

*Advances in Brief***Fez1/Lzts1 Alterations in Gastric Carcinoma<sup>1</sup>**

Andrea Vecchione,<sup>2</sup> Hideshi Ishii,<sup>2</sup>  
Yih-Horng Shiao, Francesco Trapasso,  
Massimo Rugge, Joseph F. Tamburrino,  
Yoshiki Murakumo, Hansjuerg Alder,  
Carlo M. Croce, and Raffaele Baffa<sup>3</sup>

Kimmel Cancer Center, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pennsylvania 19107 [A. V., H. I., F. T., J. F. T., Y. M., H. A., C. M. C., R. B.]; Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, NIH, Frederick, Maryland 21702 [Y.-H. S.]; and Department of Pathology, University of Padova, Padova 35126, Italy [M. R.].

**Abstract**

**Purpose:** Loss of heterozygosity (LOH) involving the short arm of chromosome 8 (8p) is a common feature of the malignant progression of human tumors, including gastric cancer. We have cloned and mapped a candidate tumor suppressor gene, *FEZ1/LZTS1*, to 8p22. Here we have analyzed whether *FEZ1/LZTS1* alterations play a role in the development and progression of gastric carcinoma.

**Experimental Design:** We examined Fez1/Lzts1 expression in 8 gastric carcinoma cell lines by Western blot, and in 88 primary gastric carcinomas by immunohistochemistry. Twenty-six of these 88 primary gastric carcinomas were also microdissected and tested for LOH at the *FEZ1/LZTS1* locus and for mutation of the *FEZ1/LZTS1* gene. Furthermore, we studied the *FEZ1/LZTS1* gene regulation and transcriptional control and the methylation status of the 5' region of the gene in all 8 gastric carcinoma cell lines.

**Results:** Fez1/Lzts1 protein was barely detectable in all of the gastric cancer cell lines tested and was absent or significantly reduced in 39 of the 88 (44.3%) gastric carcinomas analyzed by immunohistochemistry, with a significant correlation ( $P < 0.001$ ) to diffuse histotype. DNA allelotyping analysis showed allelic loss in 3 of 17 (18%) and microsatellite instability in 4 of 17 (23.5%) cases informative for *D8S261* at the *FEZ1/LZTS1* locus. When we compared the presence of LOH with Fez1/Lzts1 expression, we found loss of protein expression in all three of the tumors with allelic imbalance at *D8S261*. A missense mutation was de-

tected in one case that did not express Fez1/Lzts1. Hypermethylation of the CpG island flanking the Fez1/Lzts1 promoter was evident in six of the eight cell lines examined as well as in the normal control.

**Conclusions:** Our findings support *FEZ1/LZTS1* as a candidate tumor suppressor gene at 8p in a subtype of gastric cancer and suggest that its inactivation is attributable to several factors including genomic deletion and methylation.

**Introduction**

Gastric cancer is the second most common malignant tumor worldwide, with a much higher incidence in Asian than in Western countries (1). The international TNM<sup>4</sup> classification (2) and Lauren's system (3) classify gastric cancer into two distinct histological types: intestinal (well differentiated) and diffuse (poorly differentiated). Genetic differences have been observed in intestinal and diffuse gastric cancer, suggesting two different pathways of carcinogenesis (4). Allelotyping studies of solid tumors, determined by LOH, have shown genomic alterations in gastric cancer at specific chromosomal regions, including 3p14–21, 8p21–23, 11p11–21, and 17p11–21 (5–9). Recently, we have cloned a novel candidate tumor suppressor gene, *FEZ1/LZTS1* (hereafter called *FEZ1*), on chromosome 8p22 (10). The *FEZ1* gene encodes a putative  $M_r$  67,000 leucine-zipper protein with similarities to the cAMP-responsive Atf-5 DNA binding protein (11). Immunohistochemistry has been a valuable tool in evaluating the expression of different tumor suppressor genes, such as *p53* and *FHIT*, in gastric carcinoma (12, 13). In this report, we raise anti-Fez1 polyclonal antibody and analyze 88 cases of gastric adenocarcinoma to assess Fez1 expression. Results indicate that loss of Fez1 protein is a frequent event in gastric cancer, and reduction of its expression significantly correlates with diffuse gastric carcinoma. A somatic missense mutation in a *FEZ1* allele has been found in a case negative for Fez1 expression. To gain insight into *FEZ1* gene regulation and transcriptional control, we examined the genomic organization, identified a transcriptional start site, and analyzed a positive regulatory region of *FEZ1* transcription. In addition, we analyzed the methylation status of the region flanking the *FEZ1* promoter in gastric cancer cells. Our data suggest that altered Fez1 expression plays a role in the progression of a subset of carcinomas of the stomach, and that many factors, including DNA deletion, point mutations, and methylation seem to be involved in switching off Fez1 expression.

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<sup>2</sup> A. V. and H. I. contributed equally to this study.

<sup>3</sup> To whom requests for reprints should be addressed, at Kimmel Cancer Center, 1015 Walnut Street, Suite 1102A, Philadelphia, PA 19107. Phone: (215) 955-9072; Fax: (215) 923-1884; E-mail: R\_Baffa@lac.jci.tju.edu.

<sup>4</sup> The abbreviations used are: TNM, Tumor-Node-Metastasis; LOH, loss of heterozygosity; *LZTS1*, leucine zipper putative tumor suppressor gene 1; MI, microsatellite instability; BAC, bacterial artificial chromosome; TSS, transcriptional start site.

## Materials and Methods

**Cell Lines and Tissue Samples.** Eight human gastric carcinoma derived cell lines (Ags, KATO III, NCI-N87, RF1, RF48, SNU1, SNU5, and SNU16), transformed human kidney 293 cells, and cervical cancer HeLaS3 cells were obtained from the American Type Culture Collection and maintained in the recommended medium. *FEZ1* cDNA was ligated in a pcDNA vector (Invitrogen, Carlsbad, CA), and after confirming the DNA sequence, DNA transfection was performed with the Gene Porter reagent (Gene Therapy, Inc., San Francisco, CA) according to the manufacturer's instructions. Eighty-eight formalin-fixed, paraffin-embedded specimens of primary adenocarcinomas of the stomach, including 47 diffuse-type, 23 intestinal-type, and 18 unclassified tumors were obtained from patients who underwent radical and partial gastrectomy as described previously (14). The tumors were classified histologically and staged according to Lauren's classification (3) and to the TNM classification of malignant tumors (2), respectively.

**Immunoblot Analysis, Immunoprecipitation, and Recombinant Proteins.** Protein extraction and immunoblot analyses were performed as described (15). Briefly, the protein concentration was measured with the Protein Assay reagent (Bio-Rad Laboratories, Melville, NY). For immunoblot analysis, 50–100  $\mu$ g of proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% nonfat dry milk, the membrane was incubated with a 1:1000 dilution of rabbit anti-Fez1 polyclonal antibody. The anti-Fez1 antibody was raised in rabbits against glutathione *S*-transferase-fusion Fez1 protein corresponding with nucleotides 1–1128, which was expressed in *Escherichia coli* and purified with a glutathione column (Amersham Pharmacia, Piscataway, NJ). Mouse monoclonal anti-V5 antibody (Invitrogen) was also used. After incubation with horseradish peroxidase-conjugated secondary antibodies, membranes were subjected to the chemiluminescence detection system (Amersham Pharmacia) with X-ray film. After stripping, the blots were reprobated with anti  $\beta$ -actin (Sigma Chemical Co.-Aldrich, St. Louis, MO), followed by incubation with antimouse secondary antibody. Immunoprecipitation was performed as described (15). Cell lysates corresponding to 1 mg of the proteins were incubated with the antibody (1  $\mu$ g), after first being precleared with the protein A/G Sepharose (Amersham Pharmacia). Samples were precipitated with the protein A/G Sepharose and then subjected to SDS-PAGE for immunoblot analysis after boiling with Laemmli buffer. Recombinant Fez1 proteins were synthesized using an *in vitro* transcription/translation system (Amersham Pharmacia) with *FEZ1*/pcDNA vector.

**Immunohistochemistry.** Routine deparaffinization of all sections mounted on positively charged slides was carried out according to the standard procedures, followed by rehydration through serial ethanol treatments. The slides were immersed in citrate buffer [0.01 M sodium citrate (pH 6.0)] and heated in a microwave oven at 600 W (three times for 5 min each) to enhance antigen retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 30 min. Sections were immunostained with a 1:1000 dilution of the anti-Fez1 antibody overnight at room temperature. The primary antibody was omitted and replaced with preimmune serum in the negative

controls. Sections were reacted with biotinylated antirabbit antibody and streptavidin-biotin-peroxidase (Histostain-SP kit; Zymed Laboratories, San Francisco, CA). Diaminobenzidine was used as a chromogen substrate. Finally, sections were washed in distilled water and weakly counterstained with Harry's modified hematoxylin. All sections were examined independently by three investigators (A. V., R. B., and M. R.), and complete agreement was reached for Fez1 positivity and negativity. Any positive reaction was semiquantified into four groups: +, 96–100% Fez1-positive cells; +/-, 51–95% Fez1-positive cells; -/+, 2–50% Fez1-positive cells; and -, the tumors in which >98% of cells did not express Fez1. Associations of Fez1 expression with clinicopathological parameters were computed using a two-tailed  $\chi^2$  statistic or Fisher's exact test where appropriate. Probability of <0.05 was considered statistically significant.

**Microdissection and DNA Extraction.** We were able to process 26 of the 88 primary tumors for DNA analysis. Serial 5- $\mu$ m, formalin-fixed, paraffin-embedded sections from these 26 primary gastric cancers and matched normal tissues were subjected to microdissection. This was followed by deparaffinization and DNA extraction as described previously (14). DNA was quantified in a fluorometer using Pico green dsDNA quantitation reagent (Molecular Probes, Eugene, OR).

**LOH Study and Mutation Analysis.** PCR amplifications using 5' fluorescence-labeled primers for microsatellite loci (Research Genetics, Huntsville, AL) with tumor and normal template DNAs were performed as reported (10). MI was interpreted when a tumor sample showed a novel abnormal peak when compared with the corresponding normal control DNA (8). Mutation was analyzed by direct sequencing of PCR fragments with microdissected DNA as a template. Thirteen sets of primers covering the entire open reading frame (exons 1–3) were used for PCR in a 20- $\mu$ l reaction on a 96-well plate with a cycle of 94°C for 2 min; followed by 35 cycles of 94°C for 20 s, 62°C for 15 s, and 72°C for 1 min; and a cycle of 72°C for 1 min. The primer sequences are available upon request. The PCR products were purified with the QIAquick 96 PCR purification kit by BIO ROBOT 9600 (Qiagen, Valencia, CA). After measuring the DNA concentration with Pico green reagent (Molecular Probes), the samples were subjected to sequencing reactions and analysis by using the Applied Biosystems Prism BigDye terminator reaction chemistry on a Perkin-Elmer Gene Amp PCR system 9600 and the Applied Biosystems Prism 377 DNA sequencing system.

**FEZ1 Promoter Identification and Analysis.** By screening a human cDNA library (Clontech, Palo Alto, CA) and performing rapid amplification of cDNA ends 5' amplification using a Marathon kit (Clontech), we obtained the full-length *FEZ1* RNA. To determine the TSS, primer extension was performed as described (16), with minor modifications. BAC clones, including the *FEZ1* 5' region, were isolated by a PCR-based strategy from a normal human genomic BAC library (Research Genetics). BAC DNAs were sequenced after extraction with Maxi-prep columns (Qiagen). The 1.8-kb *HindIII* fragment (nucleotide position, -1821 to +335, counted from the TSS) containing the *FEZ1* 5' region was isolated from the BAC DNA by *HindIII* digestion and ligated to a luciferase reporter pGL3 basic vector (Promega Corp., Madison, WI).

Using unidirectional exonuclease III digestion as described (15), we performed systematic promoter deletions of human *FEZ1*. Additional mutants of the *FEZ1* 5' region were constructed by a PCR-based strategy with Pfu-turbo polymerase (Stratagene, La Jolla, CA). The promoter activity was analyzed by a luciferase vector assay kit (Promega).

#### Nuclear Protein Extraction and DNA Binding Assay.

Nuclear protein extraction and electrophoretic mobility shift assays were performed as described (15). The experiment was performed with double-stranded probes. Single-strand oligonucleotides were annealed and labeled by polynucleotide kinase reaction (Promega) with [ $\gamma$ - $^{32}$ P]ATP. Competitions were performed with 50-fold excess of nonradiolabeled, annealed, double-stranded oligonucleotides. In addition, poly(deoxyinosinic-deoxycytidylic acid) (Amersham Pharmacia, Piscataway, NJ) and sheared salmon sperm DNA (Sigma Chemical Co.-Aldrich) were used for competition.

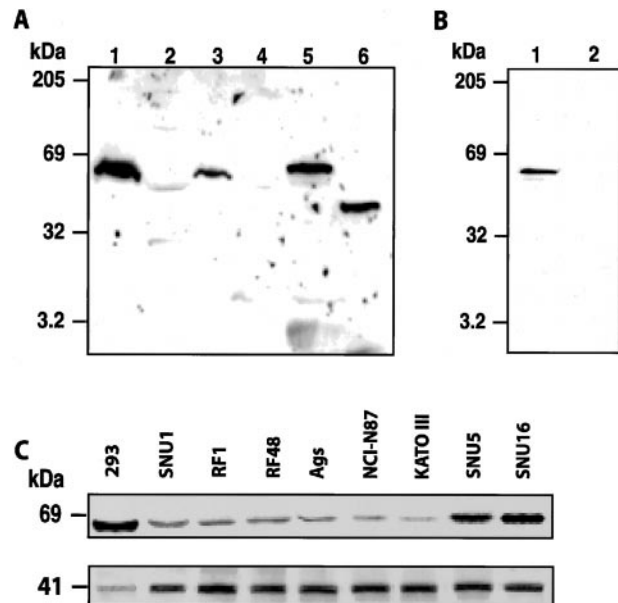
**Southern Blot Analysis.** The degree of methylation of the *FEZ1* 5' region was investigated by separating genomic DNA that had been digested with *Bam*HI, *Bam*HI/*Not*I, and *Bam*HI/*Bss*HIII on a TAE 1% agarose gel and by transferring the DNA fragments to Hybond N+ nylon membranes (Amersham Pharmacia). A 1.7-kb *FEZ1* fragment corresponding to the *FEZ1* open reading frame and containing the *Bss*HIII and *Not*I sites included in the CpG island was used as a probe. DNA extraction and Southern blot analysis were carried out as described (15).

## Results and Discussion

#### Immunoblot Analysis of Fez1 Expression.

Immunoblot analysis showed that rabbit anti-Fez1 polyclonal antibody recognized recombinant full-length and COOH-terminal truncated Fez1 proteins and detected a  $M_r$  67,000 band in brain cell lysate (Fig. 1A). When cell lysate from HeLaS3 cells transfected with the V5 tag-fusion, full-length *FEZ1* cDNA was immunoprecipitated with the anti-Fez1 antibody, immunoblot analysis with anti-V5 tag antibody detected a  $M_r$  67,000 Fez1 protein in the anti-Fez1 precipitated sample but not in the preimmune serum-precipitated sample (Fig. 1B). Results showed that the anti-Fez1 antibody specifically recognized the  $M_r$  67,000 Fez1 protein. To assess Fez1 expression in gastric carcinoma-derived cell lines, immunoblot analysis with the anti-Fez1 antibody was performed. Results showed that a single band with reduced signal intensity corresponding to endogenous Fez1 protein expression was detected in six gastric cancer-derived cell lines (SNU1, RF1, RF48, Ags, NCI-N87, and KATO III), compared with two remaining cell lines (SNU5 and SNU16) or with Fez1-positive 293 cell lines as a control. The SNU5 and SNU16 cell lines showed lower levels of Fez1 protein than the control cells when the  $\beta$ -actin level was considered (Fig. 1C). We confirmed the results by repeated experiments using cell lines with five additional passages in culture.

**Immunohistochemical Analysis of Fez1 Expression in Primary Tumors.** The 88 gastric carcinoma specimens listed in Table 1 were assessed for Fez1 expression by immunohistochemical methods. Only sections containing portions of normal gastric mucosa, representing an internal positive control, were analyzed (Fig. 2A). Fez1 protein was uniformly undetectable



**Fig. 1** Immunoblot analysis of Fez1 protein. **A**, Western blot analysis of Fez1 protein using anti-peptide rabbit serum. One hundred  $\mu$ g (Lanes 1 and 2) and 50  $\mu$ g (Lanes 3 and 4) of proteins from normal human brain (Lanes 1 and 3) and normal human testis (Lanes 2 and 4) were loaded. *In vitro* translated full-length Fez1 protein (Lane 5) and *in vitro* translated truncated Fez1 protein lacking the COOH-terminal portion (Lane 6) were also loaded. **B**, immunoprecipitation using anti-Fez1 antibody. Fez1-negative HeLaS3 cells were transfected with the *FEZ1* cDNA. After immunoprecipitation with anti-Fez1 antibody (Lane 1) or with the preimmune normal rabbit serum (Lane 2), the HeLaS3 cell lysate was blotted and probed with the anti-V5 tag antibody. **C**, Western blot analysis of Fez1 expression. *Upper panel*, Fez1 protein expression in gastric carcinoma derived cell lines (Lanes 2–9), compared with 293 cells (Lane 1). *Lower panel*,  $\beta$ -actin expression for the same membrane. *Left* (all panels), molecular weight in thousands.

(–) in 12 of 88 specimens (13.6%). Eleven tumors (12.5%) showed a mixture of Fez1 positive and negative cells, composed of 2–50% positive proportion (–/+). Sixteen tumors (18.2%) showed 51–95% positive proportion (+/–). Overall, 30.7% of the primary tumors contained a fraction (2–95%) of tumor cells with absent Fez1 expression (–/+ or +/-), where 13.6% of the cases showed a uniform absence of detectable Fez1 expression (–). Fez1 immunostaining with 96–100% positive proportion (+) was detected in 49 of the specimens (56%). Results were consistent when different areas in the same tumor were tested by immunohistochemical and allelotyping analyses. Statistical analysis showed a significant correlation ( $P < 0.001$ ) between absence/reduction of Fez1 expression (–, –/+, and +/-) and diffuse-type carcinoma, compared with either intestinal or unclassified histotype. No correlation was found between Fez1 expression and the other clinicopathological parameters tested.

Immunohistochemical analysis indicated varying fractions of gastric cancer cells with absence or reduction of Fez1 expression in 44% of the cases, whereas immunoblot analysis with 8 gastric cancer cell lines showed consistently reduced Fez1 expression in each cell line through passages or cell divisions. These data suggest that *FEZ1* gene expression would be affected or regulated depending on tumor develop-



Table 1 Clinicopathological features and Fez1 expression

Associations of Fez1 expression with clinicopathological parameters were computed using a two-tailed  $\chi^2$  statistic or Fisher's exact test as appropriate. Probability of  $<0.05$  was considered statistically significant. There was a significant correlation ( $P < 0.001$ ) between absence or reduction of Fez1 expression (–, –/+, and +/-) and diffuse-type gastric carcinoma.

Fez1 expression <sup>a</sup>	No. of cases <sup>b</sup>	Stage <sup>c</sup>	Histotype <sup>d</sup>
(–)	12 (9 M, 3 F)	2 T <sub>1</sub> , 1 T <sub>2</sub> , 9 T <sub>3</sub>	9 diffuse, 3 intestinal
(–/+)	11 (4 M, 7 F)	2 T <sub>1</sub> , 2 T <sub>2</sub> , 6 T <sub>3</sub> , 1 T <sub>4</sub>	9 diffuse, 1 intestinal, 1 unclassified
(+/-)	16 (8 M, 8 F)	1 T <sub>1</sub> , 3 T <sub>2</sub> , 12 T <sub>3</sub>	12 diffuse, 3 intestinal, 1 unclassified
(+)	49 (34 M, 15 F)	4 T <sub>1</sub> , 18 T <sub>2</sub> , 25 T <sub>3</sub> , 2 T <sub>4</sub>	17 diffuse, 16 intestinal, 16 unclassified

<sup>a</sup> Any positive immunostaining was classified into four groups: +, 96–100% Fez1-positive cells; +/-, 51–95% Fez1-positive cells, –/+, 2–50% Fez1 positive cells; and –, 0–2% Fez1-positive cells.

<sup>b</sup> M, male; F, female.

<sup>c</sup> According to the TNM classification.

<sup>d</sup> According to Lauren's classification.

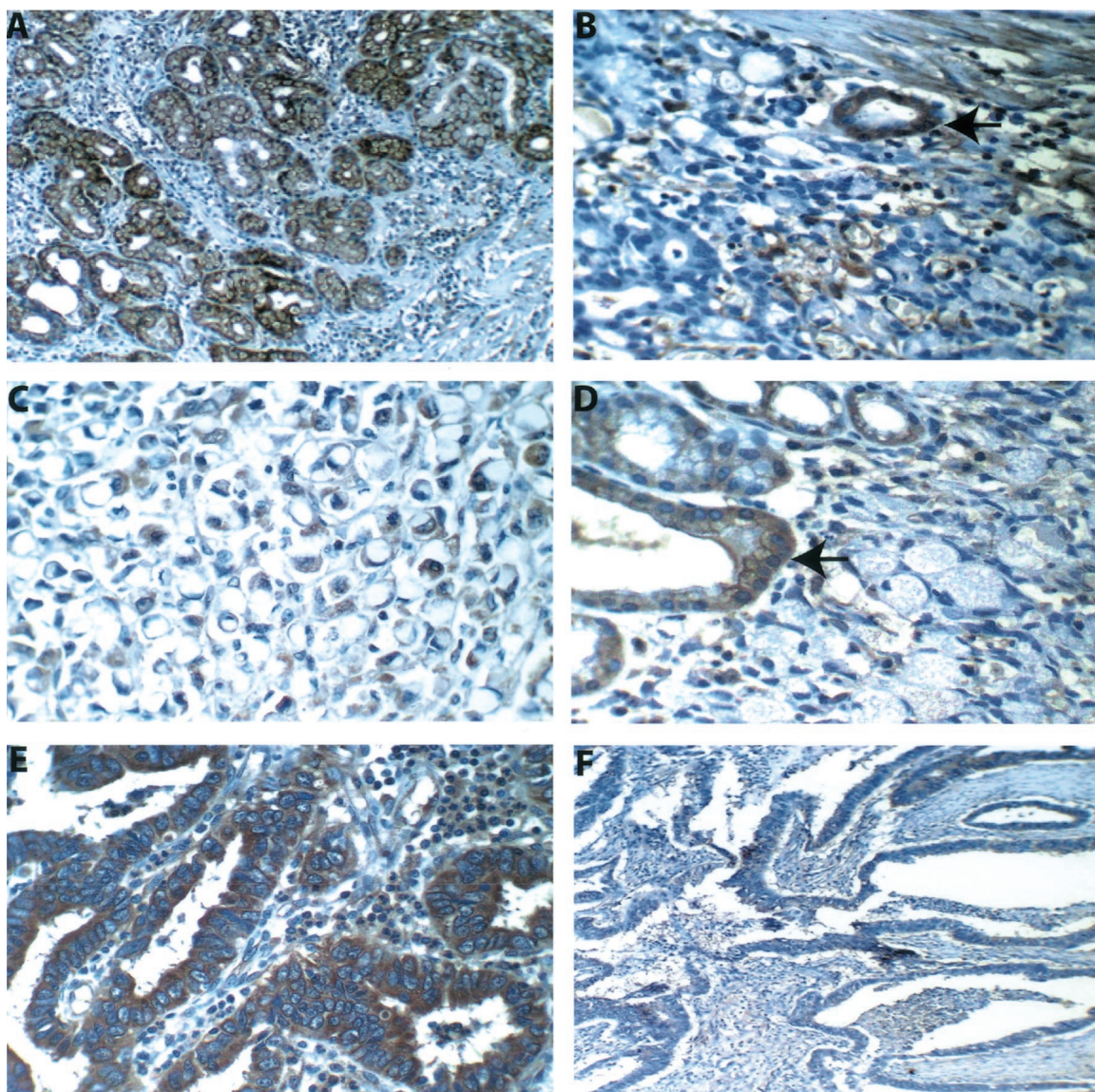
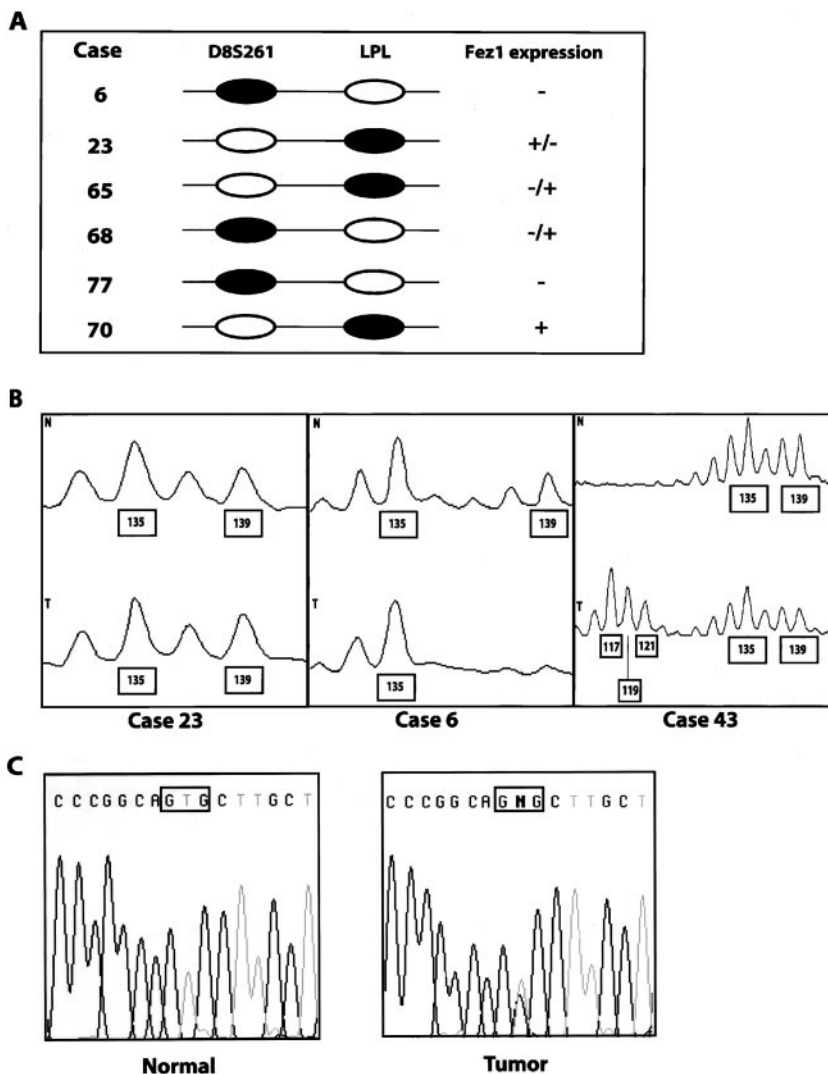


Fig. 2 Fez1 immunostaining in gastric carcinoma. A, uniform strong Fez1 staining in normal gastric glands ( $\times 250$ ). B, early gastric cancer showing loss of Fez1 protein expression (case 101;  $\times 250$ ). Fez1 is detected in a normal gland (arrow). C, diffuse carcinoma (signet-ring) showing Fez1 protein expression (case 49;  $\times 400$ ). D, signet-ring cells showing complete absence of Fez1 protein (case 19;  $\times 400$ ). Arrow, normal glands positive for Fez1. E, intestinal gastric cancer showing uniform positive staining for Fez1 protein (case 9;  $\times 400$ ). F, intestinal carcinoma showing complete absence of Fez1 protein (case 6;  $\times 250$ ).



**Fig. 3** Allelotyping study and mutation analysis. **A**, association of Fez1 expression with loss of heterozygosity at chromosome 8p21–22. *Black ovals*, loss of one allele. *Open ovals*, retention of both alleles. **B**, representative allelotyping analyses of three cases (nos. 23, 6, and 43). Fluorescent PCR product from normal (*N*) and tumor (*T*) were analyzed. DNA fragment sizes in bp compared with size marker are depicted in *boxes*. Case 23 was heterozygous for the *D8S261* polymorphic marker; case 6 showed LOH for *D8S261* (loss of the 139-bp peak in the tumor DNA); and case 43 showed MI (presence of three novel peaks 117, 119, and 121 bp, respectively, in the tumor DNA). **C**, DNA sequence chromatogram of *FEZ1* gene in case 19. A peak corresponding to a missense mutation (CAC/His to CGC/Arg) at the second base of codon 17 (*framed sequence*) was detected together with the normal allele signal in the tumor, but not in the normal DNA. Note that sequence data are shown as a reverse strand on chromatogram. Consistent with the allelotyping study that did not show LOH at the *D8S261* locus in this tumor, both wild-type and mutated *FEZ1* alleles are detected in the tumor DNA.

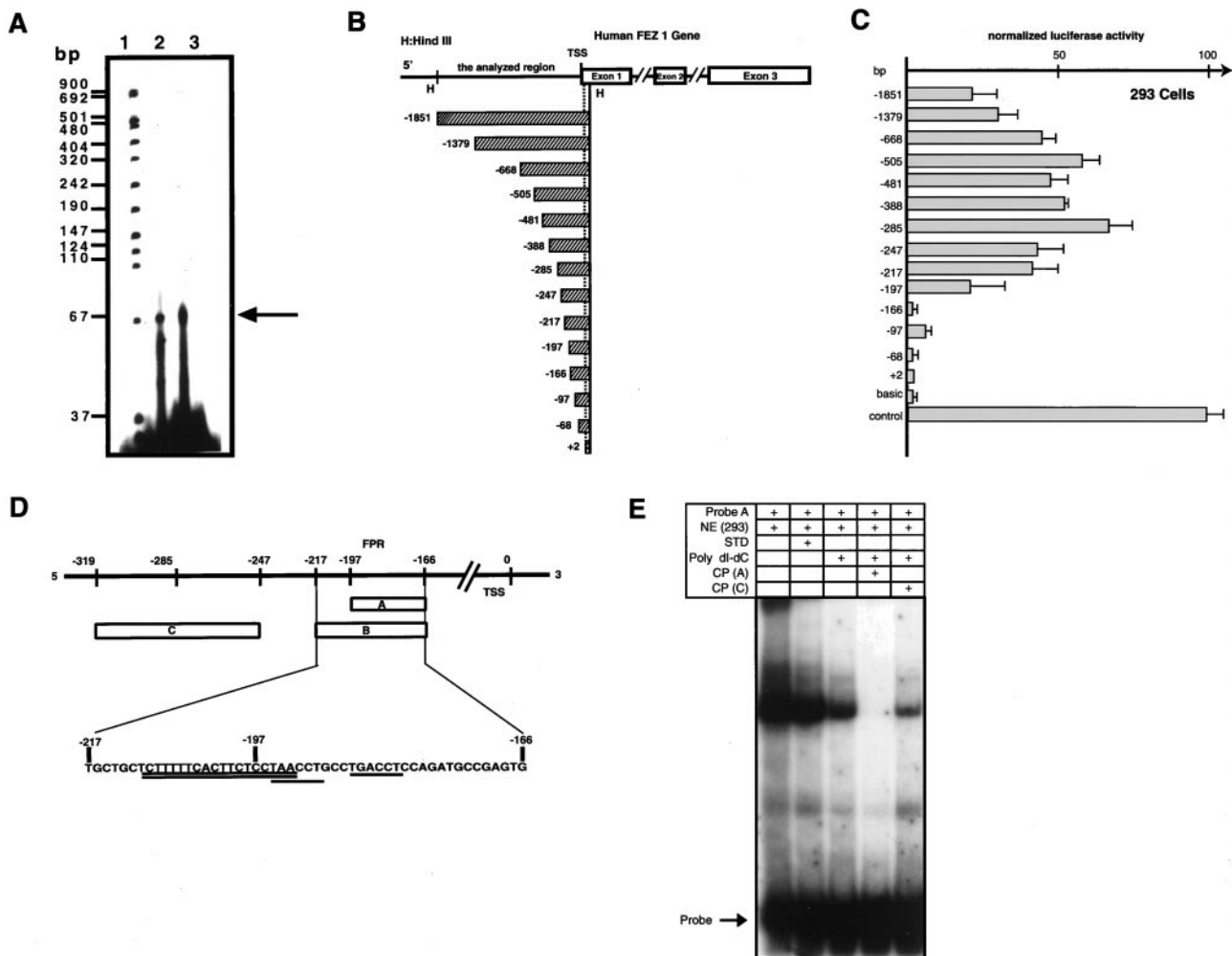
ment *in vivo*, correlated with the heterogeneity of diffuse-type carcinoma. A complete shut-down of Fez1 expression may not be essential in terms of the establishment of cell lines *in vitro*, although the expression was down-regulated in all of the cell lines examined.

**Allelotyping Study and Mutation Analysis.** Allelotyping study was performed to assess a correlation between Fez1 expression and allelic losses at chromosome 8p. Data are summarized in Fig. 3A, and representative examples are shown in Fig. 3B. Samples that were not informative for *D8S261* and *LPL* loci or did not have detectable LOH were not shown. Because a contaminant of normal cells could interfere with the analysis, microdissected DNAs from matched cancerous and normal tissues were subjected to allelotyping study using two polymorphic markers flanking the *FEZ1* gene, *D8S261* at 8p22 and *LPL* at 8p21 (10, 17). Of the 88 original cases, 26 were available for this analysis. DNA allelotyping analysis showed LOH in 3 of 17 (18%) cases informative for *D8S261* and LOH in 3 of 18 (16%) cases informative for *LPL*. MI was detected in 4 of 17 (23.5%) and in 2 of 18 (11%) cases informative for *D8S261* and *LPL*,

respectively. When we compared the presence or absence of LOH with Fez1 expression, all three cases (two diffuse and one intestinal) with LOH at *D8S261* showed absence or reduction of Fez1 protein expression (Fig. 3A), whereas 4 of the 14 cases (29%) without LOH at *D8S261* showed reduction or absence of Fez1 expression. As shown in Fig. 3A, Fez1 protein was also altered in 1 of the 3 tumors with LOH at the *LPL* locus. These results may suggest a relative correlation between allelic loss at *D8S261* and the absence or reduction of Fez1 protein, although analysis with a larger number of cases would be necessary for a definite conclusion. The genomic analysis with a Genebridge-4 radiation hybrid panel (Research Genetics) showed that the *FEZ1* gene mapped 4.5 cM telomeric to the *D8S280* marker, and that *D8S261* is located 4.5 cM telomeric to *D8S280* (data not shown). The *FEZ1* gene and *D8S261* have been shown to be located within the same yeast artificial chromosome clone, 847f8 (10, 17), although we have not completed analyzing this genomic region.

To assess direct alterations in the *FEZ1* coding sequence, PCR amplification and direct DNA sequencing were





**Fig. 4** Analysis of the 5' region of *FEZ1* gene. **A**, primer extension analysis of the transcriptional start site of human *FEZ1* gene. Poly(A)<sup>+</sup> RNAs from human brain (Lane 1) and breast (Lane 2) were reverse-transcribed with a 5'-radiolabeled, gene-specific primer corresponding with the 1524–1499-bp upstream region from the first translatable methionine. *Arrow*, TSS, which is 69 bp upstream from the 3'-end of the primer. **B**, analysis of the human *FEZ1* gene 5' region. *Bold bar* at the top of the figure indicates the *FEZ1* gene locus, in which exons are depicted in boxes. *Cross-hatched boxes*, deletion mutants used for the luciferase reporter assay. The 5'-end positions of the deleted promoter are indicated as nucleotide numbers from the TSS. **C**, luciferase reporter assay of the deleted *FEZ1* promoter regions. Luciferase reporter plasmids were transfected into Fez1-positive 293 cells to identify the positive regulatory region for *FEZ1* transcription. *Left*, 5'-end positions of the deleted promoter as nucleotide numbers from the TSS side. The experiments were performed in independently triplicated transfection assays. An average with the SD (*bars*) in the arbitrary unit, after comparing with the positive control SV40 promoter vector, is shown. **D**, map and sequence of the positive regulatory region of *FEZ1*. The *bold line* at the top indicates the promoter region around the positive regulatory region. The location is obtained by counting from the transcriptional start site of the *FEZ1* gene. *Open boxes*, position of the double-stranded oligonucleotide used for gel mobility shift assay. *Double-underlined* region in the bottom sequence is the pyrimidine-rich region. The sequence with the *bold lines* shows similarities with the vitamin D<sub>3</sub> receptor binding site. **E**, gel mobility shift assay. The gel mobility shift assays were performed using 293 cell extracts. Salmon sperm DNA (*STD*), poly(deoxyinosinic-deoxycytidylic acid), and unirradiolabeled probe C were added to the samples as nonspecific competitors. Unradiolabeled competitor probes (*CPs*) A and C were added as specific competitors for radiolabeled probe A.

performed with microdissected DNAs from the 26 gastric adenocarcinomas and matched normal tissues. Results showed a somatic missense mutation (CAC/His to CGC/Arg at *FEZ1* codon 17) of one allele in a diffuse-type gastric carcinoma (case 19; Fig. 3C), which was negative for Fez1 expression by immunohistochemistry (Fig. 2D). No LOH/MI at *D8S261* or *LPL* loci were detected in this case. We speculate that this mutation may have occurred at an early stage of carcinogenesis, which could provide dominant-negative

function of Fez1, followed by the down-regulation of Fez1 expression in an advanced stage of gastric cancer. In a previous report (8), we described the *D8S258* locus as frequently deleted in gastric cancer. On the basis of our earlier description of LOH at *D8S261* in 29% of tumors tested and the current findings of reduced or absent Fez1 protein expression in many carcinomas of the stomach, we hypothesized that *FEZ1* is a candidate tumor suppressor gene targeted by 8p deletion in a subset of gastric cancer.

### Analysis of the FEZ1 Transcription Regulatory Region.

A human brain cDNA library was screened with 5' cDNA probes, and 5' rapid amplification of cDNA ends procedure was performed to identify the 6968-bp *FEZ1* cDNA sequence. Also, we confirmed the 5'-end cDNA sequence by primer extension experiments using 5' *FEZ1* cDNA-specific primers with human poly(A)<sup>+</sup> RNAs as a template (Fig. 4A). The result showed that the TSS was located 1590 bp upstream from the first coding methionine.

Deletion mutation analysis of the *FEZ1* 5' region in Fez1-positive 293 cells allowed us to identify the *FEZ1*-positive regulatory region in the 31-bp region (−197 to −166 from the TSS). This region was essential for reporter activation, which maximally showed ~60% reporter activity as compared with an SV40 promoter control vector (Fig. 4, B–D). When deleted plasmids lacking this 31-bp region were transfected in the Fez1 positive 293 cells, the assay showed <18% activity as compared with the SV40 promoter control. Our results suggest that a resistance or refractoriness of the positive regulatory region to the *FEZ1* transcriptional process could be involved in eliminating or reducing *FEZ1* expression in cancer. Hindrance of the positive regulatory function may occur by several possible mechanisms, including alterations in the properties of regulatory factors and/or methylation of a proximate sequence, leading to suppression of *FEZ1* expression.

To characterize molecules binding to the positive regulatory region, electrophoretic mobility shift assays were performed using nuclear extract from Fez1-positive 293 cells (Fig. 4E). When a positive regulatory region probe (fragment A) was used, retarded protein-DNA complexes were detected. Addition of a 50-fold excess of a control double-stranded, nonlabeled fragment C did not compete away the protein-DNA complexes. In marked contrast, the proteins binding to the fragment A probe were reduced efficiently by a 50-fold excess of the double-stranded, nonlabeled, fragment A oligonucleotide (Fig. 4, D and E), or by a 50-fold excess of another double-stranded cold fragment B (data not shown). These data suggest that these positive regulatory region probes bind to the complexes in a sequence-specific manner.

**Methylation Analysis.** We assessed DNA methylation of the *FEZ1* gene using Southern blot analysis on genomic DNA extracted from 8 gastric cancer cell lines (see “Materials and Methods”) and on DNA from normal peripheral blood leukocytes and from normal human placenta. Our data showed that *Bss*HII and *Not*I sites into the CpG island of ~1 kb containing *FEZ1* exon 2 were methylated in six cell lines (Ags, RF1, RF48, SNU1, SNU5, and SNU16) as well as in the normal peripheral blood leukocyte DNA. The *FEZ1* CpG island was partially unmethylated in the placental DNA, suggesting either a tissue-specific methylation pattern or the methylation of only one *FEZ1* allele. No methylation was observed in the remaining two cell lines (KATO III and NCI-N87; data not shown). The status of methylation of the 5' region of *FEZ1* was not correlated with the level of Fez1 expression.

These data suggest that multiple factors, such as genomic deletions, methylation, and/or transcriptional regulation, may play a role in silencing or reducing Fez1 expression in gastric cancer. Nevertheless, additional studies will be required to define the exact role of the *FEZ1* gene in gastric carcinogenesis.

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