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Molecules and Cells

Identification of Novel Subregions of LOH in Gastric Cancer and Analysis of the HIC1 and TOB1 Tumor Suppressor Genes in These Subregions

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Previously, we identified 3 overlapping regions showing loss of heterozygosity (LOH, R_1-R_3 from 11 to 30 cM) on chromosome 17 in 45 primary gastric cancers (GCs). The data indicated the presence of tumor suppressor genes (TSGs) on chromosome 17 involved in GC. Among the putative TSGs in these regions, HIC1 (in SR_1) and TOB1 (in SR_3) remain to be examined in GC. By immunohistochemistry (IHC), methylation-specific PCR (MSP) and western blot, we evaluated the expression and regulation status for HIC1 and TOB1 protein in GC. We narrowed down the deletion intervals on chromosome 17 and defined five smaller LOH subregions, SR_1-SR_5 (0.54 to 3.42 cM), in GC. We found that HIC1 had downregulated expression in 86% (91/106) and was methylated in 87% (26/30) of primary GCs. Of the primary GCs showing downregulation of HIC1 protein, 75% (18/24) had methylated HIC1 gene. TOB1 was either absent or expressed at reduced levels in 75% (73/97) of the GC samples. In addition, a general reduction was found in total and the ratio of unphosphorylated to phosphorylated TOB1 protein levels in the differentiated GC cell lines. Further analysis revealed significant simultaneous downregulation of both HIC1 and TOB1 protein in GC tissue microarray samples (67%, 52/78) and in primary GCs (65%, 11/17). These results indicate that silencing of HIC1 and TOB1 expression is a common occurrence in GC and may contribute to the development and progression of the disease.

INTRODUCTION

Inactivation of tumor-suppressor genes (TSGs) is one of the critical events leading to gastric carcinoma (GC) development and progression. Highly polymorphic microsatellite markers have been used to determine overlapping deleted regions by comparison of tumor DNA with matched control DNA (Russell et al., 2000). Loss of heterozygosity (LOH) studies have revealed that allelic imbalance of chromosome 17 loci occurs frequently in sporadic GC. The common regions of loss have been mapped to 17p13.1 (Dockhorn et al., 1994), 17p13.3 (Wang et al., 1997), 17q21 (Semba et al., 1998) and 17q24-25 (Petty et al., 1998).

However, contradictory results have been reported concerning the incidence of LOH on chromosome 17 in GC (Noguchi et al., 2001). Furthermore, most of the LOH studies performed on chromosome 17 to date have used only a limited number of markers, and a detailed deletion map of overlapping regions on chromosome 17 has not yet been made in sporadic GC. Our previous study demonstrated three overlapping regions of deletion ranging from 11 to 30 cM (R_1 at 17p12-13.3, R_2 at 17q21.3-22 and R_3 at 17q25.3), which highlighted the association of LOH on chromosome 17 with GC pathogenesis (Yu et al., 2008). To achieve a more precise mapping of the locations of putative TSGs on the chromosome involved in GC, in the present study we narrowed these intervals using high-density genome scanning and defined five smaller overlapping subregions of LOH in GC samples. There are several putative TSGs in SR_1-SR_3, however, in our study, we focused on the hypermethylated in cancer 1 (HIC1) gene (in SR_1) and TOB1 (in SR_3) gene which are two genes that have not been investigated in GC.

The HIC1 gene is an interesting candidate TSG located in the overlapping LOH subregion of LOH in chromosome 17p13.3 (SR_1). It encodes a zinc-finger transcription regulator that contains an NH_2-terminal BTB-POZ domain characteristic of a family of repressors. The gene is ubiquitously expressed in normal tissues, but a decrease or loss of expression was found in several types of tumors (Wales et al., 1995). HIC1 is considered a likely candidate for a TSG because it has been demonstrated to have a growth suppressive effect when re-expressed in tumor cells *in vitro*, and has been implicated in the lung, ovary, brain, breast and colon tumorigenesis (Feng et al., 2008; Hayashi et al., 2001). Indeed, there is accumulating evidence indicating that a change in HIC1 expression through epigenetic mechanisms has an important role in tumor progression (Ahuja et al., 1997; Dong et al., 2001; Fujii et al., 1998; Kanai et al.,

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1999; Melki et al., 1999; Rood et al., 2002; Yamanaka et al., 2003). However, little is known about the role of HIC1 in the pathogenesis of GC.

Another notable candidate gene in the overlapping LOH subregion in chromosome 17q21.33 (SR_3) is TOB1 (transducer of ERBB-2, 1). TOB1 is a member of the TOB/BTG anti-proliferation protein family. Overexpression of the TOB family proteins results in cell growth retardation (Suzuki et al., 2001), while TOB LOH (Fong et al., 1995) and decreased TOB expression (Iwanaga et al., 2003) were observed in lung cancer tissues. Moreover, mice lacking the TOB gene developed tumors in the lung, liver, and lymph nodes (Yoshida et al., 2003), suggesting the importance of the TOB gene in these tumors. However, the role of this gene has not been determined in GC.

To determine the biological significance of HIC1 and TOB1 in gastric tumorigenesis, we investigated their expression patterns and/or promoter methylation status by immunohistochemistry (IHC) analysis, methylation-specific polymerase chain reaction (MSP)/RT-PCR, and/or Western blotting of a series of primary GCs, GC tissue microarrays and GC cell lines. We showed that aberrant methylation and reduced expression of HIC1, as well as downregulated TOB1 expression and the ratio of unphosphorylated to phosphorylated TOB1 protein are common in GC and could serve as potential markers for GC development.

MATERIALS AND METHODS

Cell lines

Four GC cell lines (moderately differentiated SGC-7901; poorly differentiated BGC-823; MGC-803; and undifferentiated HGC-27) and human embryonic kidney 293 cells (HEK 293) were obtained from the Cell Resources Center of Shanghai Life Sciences, Chinese Academy of Sciences (China). Cells were cultured according to the supplier's recommendations.

Tissue specimens

Forty-five paired specimens of GCs and corresponding non-cancerous gastric tissues for LOH analysis and thirty pairs of cancerous and adjacent non-cancerous tissues of fresh gastric adenocarcinomas, for both expression and methylation analysis, and Twenty-six pairs of formalin-fixed and paraffin-embedded specimens for immunohistochemistry analysis, were obtained at the First, Second and Third Affiliated Hospitals, Harbin Medical University in Harbin, Heilongjiang Province, People's Republic of China. All diagnoses were confirmed by routine histologic examination and patients had received no treatment before surgery.

Tissue microarrays

A set of GC tissue microarrays, purchased from Beijing Friendship Hospital (China), were used in the immunohistochemistry analysis, which contained 80 cancerous and non-cancerous tissue pairs, respectively. Each GC tissue sample came with detailed information regarding the patient, including gender, age and tumor histotype.

All the work involving human samples was carried out in accordance with The Code of the World Medical Association (Declaration of Helsinki). This project was approved by the Ethics Committee of Harbin Medical University and appropriate informed consent was obtained from all participating patients.

Microsatellite analysis for LOH

Thirty-six microsatellite markers, distributed over three overlapping deleted regions (18 in R_1, 9 in R_2 and 9 in R_3) on chromosome 17, were used for this study. Nine markers (D17S1852, D17S938, D17S831, D17S784, D17S928, D17S787, D17S785, D17S921, and D17S1868), which were used in our previous work (Yu et al., 2008), are listed in Fig. 2 and Table 1. PCR primers were synthesized according to the primer sequences obtained from the Genome Database (http://www.gdb.org; Table 1). One primer of each pair was labeled with either one of two fluorescent dyes (FAM, HEX) at the 5′-end. Multiplex PCR analysis was performed and LOH was scored as previously described (Yu et al., 2008). The LOH frequency of one site was indicated by the percentage of the allelic losses and all informative cases.

Immunohistochemistry

Immunohistochemical staining using antibodies against HIC1 (rabbit polyclonal, 1:200 dilution, Abcam, USA) and TOB1 (mouse monoclonal, 1:1200 dilution; Sigma, USA) was performed as previously described (Park et al., 2006). Global staining of HIC1 and TOB1 was scored as 0 (no staining), 1+ (focus or weak), 2+ (moderate 25%-50%), 3+ (strong 25%-50%) and 4+ (strong > 50%).

Methylation-specific PCR

Methylation-specific PCR was conducted as described previously (Herman et al., 1996). The primers for the methylated sequence of the HIC1 promoter region were 5′-TCGGTTTT CGCGTTTTGTTCGT-3′ (sense), 5′-AACCGAAAACTATCAAC CCTCG-3′ (antisense), and those for the unmethylated sequences of the promoter region for HIC1 were 5′-TTGGGTT TGGTTTTTGTGTTTTG-3′ (sense), 5′-CACCCTAACACCACC CTAAC-3′ (antisense). The 5′ ends of the unmethylated and methylated sense primers are locate in the bp 20 and 26 position of the genome sequence (GenBank accession number L41919), and -617 and -611 relative to the HIC1 transcription start site, respectively (Dong et al., 2001). The MSP products were then separated in 2% agarose gels stained with ethidium bromide and visualized under UV light. Normal lymphocyte DNA treated with Sss1 methyltransferase (New England Biolabs, USA) was used as a positive control for methylated alleles, and DNA from normal lymphocytes was used as a negative control for methylated alleles.

Reverse transcriptase-polymerase chain reaction

RT-PCR was performed according to the standard method. The primers used to amplify TOB1 (GenBank accession No.: NM_005749) mRNA were 5′-ATCAGTGTCCAGCTCTCCATC-3′ (sense) and 5′-CCATAGGCTGCAAACACATC-3′ (antisense). ACTB was used as the internal control, primer sequences were 5′-ACTCTTCCAGCCTTCCTTCC-3′ (sense) and 5′-CAT ACTCCTGCTTGCTGATCC-3′ (antisense). The PCR products were separated electrophoretically in a 1.5% agarose gel and visualized by ethidium bromide staining.

Western-blot analysis

Whole cell lysates were prepared using RIPA buffer containing 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mmol/L Tris base, 10% protease inhibitor (Sigma, USA). Protein (100 μg) was separated by SDS-PAGE (12%), transferred to PVDF membranes (Millipore, USA), blocked with 5% defatted milk at 4°C overnight, and incubated at room temperature with the primary antibodies at a dilution of 1:250 and the corresponding secondary antibodies (Sigma, USA), for 1 h for each incubation, and finally visualized using an enhanced chemiluminescence detection system (Amersham, USA). Scion Image tool software (info@scioncorp.com) was used to analyze and quantify the scanned protein bands.

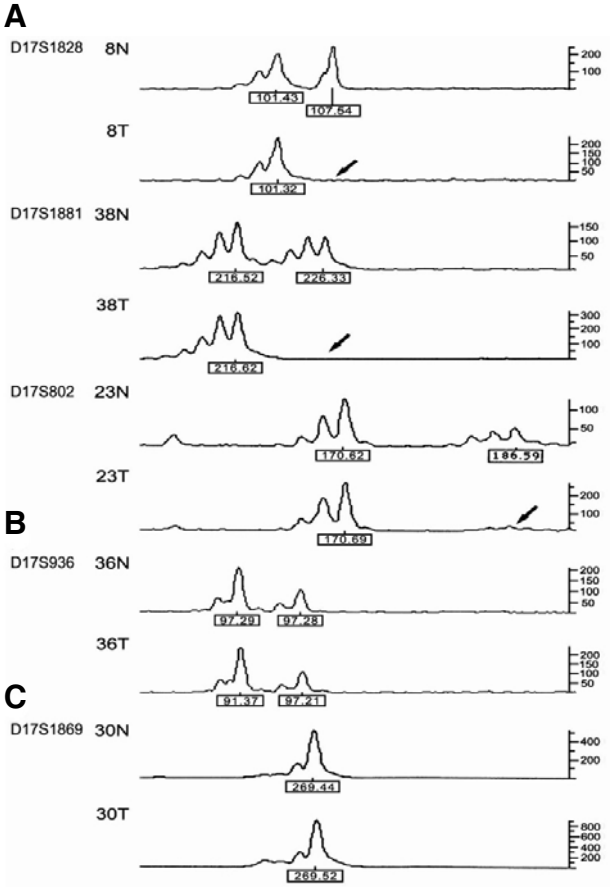

Fig. 1. Representative examples of (A) LOH, (B) retention of heterozygosity, and (C) homozygosity at several loci on chromosome 17 of paired normal (N) and tumor (T) gastric specimens. Arrows point to lost alleles. The microsatellite marker designations and sample numbers are indicated on the left side of the maps. The numbers beneath each peak indicate the length of the allele fragment.

GAPDH was used as a loading control.

Statistical analysis

Statistical comparisons were performed using the Fisher's exact test. A value of $P < 0.05$ was considered significant.

RESULTS

Identification of five LOH subregions, SR_1-SR_5, in GC

DNA from 45 primary GCs as well as their matched DNA controls was allelotyped using 36 microsatellite markers. These markers were distributed along R_1-R_3 to achieve sufficiently high resolution LOH mapping and define small areas of allelic loss. Microsatellite analysis revealed that 44 of 45 cases (98%) had partial terminal or interstitial deletions, including extensive deletions (5 cases, 11%, > 20 LOH loci), multiple deletions (10 cases, 23%, 10-20 LOH loci), a few deletions (26 cases, 59%, 2-9 LOH loci) and deletions at only one locus (3 cases, 7%). Representative LOH data are shown in Fig. 1. The allele losses were often multiple and discontinuous, with areas of LOH interspersed with areas of retention of heterozygosity. The so-called 'zebra' LOH pattern (discontinuous LOH) enabled the determination of minimal subregions of overlap with LOH frequencies of more than 20%. The deletion maps (Figs. 2A, 2B, and 2C) displayed five distinct minimal overlapping subregions (SR_1-SR_5) of LOH in R_1-R_3.

In our previous study, at 17p12-13.3 (R_1), the common overlapping deletion had been identified as approximately 30 cM between D17S831 and D17S921 (Yu et al., 2008). In this study, allelic losses at all 18 markers in R_1 were observed in 43 GCs with LOH frequencies of 21-59%. Forty-three of 45 GCs (96%) displayed allelic losses at least at one locus, and 29 (64%) at two or more loci. The highest LOH frequency we previously found is marker D17S831 with a LOH frequency of 61%. Among 29 GCs exhibiting LOH at two or more sites, LOH fragments of 9 cases (31%) were overlapped between D17S831 and D17S1828 at chromosomal band 17p13.3 (SR_1, 3.42 cM, Fig. 2A), and between D17S938 and D17S1353 at chromosomal band 17p13.2-p13.1 (SR_2, 1.08 cM, Fig. 2A).

For 17q21.3-22, the overlapping deletion (R_2) had previously been estimated to be approximately 11 cM between D17S1868 and D17S787. Here, we found that 20 out of 45 GCs (44%) exhibited allelic losses in at least one locus, and 13 (29%) in two or more loci. The LOH frequencies ranged from 3% to 36% with the highest frequency at 36% in D17S752. Among 13 GCs with LOH in two or more sites, LOH fragments were defined by markers D17S1869-D17S1820 at 17q21.33 (SR_3, 2.01 cM, Fig. 2B) in 4 cases, and by markers D17S1877-D17S787 at 17q21.33-q22 (SR_4, 0.54 cM, Fig. 2B) in 4 cases (31%).

In 17q25.3 (R_3, 23 cM), 33 of the 45 GCs (73%) showed LOH in at least one locus, and 23 (51%) in two or more loci. LOH frequencies ranged from 13% to 40% with the highest frequency observed at 40% in D17S1806. LOH fragments of 5 (22%) of 23 cases showing LOH at two or more sites were flanked by markers D17S1806 and D17S1822 at chromosomal band 17q25.3 (SR_5, 2.45 cM, Fig. 2C).

Five distinct minimal overlapping subregions of LOH (SR_1-SR_5, ranging from 0.54-3.42 cM) were identified in the three larger intervals (Table 2). In these subregions, HIC1 is located in SR_1, and TOB1 in SR_3.

Downregulated expression and aberrant methylation of the HIC1 promoter in GC

To detect HIC1 expression in GC, immunohistochemical evaluation of HIC1 was performed in 26 surgically resected primary GCs and in tissue microarrays of 80 GCs. Immunostaining of the HIC1 protein was observed primarily in the plasma and the nucleus of the parietal cells of the gastric mucosa in the non-cancerous gastric tissue (Fig. 3C). Interestingly, we observed a loss or marked reduction in immunostaining of the HIC1 protein in 91 of the 106 cases (86%). However, we found no significant correlation between HIC1 protein levels and clinicopathological features (i.e., age, gender, grade, stage and lymph node metastatic status) of GC patients (data not shown). Notably, of the 26 surgically resected primary GCs, 24 (92%) had marked reduction or loss of the HIC1 protein, indicating that GC is associated with downregulated HIC1 protein expression.

To investigate whether downregulation of HIC1 expression in GCs resulted from methylation of the HIC1 promoter region, the methylation status of the gene was analyzed by MSP of the 4 GC cell lines and 30 surgically resected primary GCs. It was expected that specimens containing methylated (M) DNA at the primer binding sites will produce PCR amplicons (95 bp) with the methylated primer set while unmethylated (U) DNA will yield PCR products (118 bp) with the unmethylated primer set. Samples with methylated and unmethylated alleles will yield PCR products with both primer sets. MSP of the GC cell lines and

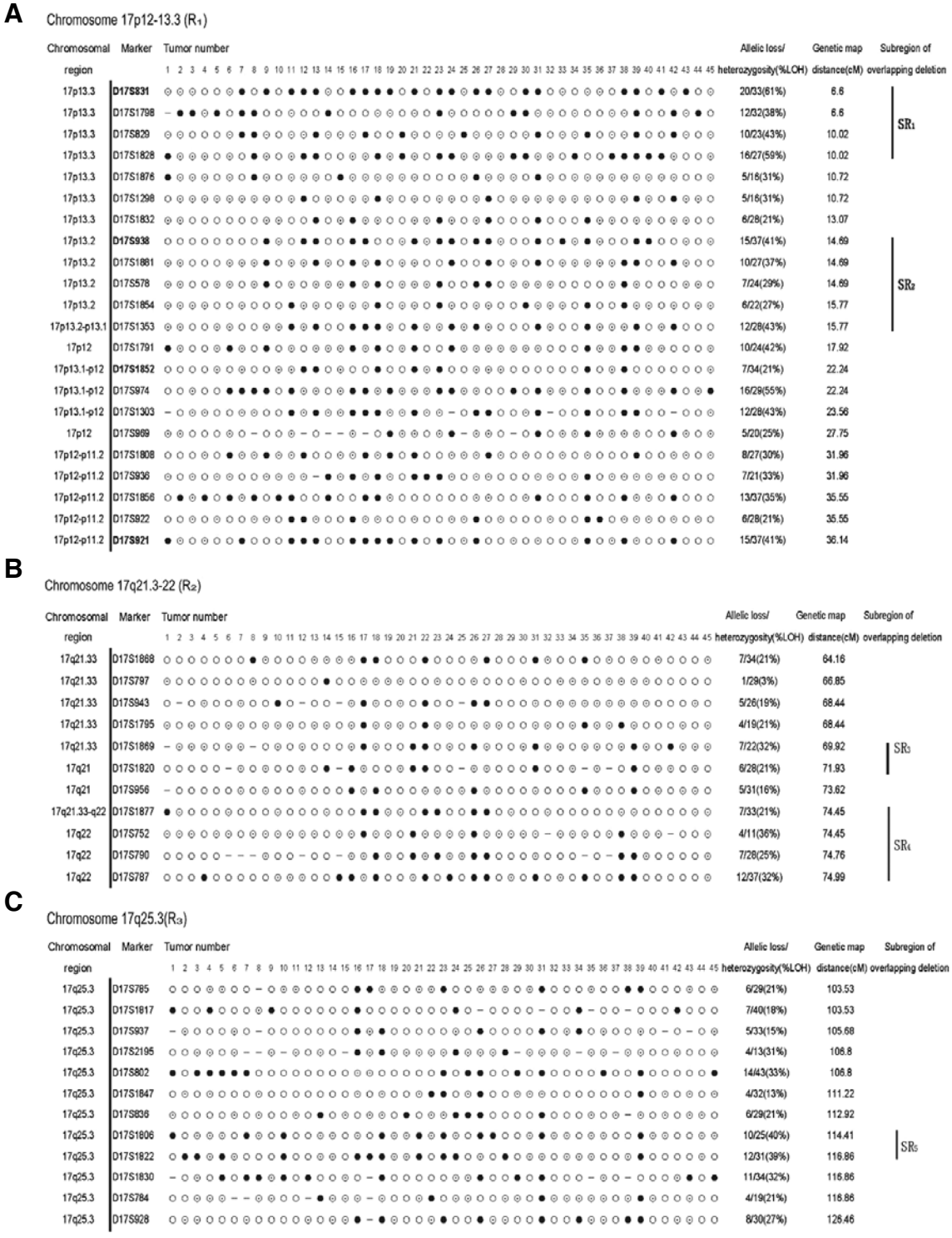

Fig. 2. Deletion maps of chromosome: (A) 17p12-13.3, (B) 17q21.3-22, and (C) 17q25.3 in 45 GCs. ●: loss of heterozygosity, ○: retention of heterozygosity, ⦿: homozygote, —: not available. Vertical bars on the right correspond to the five minimal overlapping deleted subregions (SR_1--SR_5).

tissue samples revealed monoallelic (one band for the methylated product and one band for the unmethylated product) and biallelic (one band for the methylated product) HIC1 promoter methylation (Figs. 3A and 3B). Methylation of the HIC1 promoter was observed in all GC cell lines (Fig. 3A) and in 26 of 30 (87%) primary GCs (Fig. 3B) examined. Among the samples with HIC1 promoter methylation, all 4 GC cell lines (100%) and 10 of 26 primary GCs (38%) had biallelic methylation, whereas

Table 1. Primer sequence for microsatellite markers

Marker name	Forward	Reverse
D17S831	CGCCTTTCCTCATACTCCAG	GCCAGACGGGACTTGAATTA
D17S1798	CAGTGAAATGCAATGTGATG	ATGCCCAGCCTGTGTTAG
D17S829	CTAGGGGAGCGTGTTAGCAT	TGTAAGACTGAGGCTGGAGC
D17S1828	TGCACTCACAGATTTGCC	TTAAGCCAGTTCGGATTTG
D17S1876	AGCTGCTTCTGCAAAGATG	TACAAGTCCTGGGCCAC
D17S1298	TTCACACTTTTAGTGTTGGTGG	TGACCTGAGTTTGACTGGGT
D17S1832	ACGCCTTGACATAGTTGC	TGTGTGACTGTTCAGCCTC
D17S938	GGACAGAACATGGTTAAATAGC	ATGCTGCCTCTCCCTACTTA
D17S1881	CCCAGTTTAAGGAGTTTGGC	TAGGGCAGTCAGCCTTGTG
D17S578	CTATCAATAAGCATTGGCCT	CTGGAGTTGAGACTAGCCT
D17S1854	TTTGGGAGGTCACAGACATTC	CCTTGCTCTTAGGATTTGAGGA
D17S1353	CTGAGGCACGAGAATTGCAC	TACTATTCAGCCCGAGGTGC
D17S1791	AGCTTTTGGTCAACCTG	GGGTGGGTGGAGTTAC
D17S1852	TACAGTTTCTTGTGTGCCC	AGTAACTCTGAGGACTTGCTCAT
D17S974	AGACCCTGTCTCAGATAGATGG	TAAAATAGAAAGTGCCCCTCC
D17S1303	CTCTCCAAGGCTCACTCAAA	TGGTCTTTTTCCATTCCAAA
D17S969	ATCTAATCTGTCATTCATCTATCCA	AACTGCAGTGCTGCATCATA
D17S1808	TCATTGTGTTTGACATTGGA	GCATAAACCCTCAGCACCTA
D17S936	ATTTGAAACCACAACAGCA	AGGTATATGCCCACCCC
D17S1856	AGCTGAGATGGTGCCACTG	TCATGGAAGACAATTTTGCG
D17S922	AGGCCTTCTAAGTTCAATGC	GCACCACCTTTAGAAGTGTT
D17S921	GCAACATATTACATGGGGTG	CTTTATGGCCACCATAATCA
D17S1868	GCAAAATCCATCCAAGC	AGTCAAATTCAGCCTGGG
D17S797	AGTGCTGTATAAATGTGAAGGG	GTGGCATGAACAAAGACTTG
D17S943	TGACTGTAGCCCTCACCC	TTCCAACCATCGTTTGTGTA
D17S1795	AGTGCCAGAGATATACCGTG	GTCTGCAAGGCAAGTTGTC
D17S1869	TCATACAGATGCACCCGC	ATTGGAAGGTGGCCCC
D17S1820	CATGAGGTCTTCCAGAAGG	AACACACTTGCTGATGTGC
D17S956	AGGGGGTGTAAGGCTG	CCCAAGTTGTGTGTATGTG
D17S1877	GCTCACGGTGCTCCTGTACT	TCTTGGCAGACTCTGACGTG
D17S752	GGTGACAGAGTGAGATCCTGTC	CCCATGTTTGAGAGATCAAACCTT
D17S790	AGGAAAAATGAGTGGACCAT	AGCTGGGTTATTGTTTTTCC
D17S787	TGGGCTCAACTATATGAACC	TTGATACCTTTTTGAAGGGG
D17S785	CTGGAGAGTGAAAATGGTAGGAG	GCCAACCTGAAAACTAAATCTTAATC
D17S1817	GGGGTGATGAAAGCAATCTG	CTTGAACCTGGGAAGTGGA
D17S937	CATGGAGGGACTTGCG	TTCCCAGAACCCGGTTT
D17S2195	AAATGTCACTTTGCCAGAGG	GCAAACCCTAAATGCTCAAA
D17S802	GCCACCTGCCCCTCAA	CTGCCAGCAGAGGCCA
D17S1847	GATCACCAGGAACACCC	TCTTCAGAGCTTGCCAG
D17S836	GTGCATCTGGCTTCATGTTC	AGCTTCCACACACTAGGTGC
D17S1806	GATGTGCTTATTTGAAACCTGC	TGTAACGTCCACCAGCAGAG
D17S1822	CAGGCATCTGTAATGGACCC	AACCGAGCCTAGGACTCC
D17S1830	GGGAGACTCCATCTCAAAA	ATTTGCCCATCTGCTTT
D17S784	GAGTCTCCTAAATGCTGGGG	AGCTCCTGCACAGTTCTTAAATA
D17S928	TAAAACGGCTACAACACATACA	ATTTCCCCACTGGCTG

Underlined markers are those used in our previous work (Yu et al., 2008).

Table 2. Candidate genes in subregions

Sub-region	Genes	Markers
SR_1	OVCA1, OVCA2, HIC1, ROX/MNT	D17S831, D17S1798, D17S829, D17S1828
SR_2	P53	D17S938, D17S1881, D17S578, D17S1854, D17S1353
SR_3	nm23-H1, nm23-H2, TOB1	D17S1869, D17S1820
SR_4	None	D17S1877, D17S752, D17S790, D17S787
SR_5	None	D17S1806, D17S1822

16 of the 26 primary GCs (62%) had monoallelic methylation. The incidence of HIC1 methylation was significantly higher in GCs than in the corresponding noncancerous gastric tissues ($p < 0.05$). Among the 24 primary GCs lacking or having decreased HIC1 protein, 18 (75%) showed methylation of the HIC1 gene, indicating that abnormal methylation of HIC1 is associated with downregulated gene expression, and that HIC1 may be a target for epigenetic inactivation in gastric carcinogenesis. However, since HIC1 methylation was not observed in six cases that showed loss or decreased HIC1 protein expression, there may be other pathways for inactivation of HIC1 in gastric tumorigenesis. In addition, since HIC1 methylation was observed in 19 of 30 (63%) adjacent nonmalignant gastric tissues, of which 17 (89%) showed monoallelic methylation, it is possible that methylation is an early event in gastric tumorigenesis.

Downregulated expression of the TOB1 protein in GC cells and reduced ratio of unphosphorylated to phosphorylated TOB1 protein in differentiated GC cell lines

To determine a potential link between TOB1 expression and GC, TOB1 mRNA expression was examined in the 4 GC cell lines and 30 surgically resected primary GCs by RT-PCR. No marked alterations in TOB1 mRNA levels were found in GC cells relative to HEK 293 cells (Fig. 4A), as well as in GC tissues compared to their corresponding adjacent noncancerous tissues (Fig. 4B).

We then searched for potential changes in TOB1 protein expression in GC by immunohistochemical analysis of 97 GC tissue microarrays that included 78 GCs and 19 surgically resected primary GCs. Interestingly, loss or notably decreased immunostaining of TOB1 protein in cell plasma was observed in 75% (73/97) of GCs and in 19% (11/59) of noncancerous tissues ($P < 0.01$; Fig. 4C). No significant correlation was observed between TOB1 protein expression and clinicopathological features (i.e., age, gender, grade and stage) of GC patients (data not shown).

To complement the immunohistochemistry analysis for TOB1 protein expression in GC samples, Western blot analysis was performed on the 4 GC cell lines and HEK 293 cells. Two migration patterns of TOB1 (Fig. 5A), corresponding to the unphosphorylated and phosphorylated forms, were observed in the 4 GC cell lines and HEK 293 cells. Further analysis of the blot revealed a general reduction in the total TOB1 protein in the differentiated GC cell lines (i.e., in SGC-7901, BGC-823 and MGC-803) relative to HEK 293 cells (Fig. 5B). In addition, there was a general decrease in the unphosphorylated versus the phosphorylated form of the TOB1 protein in the differentiated GC cell lines, suggesting that reduced expression of TOB1 might be related to gastric carcinogenesis at least in a certain

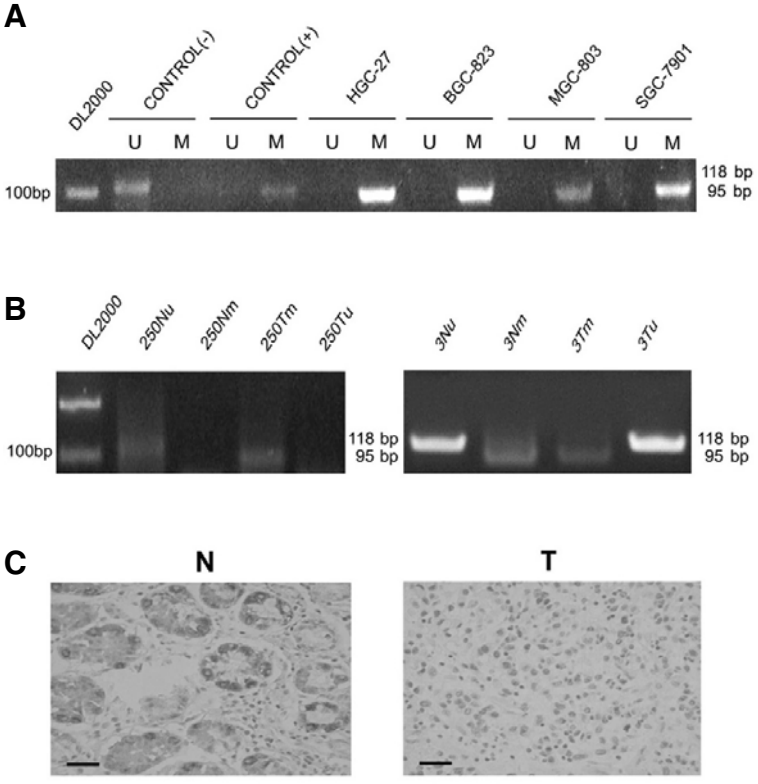

Fig. 3. MSP of HIC1 products in GC cell lines and primary GCs and HIC1 protein expression in GCs. (A) MSP analysis of HIC1 in four GC cell lines showed methylated alleles of HIC1. (B) MSP analysis of representative GC samples revealed either methylated (tumor #250) or both methylated and unmethylated (tumor #3) alleles of HIC1. U, results with primers specific for unmethylated sequences; M, results with primers specific for methylated sequences; control (+), DNA from normal peripheral leukomonocytes treated with Sss1 CpG methylase used as positive control; control (-), DNA from normal peripheral leukomonocytes. (C) Immunohistochemical analysis of HIC1 protein of formalin-fixed, paraffin-embedded specimens revealed that the HIC1 protein is either absent or expressed at very low levels in gastric tumor tissues (T) while strong expression was observed in corresponding non-cancerous gastric tissue (N). Bar = 50 μm.

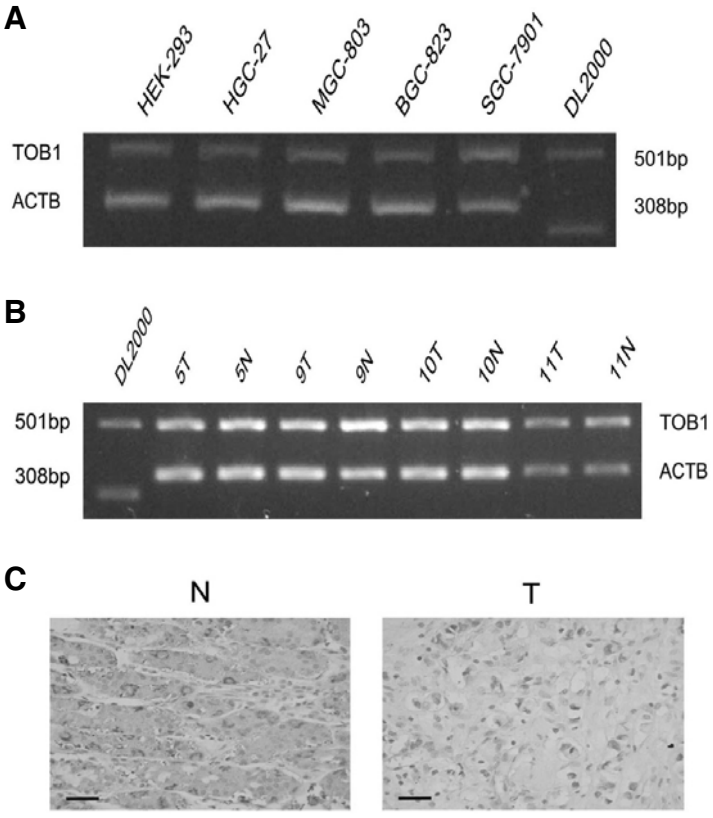

Fig. 4. TOB1 mRNA and protein expression in GC. RT-PCR analysis of TOB1 mRNA in (A) four GC cell lines and in (B) primary GCs. T and N indicate cDNA from a cancerous and non-cancerous gastric tissue, respectively. (C) Immunohistochemical analysis of TOB1 expression in formalin-fixed, paraffin-embedded specimens revealed significantly reduced TOB1 expression in cancerous (T) compared to noncancerous (N) gastric tissues. Bar = 50 μm.

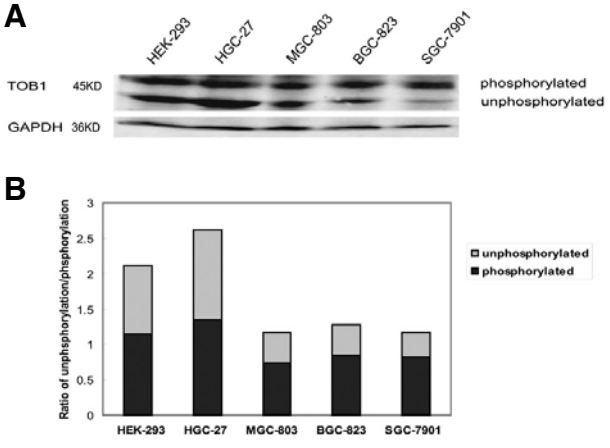

Fig. 5. Western-blot analysis of TOB1 protein expression in GC cell lines and HEK 293 cells. (A) Phosphorylated and unphosphorylated TOB1 proteins were observed in four GC cell lines. HEK 293 cells were used as a control. (B) Analysis of the ratio of unphosphorylated and phosphorylated TOB1 protein in the 4 GC cell lines using the Scion Image tool software (info@scioncorp.com). Data shown represent the results from three independent experiments.

group of GCs.

Combined downregulation of HIC1 and TOB1 proteins is common in GC

The expression of HIC1 and TOB1 proteins was simultaneously evaluated on tissue microarrays containing 78 GC samples and 17 surgically resected primary GCs. Interestingly, loss or marked reduction in both HIC1 and TOB1 proteins was observed in 67% (52/78) of tissue microarray samples and in 65% (11/17) of surgically resected primary GCs (data not shown). These results indicate that combined downregulation of HIC1 and TOB1 proteins is common in GC.

DISCUSSION

Using 36 microsatellite markers in R_1-R_3 on chromosome 17, we identified for the first time the patterns of LOH at high resolution and five distinct minimal overlapping subregions of LOH ranging from 0.54 to 3.42 cM (SR_1-SR_5), which are described here for the first time. A considerable proportion of tumors showed this so-called 'zebra' discontinuous LOH pattern, for which one possible explanation is that chromosome homologs show LOH at different non-overlapping subregions or multiple genes may be targets for LOH in a defined chromosomal region. The high frequency of LOH in these subregions indicates the existence of putative tumor suppressors here.

In the present study, we demonstrated that most (86%) of the primary GCs that we examined had none or barely detectable levels of the HIC1 protein, indicating that down-regulation of HIC1 expression was associated with the pathogenesis of primary GC. The downregulation of HIC1 protein may arise from intensive promoter methylation, since both methylation of HIC1 gene and the absent or low level of protein expression were seen in 75% of surgically resected primary GCs. The patterns of monoallelic methylation associated with a partial loss of HIC1 expression and biallelic methylation, together with a marked loss in HIC1 expression, were seen in these GCs. The concomitant loss or decreased expression and promoter methyla-

tion of HIC1 indicated that HIC1 may be silenced by aberrant DNA methylation, and could be a target for inactivation by epigenetic events in gastric tumorigenesis. According to the classical Knudson hypothesis, both alleles of a TSG have to be disrupted to achieve functional inactivation. Until recently, the two recognized mechanisms for TSG inactivation were intragenic mutation and loss of chromosomal material (LOH and homozygous deletion) (Knudson et al., 2002). Increasing evidence indicates that aberrant methylation of promoter CpG islands of TSG may also be one of the 'hits' required for TSG loss of function (Herman et al., 2003). In the present study, hypermethylation of HIC1 was accompanied by 17p13.3 LOH (SR_1), indicating that HIC1 aberrant methylation may have a role in GC progression by predisposing the SR_1 region to LOH and/or silencing of the gene. Whereas, the lack of detectable DNA methylation in six GCs with absent or decreased HIC1 protein may indicate additional mechanisms for HIC1 silencing other than the genetic and epigenetic mechanisms tested here.

It is worth noting that the methylation pattern for HIC1 has also been found in some non-cancerous samples. However, most cases displayed monoallelic HIC1 promoter methylation and the methylation incidence was much lower than that in GCs. It has been considered that promoter methylation in TSGs and tumor-related genes initially occurs in non-neoplastic gastric epithelia, increases with age, and ultimately silences gene function to constitute a field-defect that may predispose tissues to GC development (Tamura et al., 2004; Tokumaru et al., 2003). This methylation may also occur due to the infiltration of neoplastic or inflammatory cells in the surrounding tissue. Our data indicate that aberrant methylation of the HIC1 promoter may be an early epigenetic event during the multistep process of neoplastic progression in GC.

Decreased expression of TOB has been found in lung, thyroid and breast tumors and in squamous carcinoma of the skin (Ito et al., 2005; Iwanaga et al., 2003; Klebig et al., 2005; Park et al., 2006). Our IHC analysis of GC tissue samples revealed a loss of or a significant decrease in TOB1 protein levels in 75% of GCs, indicating that down-regulation of TOB1 protein expression may be associated with GC. Western-blot analysis in four GC cell lines had found that the total TOB1 protein level was decreased in three GC cell lines (SGC-7901, BGC-823 and MGC-803), accompanied by a decrease in the ratio of unphosphorylated to phosphorylated TOB1. Because phosphorylated TOB has been reported to be the inactive form of TOB protein (Iwanaga et al., 2003), we hypothesized that at least two discrete mechanisms seem to be involved in inactivation of the function of TOB in gastric carcinogenesis: a decrease in TOB expression and unphosphorylated TOB. However, our data also showed that, compared with HEK 293 cells, total TOB1 protein levels increased and the ratio of phosphorylated to unphosphorylated TOB1 protein did not change in HGC-27 cells (undifferentiated). One possible explanation for these findings may be that different genetic pathways exist for differentiated and undifferentiated histological types of GCs (Tahara et al., 2004; Tamura et al., 2002). As the significance of the modification of TOB1 by phosphorylation remains unknown, our observations need to be confirmed in further studies involving immunohistochemical analysis of the phosphorylation status of TOB1 in GC patients to understand the relationship between GC and the post-translational modification of TOB1.

In addition, our data also showed the concordant down-regulation of HIC1 and TOB1 protein expression in 67% of samples in GC tissue microarrays and 65% of surgically resected primary GCs. These results indicate that the concurrent dysfunction of HIC1 and TOB1 may be important in gastric carcinogenesis, although as yet there is no evidence to support this hypothesis. The results should therefore be considered as preliminary, and need to be investigated further to confirm the existence of functional cooperation between these two proteins.

In summary, we narrowed the three regions of LOH and identified five relevant small overlapping subregions of allele loss, indicating the existence of potential TSGs involved in GC on chromosome 17. Combined molecular and pathological investigations revealed that down-regulation of HIC1 and TOB1 protein expression, and silencing of both genes by genetic and/or epigenetic pathways may have important roles in gastric carcinogenesis. Further data are needed to demonstrate the role of other candidate TSGs (such as OVCA1, OVCA2 and ROX/Mnt) in SR_1 and investigate SR_4-SR_5 for major novel TSGs on chromosome 17 in gastric tumorigenesis.

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