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Zeb1-mediated T-cadherin repression increases the invasive potential of gallbladder cancer

Yoshihiro Adachi [a], Tamotsu Takeuchi [a,*], Tomoko Nagayama [b], Yuji Ohtsuki [c], Mutsuo Furihata [a]

[a] Department of Pathology, Kochi Medical School, Nankoku, Kochi 783-8505, Japan
[b] JST Innovation Satellite Kochi Practical Application Research, Tosayamada, Japan
[c] Division of Pathology, Matsuyama Shimin Hospital, Matsuyama, Japan

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ABSTRACT

Here, we report that the transcriptional regulator Zeb1 repressed the transcription of T-cadherin, to increase the invasive activity of gallbladder cancer cells. Zeb1 physically bound to the promoter of T-cadherin, repressed promoter activity in E-box-like sequence-dependent fashion, and suppressed T-cadherin expression. In gallbladder cancer tissues, Zeb1 was expressed at the cancer invasion front, whereas T-cadherin was exclusively expressed in non-invasive foci. Collagen gel invasion assay showed that T-cadherin was a negative regulator for gallbladder cancer invasion. These findings suggest that Zeb1 represses T-cadherin expression and thus increases the invasive activity of gallbladder cancer.

1. Introduction

Gallbladder cancer is an aggressive tumor with strong invasive activity into adjacent organs. It is well established that depth of cancer invasion is strongly correlated with prognosis in patients with gallbladder cancer [1]. Unfortunately, it is often difficult to cure advanced invasive gallbladder cancer with conventional treatment.

Genotoxic stress is, in association with long-term inflammation, important in the tumorigenesis of gallbladder cancer [2,3]. Studies focused on molecular mechanisms of tumorigenesis have revealed mutations of p53 [4–6], activating mutations of the KRAS proto-oncogene [7], and loss of cell-cycle regulation by CDK-INK4A [8] in gallbladder cancer. However, the molecular mechanisms involved in gallbladder cancer invasion remain unclear.

Recent studies have highlighted epithelial-mesenchymal transition (EMT) as a crucial process in cancer invasion and metastasis [9,10]. Cancer cells acquire fibroblast-like properties by EMT as well as increased motility. In the process of EMT, transcription factors such as Snails, bHLH, and Zebs repress the expression of various epithelial cell-related genes including E-cadherin while up-regulating the expression of mesenchymal cell-related genes [11,12].

T-cadherin (also referred to as CDH13 or H-cadherin) is a glycosyl phosphatidyl inositol (GPI)-linked surface membrane protein [13], and is expressed in various epithelial cells [14,15]. T-cadherin lacks a cytoplasmic domain, which is required for the homophilic binding activity of classical cadherins [16]. Moreover, a tryptophan conserved in all other cadherins and which plays a crucial role in adhesive function is replaced by an isoleucine in T-cadherin. Given these unique molecular structural features, and its localization on the lipid raft, which partitions various signaling molecules, T-cadherin is thought to play roles in signal transduction apart from cell-cell adhesion [17–20]. Consistent with this, NMR analysis very recently demonstrated that T-cadherin exhibits little structural evidence of homophilic adhesive activity [21].

Interestingly, many epigenetic studies focused on analysis of promoter methylation have suggested that T-cadherin acts as a tumor suppressor factor in various malignant tumors including those of breast, lung, colon, and skin [14,22–25]. We have examined the pathobiological properties of T-cadherin in various cancers, and noted the presence of a typical E-box-like (also designated as E2-box) element, 5′-CACCTG-3′ [26], to which Zeb1 could bind, in the promoter region of T-cadherin [24,27].

Here, we report that Zeb1 repressed the transcription of T-cadherin and down-regulation of T-cadherin increased cancer invasion activity in gallbladder cancer cell. We think that Zeb1-mediated repression of T-cadherin may be involved in gallbladder cancer invasion. Since restoration of T-cadherin in cultured

* Corresponding author.
E-mail address: takeutit@kochi-u.ac.jp (T. Takeuchi).

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gallbladder cancer cells markedly decreased cancer invasion activity, T-cadherin could be a possible target for corrective gene therapy.

2. Materials and methods

2.1. Antibodies

A rabbit polyclonal antibody to human Zeb1 was prepared in our laboratory using the methods of Darling et al. with modifications [28,29]. Briefly, PCR product encoding a part of human Zeb1 (amino acids 559–663) was subcloned into the PET16b vector (Novagen Inc., Madison, WI) and confirmed by sequence analysis. Recombinant histidine-tagged protein was obtained using transformed BL21 (DE3) pLyS cells by isopropyl-1-thio-beta-D-galactopyranoside induction, subsequently purified with a Ni-NTA Spin kit (Qiagen, Valencia, CA), and used for immunization of rabbits. Recombinant GST-fused Zeb1 (amino acids 559–663) protein was also obtained using the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ) and used for affinity-column preparation. The specificity of affinity-purified antibody was confirmed by Western-immunoblotting and immunohistochemical staining using well characterized Zeb-1-expressing uterine carcinosarcoma tissue (Supplementary Fig. S1). Details of the procedures used to prepare affinity-purified rabbit specific antibody to human T-cadherin have been described previously [14,17,25]. Antibodies to MHC class I and vimentin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse immunoglobulin antibodies to N-cadherin were purchased from Zymed Laboratories Inc. (San Francisco, CA) and E-cadherin from Takara Bio Inc. (Shiga, Japan). Normal control immunoglobulin was prepared in our laboratory.

2.2. Chromatin immunoprecipitation (ChIP) analysis

Zeb1-expressing but T-cadherin-negative NOZ cells were cross-linked with 1% formaldehyde for 10 min. Cells were suspended in 0.2 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0) for 10 min. Genomic DNA was fragmented to lengths of 200–1000 bp by sonication and diluted 10-fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). One-tenth of the sample was set aside as an input control, and the remainder was pre-cleared with salmon sperm DNA protein A-Sepharose beads for 30 min with agitation and subsequently incubated with rabbit polyclonal anti-Zeb1 antibody or normal anti-rabbit IgG as a negative control overnight at 4 °C. Immune complexes were pulled down with salmon sperm DNA protein A-Sepharose beads, eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$), and incubated with 5 M NaCl for 4 h at 65 °C to reverse cross-linking. The elutes were treated with proteinase K, and released DNA was purified and amplified by PCR with primers specific for the T-cadherin promoter (sense, 5′-TAC TGC TGT TCT GAG TAC CTG-3′/62220–62200; antisense, 5′-GTG GCC CAA GTC ATG ATG GTG-3′/61955–61975 of Homo sapiens chromosome 16 clone RP11-2L4).

2.3. Electrophoretic mobility shift assay (EMSA)

The entire coding region of human Zeb1 cDNA was generated by RT-PCR with SpeedStar polymerase (Takara) with the sense primer 5′-GCCATGGCGGATGGCCCCAGGTGTAAG-3′ and anti-sense primer 5′-TTAGGCTTCATTTGTCTTTTCTTCAGAC-3′, subsequently ligated into pTarget-T expression vector (Promega, Madison, WI), and confirmed by sequencing. Transfection into Cos7 cells was carried out as described previously [28]. Nuclear lysates were prepared from Cos7 cells, which were transfected with Zeb1-containing pTarget-T.

EMSA was performed using the digoxigenin Gel Shift kit according to the manufacture's protocol (Roche Applied Science, Mannheim, Germany). Briefly, a double-strand probe (−683 to −602) containing the wild E2-box 5′-CACCTG-3′, or replaced by 5′-AAATTT-3′ sequences, were end-labeled with digoxigenin-11-ddUTP. Labeled probes were added to nuclear extract, and the binding reaction was allowed to proceed for 15 min at room temperature. Unlabeled competitor at 50-fold molar excess, or antibodies, anti-Zeb1 or control antibody were added to the binding reactions 5 min before the labeled probe and allowed to incubate at room temperature.

The samples were resolved through a non-denaturing 8% acrylamide gel for separating complexes, subsequently transferred to a nylon membrane using electroblotter, and further handled for immunochemical detection as outlined by the manufacturer.

2.4. Repression of T-cadherin promoter activity by Zeb1

The procedure used for evaluation of promoter activity using the GreatEscApe™SEAP system (Takara) was described in detail previously [28]. The T-cadherin promoter sequence from −1 to −682 was cloned into pSEAP2-Basic plasmid (Takara) between BamH1 and EcoR1 restriction sites, and subsequently confirmed by sequencing. Wild-type and E2-box-mutated promoters were generated by PCR with different 5′ primers and a fixed 3′ primer, using human genomic DNA as template. The sequence of the single reverse primer was 5′-GGA ATT CAT TTT GTC CGA CTA GAA GCG-3′ (−1 to −18), while the forward primers were 5′-AGG ATC CAG ACT CTC ACC TGA GCA GTT-3′ (−663 to −682; E2-box underlined) and 5′-AGG ATC CAG ACT CTA AAT TTA GCA GTT-3′ (E2-box sequence replaced by underlined sequence) for wild-type and E2-box-mutated sequences, respectively.

Wild-type, E2-box-mutated T-cadherin promoter-containing pSEAP2-Basic vectors, or empty vector, and Zeb1-containing pTarget-T vectors were co-transfected into Cos7 cells using DEAE-Dextran. Promoter activity was measured using a GreatEscApe™SEAP chemiluminescent detection kit (Takara). Reporter assays was also performed in the cultured gallbladder cancer cell, OCUG-1, and stably Zeb1 overexpressing OCUG-1 cell, which was described below. In these assays, wild-type, E2-box-mutated T-cadherin promoter-containing pSEAP2-Basic vectors, or empty vector, was transfected.

2.5. Western-immunoblotting

Western-immunoblotting was carried out according to the modified method of Towbin et al. [30], as previously reported [14]. Briefly, equal amounts of proteins were electrophoresed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels and electroblotted to polyvinylidine difluoride membrane (Millipore, Bedford, MA). After blocking with bovine serum albumin, membranes were incubated with antibodies.

2.6. Cell culture

NOZ [31,32] and OCUG-1 [32] gallbladder cancer cells were obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). Both NOZ cell and OCUG-1 cells are derived from the malignant peritoneal effusion of patient with gallbladder adenocarcinoma. NOZ cell line composed of spindle and round shaped cells, while OCUG-1 cells proliferated in a monolayer sheet.

Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Gibco Life Technologies, NY) containing 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamycin (Gibco RL-Life Technologies, Grand Island, NY).