DLEC1 is a functional 3p22.3 tumour suppressor silenced by promoter CpG methylation in colon and gastric cancers

Promoter CpG methylation of tumour suppressor genes (TSGs) is an epigenetic biomarker for TSG identification and molecular diagnosis. We screened genome wide for novel methylated genes through methylation subtraction of a genetic demethylation model of colon cancer (double knockout of DNMT1 and DNMT3B in HCT116) and identified DLEC1 (Deleted in lung and oesophageal cancer 1), a major 3p22.3 TSG, as one of the methylated targets. We further found that DLEC1 was downregulated or silenced in most colorectal and gastric cell lines due to promoter methylation, whereas broadly expressed in normal tissues including colon and stomach, and unmethylated in expressing cell lines and immortalised normal colon epithelial cells. DLEC1 expression was reactivated through pharmacologic or genetic demethylation, indicating a DNMT1/DNMT3B-mediated methylation silencing. Aberrant methylation was further detected in primary colorectal (10 out of 34, 29%) and gastric tumours (30 out of 89, 34%), but seldom in paired normal colon (0 out of 17) and gastric (1 out of 20, 5%) samples. No correlation between DLEC1 methylation and clinical parameters of gastric cancers was found. Ectopic expression of DLEC1 in silenced HCT116 and MKN45 cells strongly inhibited their clonogenicity. Thus, DLEC1 is a functional tumour suppressor, being frequently silenced by epigenetic mechanism in gastrointestinal tumours.

Keywords: tumour suppressor gene (TSG); DLEC1; CpG island; methylation; colon and gastric cancers

Tumorigenesis is a multistep process, with colorectal cancer (CRC) as the prototype model for multi-step genetic pathogenesis (Kinzler and Vogelstein, 1996). In this model, the key molecular event is the inactivation of multiple tumour suppressor genes (TSGs) due to genetic alterations. Also, it is now well established that alternative epigenetic silencing, such as methylation of promoter CpG islands (CGIs), leads to the inactivation of TSGs in virtually all tumour types and plays significant roles in tumour initiation and progression (Jones and Baylin, 2002). In CRC, epigenetic silencing of multiple TSGs has been reported frequently, including MLH1, p16INK4A, MGMT, VHL, APC, RASSF1A, HIC1, CHFR, ADAMTS18 and PCDH10 in various percentages of CRC tumours (Herman et al, 1998; Esteller et al, 2001; Kim et al, 2005; Ying et al, 2006; Jin et al, 2007). A growing list of TSGs with CGI methylation-mediated silencing has also been reported in gastric cancer (Leung et al, 2001). It is important to identify more new TSGs that are silenced by tumour-specific methylation in CRC and gastric cancers, which could serve as valuable biomarkers for molecular diagnosis and also provide clues to the molecular pathogenesis of these tumours.

In this study, we conducted a genome-wide search for genes with promoter methylation in CRC, by utilising CpG methylation-specific subtraction, in a CRC model of HCT116 cells deficient in DNMT1 and DNMT3B (double knockout (DKO) cells). DNMT1 and DNMT3B are the two major DNA methyltransferases responsible for the maintenance and de novo CpG methylation, and the disruption of these two genes results in more than 95% loss of overall genomic methylation and CGI demethylation (Rhee et al, 2002). Virtually all known TSGs with methylation-mediated silencing in HCT116 became demethylated and reactivated in HCT116DKO, making it a good epigenetic model to identify novel candidate TSGs silenced in tumours (Rhee et al, 2002; Paz et al, 2003; Ying et al, 2003). Among the methylated target genes we
identified, one is DLEC1 (Deleted in lung and oesophageal cancer 1), located at 3p22.3 – a common tumour suppressor locus with frequent genetic abnormalities in multiple cancers (Imreh et al., 2003). The expression of DLEC1 and its regulation in digestive tumours have yet to be evaluated. We found that DLEC1 underwent promoter methylation-associated silencing in most CRC and gastric tumour cell lines and primary tumours, in a tumour-specific manner. Reintroduction of DLEC1 into silenced tumour cells significantly suppressed tumour cell clonogenicity.

MATERIALS AND METHODS

Cell lines and primary tumours

Seven CRC (HCT116, HT29, LoVo, SW480, DLD1, LS180 and SW620) and 17 gastric cancer (Kato III, YCC1, YCC2, YCC3, YCC6, YCC7, YCC9, YCC10, YCC11, YCC16, SNU719, AGS, MKN28, NCI87, SNU1, SNU16 and MKN45) cell lines were used. Cell lines were routinely maintained in RPMI-1640 medium with 10% FBS. HCT116 cell line with genetic knockout of DNA methyltransferases (DNMTs): HCT116 DNMT1−/−, DNMT3A−/−, DNMT3B−/− (DKO) (gift from Dr Bert Vogelstein, Johns Hopkins) were grown with either 0.4 mg ml−1 hygromycin or both (Rhee et al., 2002). DNA and total RNA were extracted from cell lines using TRI REAGENT (Molecular Research Center, Cincinnati, OH, USA). Genomic DNA of another five CRC cell lines (HCT15, RKO, SW48, Caco-2 and Colo205) and one immortalised normal colon epithelial cell line CCD-841 were also used. Genomic DNA samples from primary tumour tissues of 34 CRC and 89 gastric cancer patients were also used, with DNA samples of matched surgical marginal normal tissue samples from 17 CRC and 20 gastric cancer patients also available. Clinical information was available for all gastric cancer patients, including gender, differentiation, histological type and -3B) DKO of HCT116 (Rhee et al., 2002). The method was based on the principle that restriction enzymes (HpaII, SacI and NarI) have different sensitivities towards sequences containing 5-methylcytosine (CCGG, CCGCGG and GCGGCC). We further selected candidate genes with typical promoter CGI and also located at important chromosome loci commonly deleted in tumours and possibly harbouring putative TSGs for more detailed studies, such as DLEC1.

Modified methylation-sensitive representational difference analysis

To identify novel methylated TSGs, we employed a strategy of modified methylation-sensitive representational difference analysis (MS-RDA), using uracil-DNA glycosylase-based digestion during MS-RDA (Sugai et al., 1998; Kaneda et al., 2003), for DNA samples of the wild-type and DNA methyltransferases (DNMT1 and -3B) DKO of HCT116 (Rhee et al., 2002). The method was based on the principle that restriction enzymes (HpaII, SacI and NarI) have different sensitivities towards sequences containing 5-methylcytosine (CCGG, CCGCGG and GCGGCC). We further selected candidate genes with typical promoter CGI and also located at important chromosome loci commonly deleted in tumours and possibly harbouring putative TSGs for more detailed studies, such as DLEC1.

Reverse transcription PCR

Reverse transcription PCR (RT – PCR) was performed as described earlier (Tao et al., 2002; Ying et al., 2006), using GAPDH as a control. The primers for DLEC1 are listed in Table 1. The PCR programme utilised an initial denaturation at 95°C for 10 min, followed by 33 cycles of reaction (94°C for 30 s, 55°C for 30 s and 72°C for 30 s), with a final extension at 72°C for 10 min.

Bisulphite treatment and promoter methylation analysis

Bisulphite modification of DNA was carried out as described earlier using 2.4 M sodium bisulphite (Tao et al., 2002). Methylation-specific PCR (MSP) and bisulphite genomic sequencing (BGS) were conducted according to our earlier reports (Tao et al., 1999; Ying et al., 2006). Methylation-specific PCR primers are listed in Table 1. Methylation-specific PCR was conducted at 95°C for 10 min, followed by 40 cycles of reaction (94°C, 30 s; 58°C for M, 55°C for U, 30 s; 72°C, 30 s), ended by 72°C for 5 min. Methylation-specific PCR primers were tested earlier for not amplifying any non-bisulphite-treated genomic DNA and thus specific. The MSP products of selected samples have been confirmed by direct sequencing. The top strand-specific BGS primers for bisulphite-converted single-stranded DNA of the DLEC1 promoter are listed in Table 1. Amplified products were cloned into the pCR4-Topo vector (Invitrogen, Carlsbad, CA, USA), with six to eight colonies randomly chosen and sequenced.

Cloning of the human DLEC1 full-length open reading frame

Four pairs of primers were used to generate four DLEC1 fragments based on the published DLEC1 sequence (GenBank accession number AB020522): I, II, III and IV, which contain restriction enzyme sites of MfeI, NdeI and ApaLI, respectively. The sequences of primers for these fragments are listed in Table 1. Reverse transcription was carried out using normal human testis RNA as a template (BD Biosciences, Palo Alto, CA, USA). Reverse transcription PCR products were cloned into pCR II-TOPO vector (Invitrogen) with the sequences and orientations confirmed from both ends. The four fragments were then ligated to form the full-length DLEC1 cDNA, which was then cloned into the pcDNA3.1 vector, using the restriction sites BamHI and XhoI (vector and fragment 1), MfeI and NdeI (fragment II), NdeI and ApaLI (fragment III), and ApaL I and Xho I (fragment IV and vector), to generate the recombinant vector pcDNA3.1-DLEC1.

Colony formation assay

Cells (1.5 × 10⁵ per well) were plated in a 12-well plate and transfected with either expression plasmid or the empty vector (0.8 µg each), using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). Forty-eight hours post-transfection, cells were collected and plated in a six-well plate, and selected for 2 weeks with G418 (0.4 mg ml−1). Surviving colonies (>50 cells per colony) were counted after staining with Gentian Violet. Total RNA from the transfected cells was extracted, treated with TURBO DNase (Ambion, Austin, TX, USA) and analysed by RT – PCR to confirm the ectopic expression of DLEC1. All the experiments were performed in triplicate wells for three times.

Statistical analysis

Chi square test was used to analyse possible correlation between clinical parameters and DLEC1 methylation status of tumour and non-tumour samples. For colony formation assay, experimental differences were tested for statistical significance using t-test. All analyses were performed using SAS for windows, version 9 software (SAS Institute Inc., Cary, NC, USA). A P-value of <0.05 was considered significant.
RESULTS

Epigenetic identification of DLEC1 as a methylated gene in CRC

Using a modified MS-RDA to screen genome wide for methylated sequences in HCT116 and its demethylated DKO cells, we identified 22 hypermethylated DNA fragments/genes (Ying and Tao, manuscript in preparation). Among these identified sequences, one of particular interest is DLEC1, a candidate TSG previously identified in lung cancer (Daigo et al., 1999). Although no methylation was detected in this first report, the region spanning the putative promoter and exon 1 of DLEC1 is a typical CGI (Gardiner-Garden and Frommer, 1987) (Figure 1A) that is susceptible to epigenetic silencing. We designed MSP and BGS primers to analyse its methylation status. Methylation-specific PCR analyses showed that DLEC1 was completely methylated in HCT116 and became completely demethylated in DKO cells, but only marginally demethylated in DNMT1KO and not demethylated in DNMT3BKO cells. Correlated with its methylation status, DLEC1 was silenced in HCT116, and only reactivated in DKO cells, but not in DNMT1KO or DNMT3BKO cells (Figure 1B). Detailed BGS analysis, revealing the methylation status of individual CpG site of the DLEC1 promoter, showed that only few scattered CpG sites remained methylated in DKO cells whereas HCT116 was almost completely methylated (Figure 1C). These results thus demonstrate a close relationship between the silencing of DLEC1 and its promoter methylation in HCT116 and DKO cells.

Frequent methylation-associated silencing of DLEC1 in CRC and gastric cell lines

To further examine the correlation of DLEC1 methylation and silencing, we investigated additional human tissues and gastrointestinal cell lines. DLEC1 was found to be readily expressed in all 22 normal adult and 9 foetal tissues including colon, rectum and stomach, with the highest level in testis and weak expression in skeletal muscle and pancreas (Figure 2A), in agreement with the earlier study that this gene is expressed in all tissues examined and abundantly in testis (Daigo et al., 1999). In contrast, DLEC1 was silenced or downregulated in six of seven CRC and 15 of 17 gastric cancer cell lines (Figure 2B). By MSP, DLEC1 methylation was

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**Table 1** List of primers used in this study

| Method  | Primer  | Sequence                  | Location | Product size | PCR cycles | Annealing temperature (°C) |
|---------|---------|---------------------------|----------|--------------|------------|-----------------------------|
| RT–PCR  | DLEC1A  | ttcctccctggcctgcttc       | Exon 1   | 309 bp       | 33         | 55                          |
|         | DLEC1B  | aaacctctacgacgcgctg       | Exon 2   | ~ 1.1 kb     | 35         | 53                          |
| cDNA cloning | Fragment I | gcgcgtcattgagacgccg     | Exon 6   | ~ 1.2 kb     | 35         | 53                          |
|         | Fragment II | agtgtaacgtgtaagcc         | Exon 14  | ~ 1.9 kb     | 35         | 53                          |
|         | Fragment III | ccggcgccatagctggagt      | Exon 1   | ~ 1.6 kb     | 35         | 53                          |
|         | Fragment IV | ccagggagacccagtgggg        | Exon 25  | ~ 1.8 kb     | 35         | 53                          |
| MSP     | DLEC1m1 | gcgcggggcggctggcttc       | Exon 1   | 107 bp       | 40         | 58                          |
|         | DLEC1m2 | ccacggtgcgttcagcc         | Exon 1   | 110 bp       | 40         | 55                          |
|         | DLEC1u1 | tagtaacggttcagcc          | Exon 1   | 110 bp       | 40         | 55                          |
|         | DLEC1u2 | cagacgccggttcagcc         | Exon 1   | 110 bp       | 40         | 55                          |
| BGS     | DLEC1BGS1 | ccgccgccgttcagcc         | Promoter | 597 bp       | 40         | 55                          |
|         | DLEC1BGS4 | caacctccaccccccaccaagaa   | Intron 1 |             |            |                             |

BGS = bisulphite genomic sequencing; MSP = methylation-specific PCR.
detected in 83% (10 out of 12) of CRC and 100% (17 out of 17) of gastric cancer cell lines, with complete methylation detected in most cell lines, whereas no methylation was seen in the normal colon epithelial cell line CCD-841 (Figure 2B). Further BGS methylation analysis for one CRC, two gastric cell lines and CCD-841 confirmed the MSP results, with a high density of methylated CpG sites detected in all tumour cell lines, but not in CCD-841 (Figure 2C). Thus, the results revealed a strong correlation between DLEC1 transcriptional silencing and its promoter methylation in virtually all CRC and gastric cancer cell lines examined, except for one CRC cell line LS180, which has both methylated and unmethylated promoter alleles but DLEC1 expression is totally silenced, indicating that other mechanisms such as histone modification also could not be excluded.

Restoration of DLEC1 expression by pharmacologic demethylation

To determine whether methylation directly mediates the silencing of DLEC1, two CRC (HCT116 - methylated, SW480 - unmethylated) and two methylated gastric (YCC10 and SNU719) cancer cell lines were treated with Aza, a DNA methyltransferase inhibitor. After the treatment, DLEC1 expression was restored in methylated cell lines along with an obvious increase of unmethylated promoter alleles (Figure 2D). In contrast, no significant change of DLEC1 expression and methylation levels was observed in the unmethylated cell line SW480, indicating that Aza treatment did not cause indirect reactivation effect. Together with earlier results of DLEC1 reactivation after genetic demethylation in DKO cells, these results showed that methylation of the DLEC1 promoter directly leads to its silencing in CRC and gastric cancers.

Frequent DLEC1 methylation in primary CRC and gastric tumours

The methylation status of DLEC1 was further examined in primary CRC and gastric tumour samples using the well-validated MSP analysis. Aberrant methylation was detected in 10 out of 34 (29%) of CRC and 30 out of 89 (34%) of gastric tumours, but seldom in paired normal gastric tissues (1 out of 20, 5%) nor any of the paired normal colon tissue samples (0 out of 17) (Figure 3A). Further BGS analysis revealed densely methylated promoter alleles in primary tumours, whereas only scattered methylated CpG sites in paired normal tissues (Figure 3B). Thus, promoter methylation of DLEC1 is a frequent and tumour-specific epigenetic abnormality in CRC and gastric cancer.

Although the frequency of DLEC1 methylation in gastric cancer was high (34%), no correlations between DLEC1 methylation status and gender, tumour location, Lauren type, tumour differentiation and TNM stage were found (Table 2).
Ectopic expression of \textit{DLEC1} suppresses colorectal and gastric tumour cell clonogenicity

The frequent silencing of \textit{DLEC1} by methylation in colon and gastric cancer cell lines as well as primary tumours suggested that \textit{DLEC1} is a potential tumour suppressor for these tumours. We thus investigated the tumour suppressor function of \textit{DLEC1} by colony formation assay. The CRC cell line HCT116 and gastric carcinoma cell line MKN45 with silenced \textit{DLEC1} were transfected with \textit{DLEC1}-expressing vector pcDNA3.1-\textit{DLEC1}. A strong reduction of colonies (i.e., down to 17 and 37\% of the controls in HCT116 and MKN45 cells, respectively, \textit{P}\textless 0.01) was observed in cells transfected with pcDNA3.1-\textit{DLEC1}, compared with the empty vector control (Figure 4). These results indicate that \textit{DLEC1} indeed has growth inhibitory activities and could function as a tumour suppressor for colorectal and gastric cancer cells.

DISCUSSION

This is the first report to identify \textit{DLEC1} as a methylated candidate TSG for CRC and gastric cancer. We demonstrated that \textit{DLEC1} was absent in most colorectal and gastric cancer cell lines due to promoter methylation, and methylated in a significant part of primary CRC and gastric tumours in a tumour-specific manner. In addition, the

| Variable                     | Methylated (\textit{n} = 30) | Non-methylated (\textit{n} = 59) | \textit{P}-value |
|------------------------------|-------------------------------|-----------------------------------|-----------------|
| Gender                       |                               |                                   |                 |
| Male                         | 20                            | 29                                | 0.843           |
| Female                       | 10                            | 16                                | 61.5            |
| Location                     |                               |                                   |                 |
| Corpus                       | 23                            | 41                                | 0.476           |
| Antrum                       | 7                             | 18                                | 72.0            |
| Lauren                       |                               |                                   |                 |
| Diffuse                      | 4                             | 18                                | 81.8            |
| Intestinal                   | 19                            | 34                                | 64.2            |
| Differentiation              |                               |                                   |                 |
| Poor (or no differentiation) | 19                            | 31                                | 62.0            |
| Well or moderate             | 11                            | 26                                | 70.3            |
| TNM (tumour, node, metastasis) |                     |                                   |                 |
| i and ii                     | 15                            | 24                                | 61.5            |
| iii and iv                   | 15                            | 34                                | 69.4            |
ectopic expression of DLEC1 significantly suppressed colorectal and gastric carcinoma cell clonogenicity. Our results indicated that DLEC1 is a functional TSG for CRC and gastric cancers, but is frequently inactivated by methylation-mediated silencing in these tumours.

Given the critical role of DNA methylation in the inactivation of TSGs as well as its potential application as tumour biomarkers for cancer diagnosis and prognosis assessment, various genome-wide techniques have been developed to screen for methylated genes in cancer cells. Among them, one is MS-RDA, which has been successfully used to identify methylated targets in multiple tumours (Kaneda et al., 2003; Ying et al., 2005). We used an improved MS-RDA to identify methylated targets in HCT116 comparing with DKO, in which virtually all known epigenetically silenced TSGs were reactivated with demethylation (Paz et al., 2003). The re-identification of multiple genes that had been shown earlier to be methylated in tumours represented a successful validation of this approach (Ying and Tao, manuscript in preparation). Considering promoter CGI and the chromosome location of identified genes, among the 22 targets identified, one regarded to be of particular interest is DLEC1 (Daigo et al., 1999), which is located at the commonly deleted locus 3p22.3 and recently reported to be methylated in lung, ovarian and nasopharyngeal carcinomas (Kwong et al., 2006, 2007). Here, we provided solid evidence that DLEC1 is also frequently methylated and acts as a functional TSG in CRC and gastric cancers.

Frequent deletion of 3p is one of the earliest molecular changes in tumours of the lung, nasopharynx, oesophagus, kidney, head and neck, breast, cervix and gastrointestinal tract (Hung et al., 1995; Kok et al., 1997; Wistuba et al., 2000; Hesson et al., 2007). Identification of TSGs in the gene-rich 3p22–21.3 region has been challenging, although several candidate TSGs within this region showed tumour suppressor functions, such as RASSF1A (Pfeifer et al., 2002), SEMA3B (Tomizawa et al., 2001), BLUZMYND10 (Qiu et al., 2004), FUS1 (Zabarovsky et al., 2002) and HYA22 (RBSP3) (Kashuba et al., 2004), with some of them (RASSF1A and BLU) frequently inactivated by promoter methylation-mediated silencing. DLEC1, also located at this region, contains 37 exons, spans ~59 kb and encodes a 1755-amino-acid protein. DLEC1 was first identified as a potential TSG involved in lung, oesophageal and renal cancers, but with no methylation detected (Daigo et al., 1999). Recently, DLEC1 was reported to be frequently downregulated by methylation in ovarian and nasopharyngeal cancer (Kwong et al., 2006, 2007). Together with our results of DLEC1 methylation in CRC and gastric cancers, DLEC1 is likely to be inactivated in a wide range of tumours by promoter methylation and plays an important role in multiple tumorigenesis.

The function of DLEC1 was further explored by examining the inhibitory effect of DLEC1 expression on tumour cell growth. Introduction of DLEC1 to silenced tumour cell lines HCT116 and MKN45 strongly suppressed their growth in colony formation assays. Similar tumour-suppressive properties were observed in oesophageal, renal and lung cancer cell lines (Daigo et al., 1999; Kwong et al., 2006). The proliferation and invasiveness of DLEC1-expressing cells were also greatly reduced with a dramatic reduction in tumorigenic potential in in vivo animal models (Kwong et al., 2007). These findings support that DLEC1 is a functional TSG involved in multiple tumorigenesis; however, the mechanism underlying this role remains largely unknown. The predicted protein sequence of DLEC1 has no significant homology to any known proteins or domains. Earlier report showed that 27 potential CK2 (formerly known as casein kinase II) phosphorylation sites are present in its predicted sequence (Daigo et al., 1999). Protein kinase CK2 is a pleiotropic, ubiquitous, constitutively active and second message-independent protein kinase and known to phosphorylate more than 100 substrates, many of which are involved in the control of cell division, signal transduction and many other cellular functions (Litchfield, 2003). CK2 is required at multiple transition in the cell cycle, including G0/G1, G1/S and G2/M (Litchfield, 2003), indicating that DLEC1 may be one of the increasing CK2 targets, modulated by CK2 and involved in cell cycle arrest.

Although DLEC1 methylation has been shown to be associated with tumour stages in hepatocellular carcinoma (Qiu et al., 2008), we did not find any correlation between DLEC1 methylation and clinical parameters in gastric tumours. A further larger scale study is needed to confirm this negative finding.

Tumour-specific promoter methylation can serve as a biomarker for tumour early diagnosis. The tumour-specific methylation of DLEC1 in CRC and gastric cancers indicates that it could be used for such purposes in future. Our results revealed a higher frequency of methylation in cell lines (83–100%) but lower in primary tumours (29–34%), indicating that some cell lines may have acquired methylation during their establishment or maintenance process. Similar phenomenon has been reported for some other TSGs in other tumours as well (Smiraglia et al., 2001; Paz et al., 2003; Ying et al., 2005).

In summary, we found that DLEC1 is frequently silenced by promoter methylation in colorectal and gastric cancers in a tumour-specific manner. We also showed that the methylation-mediated silencing of DLEC1 could be reversed by genetic or
pharmacologic demethylation, and restoration of DLEC1 suppressed tumour cell clonogenicity, providing significant evidence that DLEC1 functions as a tumour suppressor in these tumours. It would thus be worthy further exploring the possible use of DLEC1 methylation as an epigenetic biomarker for molecular diagnosis.

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