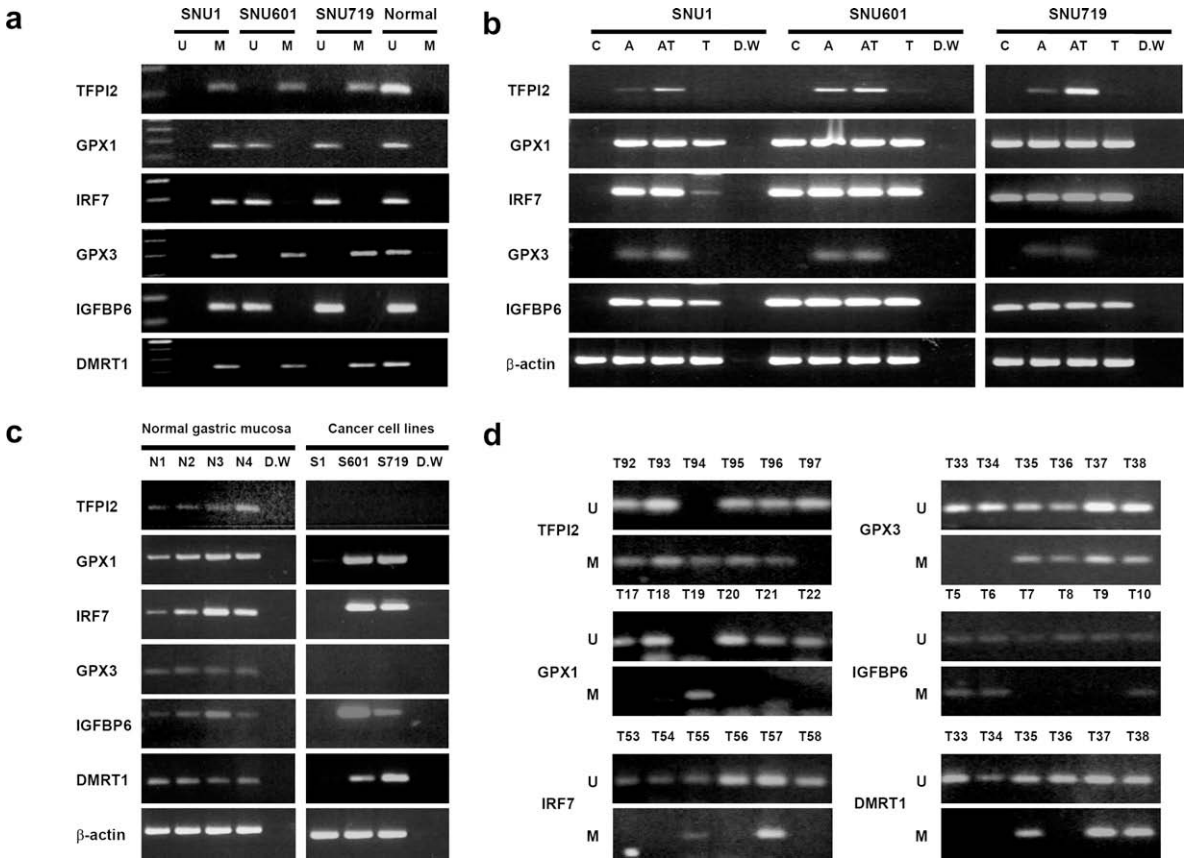

Fig. 3 – (a) Methylation-specific PCR (MSP) of *GPX3*, *DMRT1*, *GPX1*, *IGFBP6*, *TFPI2* and *IRF7* in gastric cancer cell lines. U, unmethylated; M, methylated. (b) RT-PCR validation of up-regulation after 5Aza-dC and/or TSA treatment. Re-expressions of five genes (*TFPI2*, *GPX1*, *GPX3*, *IGFBP6* and *IRF7*) after treatment with 5Aza-dC and/or TSA. C, untreated; A, 5Aza-dC (5 μM) treatment for 4 d; T, TSA (300 nM) treatment for 24 h; AT, 3 d treatment of 5Aza-dC followed by treatment with TSA for 24 h; D.W, negative control. (c) RT-PCR analysis of the indicated genes in normal gastric tissue samples and in the three gastric cancer cell lines. N1–N4 samples represent normal gastric tissues from four different donors, β-actin was used as a loading standard. (d) Methylation-specific PCR analyses of *TFPI2*, *GPX1*, *IRF7*, *GPX3*, *IGFBP6* and *DMRT1* in gastric cancer samples. Two primers sets (U, unmethylated; M, methylated) specific for each indicated gene were used. The PCR product in the unmethylated lane (U) from tumour DNA probably arose from normal cells in the tumour specimen.

in 566 consecutive gastric cancer tissue samples (tumour tissues and normal tissues were simultaneously stained on same tissue-array slides). TFPI2 immunostaining was either cytoplasmic or cytoplasmic/nuclear in normal and in tumour cells (Fig. 5). Moreover, strong positive TFPI2 staining was observed throughout normal gastric glands in all specimens, but its expression was absent in 185 (32.7%) of the 566 consecutive gastric cancer tissues. Since 124 cases have both methylation status and immunostaining results, association between methylation status and loss of TFPI2 expression was examined in 124 gastric cancer tissue samples. The application of Fisher's exact test showed that promoter hypermethylation of *TFPI2* was significantly associated with its expressional loss in gastric cancer (Table 3, $p < 0.036$).

Table 4 shows associations between clinicopathologic parameters and TFPI2 expressional status. Furthermore, TFPI2 expressional modulations were found to be significantly associated with the WHO classification ($p < 0.001$), and with a mixed subtype, as defined by Lauren's classification ($p < 0.001$). However, no association was found between TFPI2 expression and pTNM stage or distant metastasis.

4. Discussion

Methods of detecting DNA methylation are based on the differentiation of cytosine and 5-methylcytosine in DNA. Three strategies are currently used: (1) DNA digestion using a methylation-sensitive or -insensitive restriction endonuclease, (2) chemical modification of DNA by sodium bisulphite or metabisulphite and (3) immunoprecipitation of 5-methylcytosine to directly separate unmethylated and methylated genomic fractions. Recently, all three of these approaches have been coupled to high-throughput technologies, such as, high density oligonucleotide microarray analysis.[29] One of the challenges that remains to be overcome involves the large-scale detection of methylation variation, without completely sacrificing the ability to resolve subtle changes in individual genes.

In an attempt to identify novel targets aberrantly methylated in gastric cancer, we analysed the expression profiles

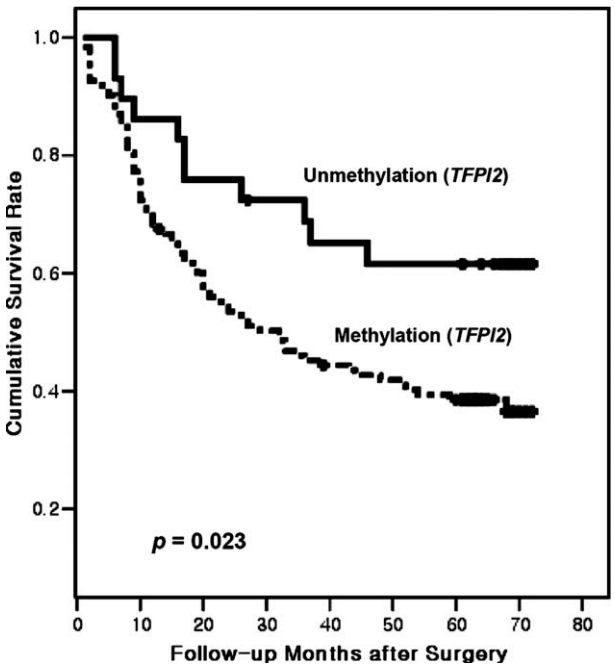

Fig. 4 – Kaplan–Meier survival curves for gastric cancer patients versus *TFPI2* methylation status. The survival curves show that *TFPI2* methylation is associated with an unfavourable prognosis ($p = 0.023$).

Table 2 – Multivariate analysis findings concerning predictive factors of survival (Cox proportional hazards model).

Factors	Hazard ratio (95% CI)	*p*-value
TFPI2		
Unmethylation	1.000 (Reference)	0.042
Methylation	1.959 (1.025–3.745)	
pTNM stage		
I	1.000 (Reference)	<0.001
II	2.321 (0.658–8.182)	
III	5.293 (1.598–17.529)	
IV	8.660 (2.570–29.183)	
Radical surgery		
R0	1.000 (Reference)	0.074
R1	1.580 (0.660–3.778)	
R2	2.225 (1.048–4.723)	
Lauren's classification		
Intestinal	1.000 (Reference)	0.178
Diffuse	0.646 (0.385–1.085)	
Mixed	0.967 (0.462–2.025)	
Sex		
Male	1.000 (Reference)	0.652
Female	1.118 (0.688–1.819)	

CI, confidence interval; R0, no residual tumour; R1, microscopic residual tumour; R2, microscopic residual tumour.

of three gastric cancer cell lines, i.e. SNU-1, -601 and -719 after exposure to 5Aza-dC (a demethylating drug) using an oligonucleotide microarray. After 5Aza-dC treatment, 361 transcripts were found to be substantially up-regulated by >twofold in at least one of these cell lines. Candidate gene numbers were then reduced by excluding unknown genes, imprinted genes and genes without CpG islands. Finally, we included 143 candidate genes in our further study. Several of these genes have been previously reported to be aberrantly methylated in gastric cancer, e.g. *AKAP12*, *ID4*, CDKN1C/*p57KIP2*, *stratifin/14-3-3σ* and *TIMP3*, which supports the validity of our screening approach.[30–34] Of the 143 genes selected, we chose 18 genes with a known cancer-related function. Subsequently, using bisulphite sequencing and MSP, we were able to confirm the aberrant methylation patterns of six of these selected genes (*TFPI2*, *GPX1*, *GPX3*, *IGFBP6*, *IRF7* and *DMRT1*) in the three gastric cancer cell lines and in a series of resected primary gastric cancer tissues. Moreover, these genes have not been previously identified as sites of aberrant methylation in gastric cancer. These results demonstrate that gene expression profiling can be used to identify novel target genes that display aberrant methylation in gastric carcinoma. Furthermore, it was found that these aberrantly methylated genes were present in gastric cancer samples but not in normal tissue samples. Our results support the findings of previous studies that aberrant hypermethylation of multiple genes is a common event in gastric cancer, and suggest that some of these genes may be sensitive and specific markers for diagnosis and prognostic determinations of gastric cancer.

High-throughput oligonucleotide microarray data from multiple cell lines allow the identification of substantial numbers of genes aberrantly methylated in cancer. In addition, they enable us to conservatively estimate the number of genes directly affected. In the present study, we found that 5Aza-dC induced an average of 166 transcripts (range, 108–242) per cell line. Eighteen genes with a CpG island induced by 5Aza-dC were selected in the three cell lines, and ~61% (11 of 18) of these genes were found to be aberrantly methylated in the gastric cancer cell lines. Thus, extrapolation suggests that about 100 genes (61% of 166 genes) are aberrantly methylated in at least one of the three gastric cancer cell lines examined, though it should be noted that differences in CpG island methylation density and different transcriptional cofactor levels in the three cell lines modulate gene expression responses to 5Aza-dC.

To identify genes epigenetically silenced in cancer, genes were screened based on; (i) their known anti-oncogenic functions at the cellular level or (ii) their locations in genomic regions frequently demonstrating LOH in gastric cancer. Candidate tumour-related genes were further selected based on (a) the presence of promoter CpG island methylation in primary gastric cancer tissues and (b) on their expressions in normal gastric tissues. Six of these 11 genes met these criteria, and were considered good tumour-related candidates. It is possible that the silencing of these genes is related to development and progression of gastric cancer, but in view of the large number of methylation-silenced genes detected, we believe it likely that the majority of genes silenced in the three gastric cancer cell lines did not participate in gastric carcinogenesis.

Glutathione peroxidase 3 (*GPX3*) catalyses the reduction of peroxides by glutathione and protects cells against oxidative damage. Thus, the silencing of *GPX3* may impair defences against endogenous and exogenous genotoxic compounds,

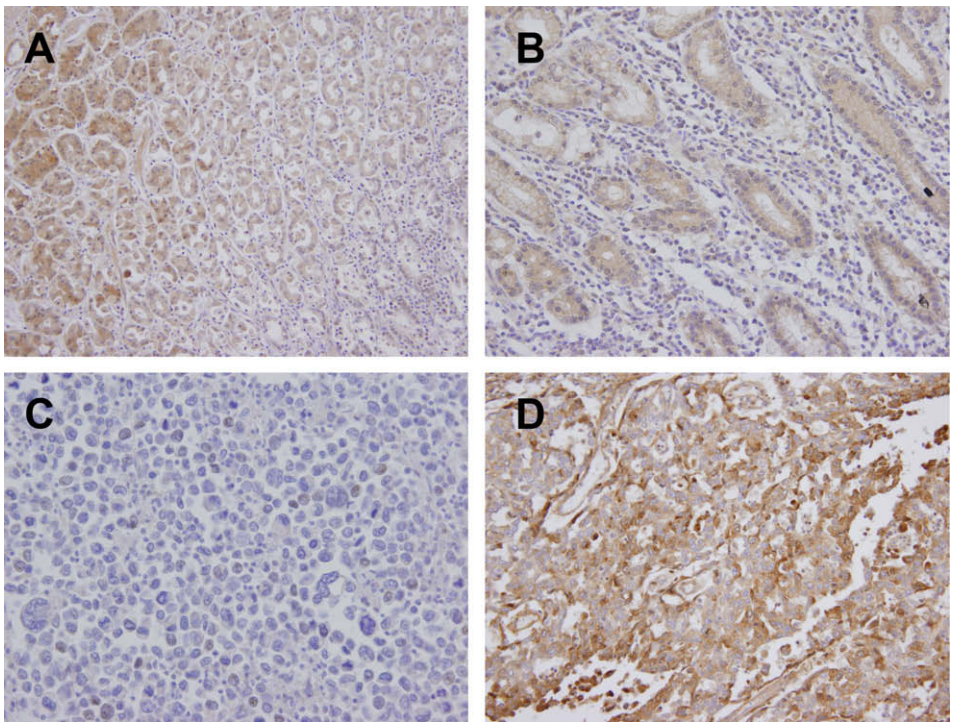

Fig. 5 – TFPI2 immunostaining findings in consecutive gastric carcinomas. All normal gastric tissue sections examined displayed cytoplasmic staining (A and B), but TFPI2 immunostaining was uncommon in tumour cells (C). A case showing positive staining in tumour cells (D). Original magnification ×200.

Table 3 – TFPI2 Promoter methylation and its protein expression in 124 gastric carcinoma tissue samples.

Expression (IHC)	TFPI2		Total	*p*-value[a]
	Unmethylation	Methylation		
Positive	18	59	77	0.036
Negative	4	43	47	
Total	22	102	124	

a Fisher's exact test.

which could increase gene mutation rates. *GPX3* has been found to be frequently hypermethylated in prostate and oesophageal carcinoma.[35] Furthermore, *GPX3* expression was recently reported to be down-regulated in Barrett's dysplasia and adenocarcinoma[36], and the present study suggests that this down-regulation is caused by epigenetic silencing. Promoter hypermethylation of *TFPI2* occurs frequently in cervical carcinoma, pancreatic ductal adenocarcinoma, and non-small cell lung cancer.[37–39] *IRF7* is inactivated by promoter hypermethylation in lung cancer and hepatocellular carcinoma and is associated with loss of expression.[40,41] *IGFBP6* was found to be inactivated by promoter region hypermethylation in osteosarcoma and shown to be a potential regulators of IGFs with anti-growth properties.[42]

In the present study, we examined the methylation statuses of six genes, i.e. *TFPI2, GPX3, DMRT1, GPX1, IGFBP6* and *IRF7*, in 152 surgically resected gastric cancer samples using the MSP method. Aberrant methylation of these genes in cancer tissue samples was detected at the following frequencies; *TFPI2* 80.9%, *GPX3* 30.1%, *DMRT1* 46.9%, *GPX1* 16.7%, *IGFBP6* 22.6% and *IRF7* 32.1%. In terms of statistics, the only significant relation found was between *TFPI2* methylation status and survival, i.e. patients with methylated *TFPI2* (123/152, 80.9%) showed poorer survival that those without ($p = 0.023$). Furthermore, multivariate analysis confirmed that *TFPI2* methylation is a significant and independent prognostic indicator in gastric carcinoma. Immunohistochemical analyses demonstrated the significant correlation between *TFPI2* methylation and negative expression of TFPI2 ($p = 0.036$). In this point, patients with the loss of TFPI2 expression also showed a trend towards an association with a poorer survival but this was not statistically significant.

In this study, we showed that *TFPI2* is highly methylated (80.9%) in 152 gastric cancer tissues and conclude that the methylation is cancer specific because of no methylation in normal mucosa. Unfortunately, the corresponding normal samples were not available in many cases at the time, we investigated *TFPI2* methylation in only 21 matched normal samples. Several reports have recently showed aberrant gene methylation such as RUNX3, E-cadherin even in non-cancer-

Table 4 – Clinicopathologic characteristics versus TFPI2 expression in 566 gastric carcinoma samples.

Characteristic	TFPI2 negative	TFPI2 positive	Total	*p*-value
	(*n* = 185)	(*n* = 381)	(*n* = 566)	
Gender				0.003
Male	107	268	375	
Female	78	113	191	
Mean age (years)	58.1 ± 12.5	53.9 ± 13.1	54.7 ± 13.0	0.296
WHO				<0.001
W/D and M/D	48	167	215	
P/D	82	162	244	
Mucinous	17	19	36	
Signet ring cell	38	33	71	
Lauren's classification				<0.001
Intestinal	41	166	207	
Diffuse	131	185	316	
Mixed	13	30	43	
Tumour invasion				0.589
EGC	57	109	166	
AGC	128	272	400	
Lymph node metastasis				0.103
Absent	64	159	223	
Present	121	222	343	
Distant metastasis				0.598
Absent	169	350	519	
Present	15	26	41	
pTNM stage				0.804
I	69	157	226	
II	42	85	127	
III	41	80	121	
IV	33	59	92	

W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; EGC, early gastric carcinoma; AGC, advanced gastric carcinoma.

ous gastric mucosa with chronic gastritis or intestinal metaplasia. In order to conclude cancer-specific *TFPI2* methylation, further work is required to confirm methylation status of *TFPI2* in more normal samples.

TFPI2 (also known as PP5 and MSPI) is a member of the Kunitz-type serine protease inhibitors, which negatively regulate the enzymatic activities of trypsin, plasmin and VIIa-tissue factor complex.[43] Furthermore, promoter methylation and TFPI2 under-expression are commonly observed in human cancer, and it has been proposed that *TFPI2* inactivation is implicated in human carcinogenesis and metastasis.[39] Nevertheless, *TFPI2* promoter methylation has not been investigated in gastric cancer, and the function of TFPI2 is unclear in human gastric cancer. In the present study, aberrant *TFPI2* promoter methylation was found in 80.9% of primary gastric cancer tissues, but only 32.7% failed to express TFPI2 at the protein level. Thus, promoter hypermethylation may not be the only factor that causes TFPI2 under-expression in gastric cancer. We utilised a methylation-specific PCR (MSP) assay to determine the methylation status of *TFPI2* promoter-associated CpG island. Methylation density in promoter CpG islands was indicated to be important for gene silencing rather than methylation that occurred at a limited number of CpG islands in the promoter. Accordingly, MSP does not fulfil the need to quantify the level of methylation relating to the gene silencing. Due to high sensitivity of MSP, the methylated band may be observed even if the methylated DNA represents only a minor portion (partially methylated).[44]

Summarising, a considerable number of candidate methylated genes were found in the three gastric cancer cell lines investigated using a microarray screening approach. The chemical genomic screening results obtained contribute to our understanding of the multiple methylation events that occur in gastric cancer, and of their possible roles during cancer progression. The identification of these genes provides further insight of tumour biology, and may lead to the identifications of novel diagnostic, prognostic, and therapeutic targets.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.12.027.

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