FEZ1/LZTS1 is Down-Regulated in High-Grade Bladder Cancer, and Its Restoration Suppresses Tumorigenicity in Transitional Cell Carcinoma Cells

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FEZ1/LZTS1 is a tumor suppressor gene that maps to chromosome 8p22, a chromosomal region frequently deleted in many human malignancies, including transitional cell carcinoma (TCC) of the urinary bladder. FEZ1/LZTS1 alterations have been reported in esophageal, breast, prostate, and gastric carcinomas. Fez1 expression was studied in five TCC-derived cancer cell lines by Western blot analysis and in 60 primary TCCs of the urinary bladder by immunohistochemistry. Fez1 protein was absent or reduced in four of five cell lines and in 37 of 60 primary TCC examined. We also restored Fez1 protein expression in human SW780 TCC-derived cells lacking endogenous Fez1 protein to study the effects of Fez1 expression on cell proliferation, cell kinetics, and tumorigenicity in BALB/c nude mice. In vitro transduction of SW780 Fez1-negative cell, with Ad-FEZ1, inhibited cell growth, altered cell cycle progression, and suppressed subcutaneous tumor growth in nude mice. These results suggest that FEZ1/LZTS1 gene plays a role in the development of TCC of the urinary bladder by acting as a bona fide tumor suppressor gene both in vitro and in vivo. (Am J Pathol 2002, 160:1345–1352)

Transitional cell carcinoma (TCC) comprises 90% of primary malignant tumors of the urinary bladder. Currently in the United States, TCC affects 54,300 individuals, 25% of whom will die of this disease.1 TCC displays a great variability in morphological and biological behavior. Multifocal growth of the primary neoplastic cells, invasion, metastases, and recurrence, even after a seemingly successful treatment at very early stage, are characteristics. How a normal urothelial cell transforms into a malignant cell and metastasizes is a complex multistep process that involves multiple genetics alterations. Recently, a two-pathway model for bladder tumor development has been proposed.5 This model suggests that different genetic alterations are involved in the development of the 70 to 80% of bladder TCC which are first diagnosed as non-invasive papillary tumors and in the remaining 20 to 30% of tumors that grow invasively at presentation. According to this model several genetic abnormalities occur more frequently in early invasive carcinoma (pT1) than in low-grade tumors.3,4

Loss of heterozygosity (LOH) at chromosome 9p occurs in more than 80% of early and superficial TCC and is believed to be an initial genetic event.5 Conversely, LOH at chromosome 8p is a late event in TCC tumorigenesis, and shows association with invasive tumors.6–8 Interestingly, LOH at 8p21–22 is associated with the invasive behavior of breast cancer9 and the progression of prostate cancer.10 These observations suggest that a tumor suppressor gene(s) on chromosome 8p21–22 plays an important role in the progression of TCC of the urinary bladder, as well as of other human tumors. Chromosome transfer into tumor cells has provided functional evidence of the presence of tumor suppressor genes on 8p.11,12 Using a combination of genetic approaches, we have recently identified FEZ1/LZTS1 (leucine zipper putative tumor suppressor 1), tumor suppressor gene at 8p22.13 FEZ1 is mutated in solid tumors, including prostate, breast, esophageal, and gastric carcinomas13,14 while Fez1 expression is absent or reduced in a sub-type of gastric cancer.14 FEZ1 encodes a 67-kd leucine-zipper protein with a region of similarity to cAMP-dependent activated protein.13 Yeast two-hybrid screening has allowed the identification of Fez1 binding partners, including elongation factor (EF) 1γ.15 In Fez1 null cancer cells, introduction of Fez1 reduced cell growth with the accumulation of cells at late S to G2/M phase of the cell cycle. On the contrary, inhibition of Fez1 expression stimulated cell growth.12,15 These data are consistent with a role of FEZ1 in human cancer. The present study investigated

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whether \( \text{FEZ1} \) alterations play a role in the development and progression of bladder cancer by studying \( \text{Fez1} \) expression in TCC-derived cancer cell lines and in primary transitional cell carcinomas. In addition we analyzed the effects of \( \text{Fez1} \) adenoviral-transduced protein expression in TCC-derived cells that lack endogenous \( \text{Fez1} \) protein.

**Materials and Methods**

**Cell Lines and Tissue Samples**

TCC cell lines T24, HTB9, CRL7833, CRL7193, and SW780, transformed human kidney 293 cells, and HeLa were obtained from the American Type Culture Collection (ATCC) and maintained as recommended. Sixty formalin-fixed paraffin-embedded specimens of primary TCC of the bladder were obtained from archival blocks of radical cystectomy cases. Thirty-one of these specimens were collected in the Department of Urology, University of Padova (Padua, Italy), and 29 specimens in the Department of Urology, Jefferson Medical College, Thomas Jefferson University (Philadelphia, PA). Six of these latter 29 samples were also macrodissected immediately after resection and fragments of tumors and normal bladder mucosa were snap frozen in liquid nitrogen. All samples were obtained from patients who gave informed consent to use excess pathological specimens for research purposes. Tumors were classified histologically and staged according to tumor-node-metastasis (TNM) classification of malignant tumors,\(^ {16} \) and tumor grade was assessed according to standard criteria.\(^ {17} \)

**Immunoblot Analysis and Immunoprecipitation**

Protein extraction and immunoblot analyses were performed as described.\(^ {18} \) Briefly, cells and tissues were lysed in NP-40 lysis buffer, clarified by centrifugation, resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Melville, NY). After blocking in 5% nonfat dry milk, membranes were incubated with primary and secondary antibodies. Specific signals were revealed using a chemiluminescence detection system (Amersham Life Sciences, Piscataway, NJ). The primary antibodies used were rabbit anti-\( \text{Fez1} \) polyclonal antibody,\(^ {14} \) mouse monoclonal anti-cdc2 (Santa Cruz, Santa Cruz, CA), and anti-actin (Sigma, St. Louis, MO). Immunoprecipitation was performed as previously described.\(^ {18} \) Briefly, 300 \( \mu \)g of proteins were incubated overnight at 4°C in presence of protein A/G sepharose (Amersham) with anti-cdc2 antibody (2 \( \mu \)g). After several washes in lysis buffer, pellets were resuspended in 2X Laemmli buffer, boiled, resolved on SDS-PAGE, and processed as described above. For cdc2-kinase assay, 10% of the immunoprecipitate was washed twice in 10 mmol/L Tris-HCl (pH 6.8) and 25 mmol/L MgCl\(_2\), and incubated in kinase assay buffer in presence of \( \gamma ^{32} \text{P} \)ATP using the cdc-2 kinase assay kit (Upstate Biotechnology, Waltham, MA) as previously reported.\(^ {19} \) Reactions were incubated for 20 minutes at 30°C and stopped by adding 2X Laemmli buffer. Samples were then boiled, loaded on polyacrylamide gels, dried, and exposed to X-ray films.

**Immunohistochemistry and Statistical Analysis**

Immunohistochemistry was performed as previously described.\(^ {14} \) Briefly, after deparaffinization, all sections were immunostained overnight at room temperature with a 1:1000 dilution of the anti-\( \text{Fez1} \) antibody. The primary antibody was omitted and replaced with preimmune serum in the negative control. Sections were reacted with biotinylated anti-rabbit antibody and streptavidin-biotin-peroxidase (Histostain-SP Kit, Zymed Laboratories, San Francisco, CA). Diaminobenzidine was used as a chromogene substrate. Finally, sections were washed in distilled water and weakly counterstained with Harry’s modified hematoxylin. All sections were examined independently by two investigators (A.V., R.B.). Any positive reaction was semiquantified with a four-tier system: +, 96 to 100% \( \text{Fez1} \) positive cells; +/−, 51 to 95% \( \text{Fez1} \) positive cells, −/+ , 2 to 50% \( \text{Fez1} \) positive cells; and −, tumors in which more than 98% of cells did not express \( \text{Fez1} \). Associations of \( \text{Fez1} \) expression with clinicopathological parameters were computed using two-tailed \( \chi ^2 \) statistic or Fisher’s exact test as appropriate. Probability of less than 0.05 was considered statistically significant.

**Recombinant Adenoviral Vector Construction and Gene Transduction**

Adenoviral full-length \( \text{FEZ1} \) expression vector was developed as described.\(^ {20} \) Briefly, full-length \( \text{FEZ1} \) cDNA was isolated from human normal brain cDNA (Clontech, Palo Alto, CA) by reverse transcription-polymerase chain reaction strategy and confirmed by DNA sequencing. cDNA for green fluorescent protein (GFP) was obtained from expression vector (Clontech). Each cDNA was ligated into an adenoviral backbone DNA (Quantum, Montreal, Canada). Two adenoviral vectors, an adenoviral-\( \text{FEZ1-GFP} \) vector that encodes two separate proteins through the internal ribosome entry site, and an adenoviral-\( \text{GFP} \) vector were constructed as recommended (Quantum). cDNAs were expressed under control of a cytomegalovirus promoter (CMV5) in each vector. Each adenoviral vector plasmid in which cDNA was ligated, was transfected into human fetal kidney 293 cells (Microbix, Toronto, Canada); after 14 to 21 days homologous recombination occurred in cells leading to plaque formation. Plaques were then isolated and eluted to infect 293 cells in 24-well culture plates. \( \text{Fez1} \) negative HeLa cells were infected to check transgene expression by immunoblot analysis and confocal microscopy for GFP. After viral clones were selected, 293 cells were infected to amplify the virus, which was purified by CsCl gradient centrifugation. Viral titers were determined by plaque assay, absorbance measurement, and serially diluted infection of GFP vector, then observed in a confocal microscope.
Figure 1. Immunohistochemical analysis of primary TCC. A: Normal urothelium showing uniform, cytoplasmic positive staining for Fez1 (magnification, ×400). Fez1 staining is also detected in normal fibroblasts and endothelial cells. B: Grade 3 infiltrating TCC showing complete absence of Fez1 (magnification, ×250). C: Different levels of Fez1 expression are detected in dysplastic superficial urothelium (magnification, ×250). Black box: Fez1 positive cells. Red box: decreased/absent Fez1 expression. D and E: A high-power view of the dysplastic urothelium depicted in the black and red boxes, respectively (magnification, ×400). F: Papillary superficial TCC showing Fez1 expression (magnification, ×400).
SW780 TCC-derived cell lines were transduced using standard techniques.20

Flow Cytometry and Cell Counting

Flow cytometry analysis was performed as described.21 Briefly, 1 × 10^7 cells were fixed in 3% paraformaldehyde, washed in phosphate-buffered saline (PBS) and stained in propidium iodide, 5 µg/ml in PBS supplemented with RNase A (Roche, Indianapolis, IN) for 30 minutes at room temperature. Data were collected using a Coulter counter (Becton Dickinson, Bedford, MA) and analyzed using the XL II System (Becton Dickinson) and the WinMDI 2.8 computer programs. For growth curve experiments 5 × 10^5 cells were plated in triplicate in 6-well plates and counted each day for 5 days. Dead cells were excluded by trypan blue staining.

Tumorigenicity

Animal experiments were conducted under institutional guidelines established for the Animal Facility at the Kimmel Cancer Center, Thomas Jefferson University. BALB/c nude mice were obtained from Jackson Laboratories (Bar Harbor, ME). For tumorigenicity assay, SW780 cells were transduced in vitro with Ad-Fez1 at multiplicity of infection (MOI) of 30, with Ad-GFP as a negative control. Transduced cells were harvested 48 hours after transduction. After determining the viability of the cell by trypan blue staining, viable cells (1 × 10^5) were injected subcutaneously (s.c.) into the right flank of three 8-week-old male BALB/c nude mice in each experimental group. Tumor formation was observed every other day up to 9 days. Tumor volume for each mouse was determined by measuring in two directions and calculated as: tumor volume = length × (width)^2/2.22

Results

Fez1 Expression in Primary TCC

Sixty TCC specimens listed in Table 1 were assessed for Fez1 expression by immunohistochemistry. Sections containing portions of normal transitional epithelium, representing an internal positive control, were analyzed (Figure 2A). Normal urothelial cells were uniformly positive in all cases. Fez1 protein was uniformly undetectable (−) in 15 of 60 specimens (25%) (Figure 2B). Sixteen tumors (27%) showed 2 to 50% positive cells (−/+ ) (Figure 1, C, D, and E). Six tumors (10%) showed a mixture of Fez1 positive and negative cells (51 to 95%, +/−) (Figure 1, F, G, and H). Fez1 positive immunostaining of 96 to 100% of the cells was detected in 23 specimens (38%) (Figure 1F). Overall, 62% of the primary tumors contain a fraction of tumor cells with reduced or absent Fez1 expression, compared with normal transitional epithelium. Statistical analysis showed a significant correlation (P < 0.005) between absence and reduction of Fez1 expression and tumor grade; no correlation was found between Fez1 expression and the other pathological parameters tested.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/No.</th>
<th>Grade †/No.</th>
<th>Fez1 expression</th>
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<tr>
<td>15 (25%)</td>
<td>M/15</td>
<td>G1/0</td>
<td>−</td>
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<td>G2/4</td>
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<tr>
<td>16 (27%)</td>
<td>M/11</td>
<td>G1/1</td>
<td>+/−</td>
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<td></td>
<td>G2/3</td>
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<td>G4/1</td>
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<td>6 (10%)</td>
<td>M/5</td>
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<td>23 (38%)</td>
<td>M/19</td>
<td>G1/6</td>
<td>+</td>
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† According to the WHO classification.
‡ Any positive immunostaining was classified into four groups: +, 96–100% Fez1 positive cells; +/−, 51–95% Fez1 positive cells; −/+ , 2–50% Fez1 positive cells; and −, tumors in which more than 98% of cells did not express Fez1.

Adenoviral Fez1 Expression Affects Cell Cycle and Cell Growth in TCC Cells in Vitro

We assessed Fez1 expression in five TCC-derived cell lines, in six primary TCCs, and in a normal bladder mucosa performing immunoblot analysis with anti-Fez1 antibody. Three cell lines (HTB9, CRL7833, and CRL7193) showed a reduction of Fez1 protein expression as compared to transformed human kidney 293 cells that have been shown previously to express high levels of endogenous Fez1 (Figure 2A).14 The SW780 cell line showed complete absence of Fez1 expression. As control of Fez1 normal level of expression, we tested a normal bladder mucosa and six primary TCC of the bladder with different percentages of Fez1 positive cells detected by immunohistochemistry.

Cases 4, 3, 7, 8, 26, and 15 depicted in Figure 2B were classified, according to the Fez1 immunoreactivity, as −/+ , − , −/− , −/+ , and −/+ , respectively. The same blots were reprobed with an anti-actin antibody to normalize the amount of loaded proteins (Figure 2A and lower panel of Figure 2B). To investigate the effects of Fez1 replacement in Fez1 null cancer cells, SW780 cells were infected with an adenoviral vector expressing both Gfp and Fez1 proteins (Ad-Fez1). As a control of viral
infection, the same cells were infected with the same adenovirus carrying only the GFP cDNA (Ad-GFP). Immunoblot analysis showed that, 48 hours after treatment, Ad-FEZ1 and Ad-GFP infections resulted in substantial expression of the transgene in the SW780 cell line (Figure 3A and B).

To define the functional consequences of this overexpression, SW780 cells were plated on 6-well plates and 24 hours later were infected once with a MOI 30 of Ad-FEZ1 or Ad-GFP. The cells were then counted each day for 7 days. Growth of SW780 cells transduced with Ad-FEZ1 was inhibited starting from 48 hours after treatment, when compared to the growth of cells infected with Ad-GFP vector (Figure 4A). Flow cytometry analysis indicated that the cell cycle distribution of SW780 cells was significantly affected by Fez1 overexpression at 48 hours of transduction compared with the untransduced and the Ad-GFP-transduced controls (Figure 4B). Indeed, cell cycle profile indicates that 61.3% of the SW780 cells transduced with Ad-FEZ1 were arrested at G2/M phase at 48 hours post-transduction (Figure 4B), whereas only 21.4% of untransduced control cells and 20.6% of Ad-GFP-transduced cells were arrested at the G2/M phase. Interestingly, at 72 hours post-transduction, 34.9% of the SW780 cells infected with Ad-FEZ1 were arrested at G2/M phase with a 39.6% of cells in apoptosis, whereas no apoptotic effect was observed in the untransduced and in Ad-GFP-transduced cells (Figure 4B). In conclusion, Fez1 replacement inhibited the growth of SW780 cells by causing a G2/M block of the cell cycle followed by apoptosis.

**Fez1 Protein Binds p34<sup>cdc2</sup> Mitotic Kinase**

SW780 TCC cell line was infected with Ad-FEZ1 for 48 hours as described above. After restoration of Fez1 expression (Figure 5A, left panel), we looked at the expression levels of cdc2 protein. As shown, no differences where found in the expression of cdc2 in cells infected with Ad-FEZ1 or control adenovirus (Figure 5A, right panel). The same lysates were immunoprecipitated with an anti-cdc2 antibody and probed with anti-Fez1 and anti-cdc2 antibodies. As shown in Figure 5B (upper panel), Fez1 coprecipitated with cdc2. Comparable amounts of the two proteins were detected by immunoblot, using the respective antibody, suggesting a stoichiometric interaction between these proteins. Interestingly, when we looked at the kinase activity of cdc2, we found a two- to threefold increase in the ability of cdc2 immunoprecipitated from Ad-FEZ1-transduced cells to phosphorylate <em>in vitro</em> its substrate Histone H1 (Figure 5C).

**Inhibition of Tumorigenicity in Ad-FEZ1-Transduced Cancer Cells**

Because <em>in vitro</em> results do not necessarily predict <em>in vivo</em> behavior we have to rely on mouse models for <em>in vivo</em> tumor growth. Although there are different models, the subcutaneously implanted tumor-bearing mouse model is the most widely used and provide the first line of evidence of possible reduced tumorigenicity.

To further determine the observed inhibitory effects of Fez1 on tumor cell proliferation <em>in vitro</em>, we tested the tumorigenic potential of Ad-FEZ1-transduced SW780 in nude mice. Viable SW780 cells (1 x 10<sup>7</sup>) transduced <em>in vitro</em> at MOI 30 with Ad-FEZ1 or Ad-GFP for 48 hours were injected s.c. into three mice in each treatment group. Tumors started to form around 3 days after injection and were observed in all of the mice that received the injection of Ad-GFP-transduced cells. Conversely only two of three mice that received injection of Ad-FEZ1 developed a tumor. Once the tumors were established they rapidly started growing, reaching an average volume of about 0.7 cm<sup>3</sup> in mice who received injection of Ad-GFP and 0.2 cm<sup>3</sup> in mice who received injection of Ad-Fez1. As depicted in Figure 6, we observed a 70% inhibition of tumorigenicity when compared to Ad-GFP control mice. Thus, these results suggested that treatment with Ad-FEZ1 not only reduced tumor size, but also prevented tumor growth.

**Discussion**

In this study we have demonstrated that Fez1 protein is not expressed in 20% of TCC-derived cell lines as well as in 25% of primary TCC. An additional 37% of the primary TCCs, and 70% of the TCC-derived cell lines analyzed showed a substantial decrease of Fez1 protein. Statistical analysis showed a significant association between decrease and absence of Fez1 and poor grade, with 79% of G3-TCC with decreased Fez1 expression, in contrast to 25% of G1-TCC and 53% G2-TCC, respectively. These results suggest that <em>FEZ1</em> may be the tumor suppressor gene targeted by genomic loss at 8p22 during bladder tumor progression from low-grade superficial to high-grade invasive tumors. After restoring Fez1 expression into SW780 Fez1-negative cells via Ad-FEZ1 transduction, we observed inhibition of cell growth and marked
alterations in the cell cycle with a predominant arrest in the G2/M phase, followed by a large fraction of apoptotic cells. Similar results were obtained in our previous studies on MCF7 breast cancer cell line in which we observed an accumulation in G2/M phase after 9 to 12 hours of Fez1 induction.15 To further address cell growth regulatory function of Fez1, we studied its interactions with other cell cycle regulators. Yeast-two-hybrid has allowed us to isolated Fez1 binding partners, including EF 1y, which has been shown to associate with p34cdc2 kinase.23 Since we observed a block in G2/M phase of the cell cycle in cells transduced with Fez1, we explored the possibility of a direct interaction between Fez1 and cdc2. Immunoprecipitation experiments showed a binding of Fez1 to cdc2 in Fez1-transduced SW780 cell line. Interestingly, when Fez1 and p34cdc2 were co-expressed, a higher activity of p34cdc2 was detected. In our previous report, we demonstrated that this interaction takes place predominantly in the late S-G2/M phase and that Fez1 is involved in the stability of active p34cdc2-cyclin B1 complex.15 We can speculate that, since the destruction of the p34cdc2-cyclin B1 complex is necessary to allow the cell to exit from mitosis,24 Fez1 overexpression could hold the cells in G2/M phase of the cell cycle by inhibiting the dissociation of p34cdc2-cyclin B1 complex. The presence of different and divergent stimuli could then determine the massive apoptotic effect displayed from Fez1 infected cells. It is noteworthy that a G2/M block of the cell cycle was also found on a mammary carcinoma cell line15 suggesting Fez1 overexpression activates the same intracellular events independently of the cellular type and could represent a new mechanism in the regu-

Figure 4. Cell counting and flow cytometry of infected TCC cancer cell lines. A: In vitro cell growth of TCC cancer cell line SW780. The cell numbers was counted by trypan blue exclusion at the indicated times after transduction with Ad-FEZ1 (○), and Ad-GFP (●) vectors at MOI 50. B: Flow cytometry analysis of TCC cancer cell line SW780 untreated and infected with Ad-GFP or Ad-FEZ1 at 48 hours (top row) and at 72 hours (bottom row). The percentage of cell cycle distribution is described in the text.
Figure 5. Association of Fez1 with p34\(^{cdc2}\) 

A: Left panel shows Fez1 expression in SW780 cancer cells mock infected (lane 1) or infected with Ad-FEZ1 (lane 2). Right panel shows expression of cdc2 protein in the same samples. B: Immunoprecipitation analysis of SW780 cancer cells mock infected (lane 2) or infected with Ad-FEZ1 (lanes 1 and 3) immunoprecipitated with anti-p34\(^{cdc2}\) antibody (lanes 2 and 3) or with normal rabbit serum (lane 1). The blot was hybridized with anti-Fez1 (upper panel) and with anti-cdc2 (lower panel) antibodies. C: cdc2-kinase assay performed using lysates from SW780 cells mock infected (lane 1) or infected with Ad-FEZ1 (lane 2). Phosphorylation of histone H1 is shown.

Figure 6. Tumorigenicity of Ad-FEZ1-transduced SW780 cells. SW780 cells were infected by Ad-FEZ1\((\bullet)\) and Ad-GFP\((\bigodot)\), and injected s.c. into nude mice as described in the text. Tumor size measurement is shown.

Acknowledgments

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References


