Invasion and Metastasis of Oral Cancer Cells Require Methylation of E-Cadherin and/or Degradation of Membranous β-Catenin

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ABSTRACT

The extent of lymph node metastasis is a major determinant in the prognosis of oral squamous cell carcinoma (OSCC). Abnormalities of cell adhesion molecules are known to play an important role in invasion and metastasis of cancer cells through the loss of cell-to-cell adhesion. In this study, we isolated highly invasive clones from an OSCC cell line established from a lymph node metastasis by using an in vitro invasion assay method and compared the abnormalities of cell adhesion molecule E-cadherin and β-catenin in these cells. The isolated, highly invasive clones showed significant invasive capacity and reduction of E-cadherin and membranous β-catenin protein in comparison with parent cells. We found that reduced expression of E-cadherin was due to methylation of its promoter region. In fact, most invasive and metastatic area of OSCCs showed reduced expression and methylation of E-cadherin. Moreover, we found that reduced expression of membranous β-catenin was due to its protein degradation. Reduced expression of membranous β-catenin was also found frequently in invasive and metastatic areas of OSCCs. In summary, invasion and metastasis of OSCC cells require methylation of E-cadherin and/or degradation of membranous β-catenin. In addition, we suggest that the method of isolation of highly invasive clones may be useful for studies aimed at discovering novel genes involved in invasion and metastasis.

INTRODUCTION

Squamous cell carcinoma is the most common malignant neoplasm of the oral cavity. The most important prognostic indicator for patients with oral squamous cell carcinoma (OSCC) is metastasis to cervical lymph nodes or distant organs (1). The process of metastasis consists of sequential and selective steps including proliferation, induction of angiogenesis, detachment, motility, invasion into circulation, aggregation and survival in the circulation, cell arrest in distant capillary beds, and extravasation into organ parenchyma (2). The development of a metastasis depends on interplay between host factors and intrinsic characteristics of cancer cells, and the metastatic lesion represents the end point of many destructive events that only a few cells can survive (3). Moreover, neoplasms contain a variety of subpopulations of cells with differing metastatic potential, and highly metastatic clones may exist within a primary tumor (3).

The cell adhesion molecule E-cadherin is a transmembrane glycoprotein responsible for homotypic binding and morphogenesis of epithelial tissues (4, 5). E-cadherin is arguably the prototypic member of the classical cadherin family and plays a critical role in cell-to-cell adhesion. The cytoplasmic domain of E-cadherin is linked to the actin cytoskeleton through critical interactions with catenins (6). Disruption of the cadherin-catenin complex has been demonstrated in various cancers and has been correlated with tumor differentiation, invasion, metastasis, and prognosis. Immunohistochemical studies have demonstrated that loss of E-cadherin expression is a frequent event in various types of cancers (6). In OSCC, E-cadherin also correlates well with invasion and metastasis (7–10). However, mutations of the CDH1 gene encoding E-cadherin are rare or absent, and CDH1 gene mutations that compromise the nonadhesive function of E-cadherin have been observed only in human gastric carcinoma cell lines, lobular breast cancer, and familial gastric cancer (11). In addition, loss of E-cadherin expression is heterogeneous in cancers and may be modulated by the tumor microenvironment (11). These results suggest that the mechanism of E-cadherin loss in these tumors does not involve irreversible genetic alterations. Methylation associated with E-cadherin loss in breast cancers is heterogeneous and unstable (12). Proposed epigenetic mechanisms for E-cadherin loss include alterations in the expression and/or function of trans-acting factors that regulate CDH1 gene transcription, hypermethylation of the CDH1 promoter, and chromatin-mediated effects. Such epigenetic plasticity may contribute to the dynamic, phenotypic heterogeneity that drives metastatic progression.

In adherens junctions, β-catenin links E-cadherin and the actin cytoskeleton through interaction with α-catenin (6). A human signet ring gastric cancer cell line has a homozygous deletion in the β-catenin gene CTNNBI, which results in impaired cell-to-cell adhesion (13). In addition to its critical role in cellular adhesion, β-catenin functions in the Wnt signaling pathway (14). Consistent with its ostensibly independent functions in cell adhesion and signal transduction, at least two distinct pools of β-catenin are supposed to exist in cells: a cell membrane-associated pool, and a pool involved in Wnt signal-
ing and gene transcription. In Wnt signaling, β-catenin is regulated in part by the adenomatous polyposis coli (APC) protein AXIN and glycogen synthase kinase (GSK) 3β (14). A pool of β-catenin involved in Wnt signaling and gene transcription is degraded by the ubiquitin-proteasome pathway. In the absence of a Wnt signal, β-catenin is phosphorylated by a multimolecule complex consisting of GSK3, APC, and AXIN, and then the phosphorylated β-catenin is recognized and ubiquitinated by the E3 ubiquitin ligase complex SCF^P^Trp (15). Activation of the Wnt signaling pathway leads to inhibition of β-catenin degradation by decreasing the ability of GSK3β to phosphorylate β-catenin, and stabilization of β-catenin enhances the interaction with members of the T-cell factor/lymphoid enhancer factor family of transcription factors (16). Numerous studies have suggested that β-catenin is a potent oncogene product, and its accumulation has been implicated in tumorigenesis in various cancers (14). In contrast, decreased expression of β-catenin is found in esophageal, colon, gastric, and oral cancers (17–19).

In the present study, we isolated highly invasive clones (MSCC-Inv1 and MSCC-Inv2) from MSCC-1 cells using an in vitro invasion assay and found E-cadherin methylation and β-catenin degradation in these highly invasive clones. Moreover, we also found frequent E-cadherin methylation and reduced expression of membranous β-catenin in the invasive and metastatic areas of OSCCs.

**MATERIALS AND METHODS**

**Cell Culture and Transfection.** The MSCC-1 cell line was established previously from lymph node metastasis of gingival OSCC by our laboratory (20). MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells were maintained in Keratinocyte-SFM (In-19) and then the phosphorylated β-catenin is recognized and ubiquitinated by the E3 ubiquitin ligase complex SCF^P^Trp (15). Activation of the Wnt signaling pathway leads to inhibition of β-catenin degradation by decreasing the ability of GSK3β to phosphorylate β-catenin, and stabilization of β-catenin enhances the interaction with members of the T-cell factor/lymphoid enhancer factor family of transcription factors (16). Numerous studies have suggested that β-catenin is a potent oncogene product, and its accumulation has been implicated in tumorigenesis in various cancers (14). In contrast, decreased expression of β-catenin is found in esophageal, colon, gastric, and oral cancers (17–19).

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**Preparation of Membrane Fraction.** To obtain the membrane fraction, we used the Mem-Per Eukaryotic Membrane Protein Extraction Kit (Pierce). Cells were lysed according to the manufacturer’s instructions.

**Western Blot Analysis.** Cells were lysed in cold lysis buffer containing 30 mM Tris-Cl (pH 7.5), 250 mM NaCl, 0.1% Triton X-100 (Roche), 1 mM EDTA, 50 mM NaF, 0.1 mM Na3VO4, 1 mM diethiothreitol, 0.1 mM leupeptin, 0.1 μg/ml soybean trypsin inhibitor, 10 μg/ml 1,1-chlor-3-(4-tosylamido)-4-phenyl-2-butanon, 10 μg/ml 1,1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride, 10 μg/ml aprotinin, and 50 μg/ml phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 30 min and spun down at 15,000 rpm for 20 min at 4°C. The protein concentration was determined by Bradford protein assay (Bio-Rad) using bovine serum albumin (Sigma) as a standard. Western blot analysis was performed as described previously (22).

**Immunohistochemistry.** Immunohistochemical detection of E-cadherin and β-catenin in OSCCs was performed on 4.5-μm sections mounted on silicon-coated glass slides, using a streptavidin-biotin peroxidase technique as described previously (22). For immunohistochemical detection of β-catenin in cells, cells were plated on coverslips and fixed with formalin for 30 min after treatment, and then immunohistochemistry was performed as described above.

**Laser Capture Microdissection and DNA Extraction.** Ten-micrometer sections mounted on glass slides covered with PEN foil (Leica Microsystems) were used for DNA extraction. The sections were stained slightly with H&E and air-dried. The
areas showing no expression or reduced expression of E-cadherin were selected by comparison with the immunostained sections. The selected areas were dissected from the sections with Laser Microdissection systems (Leica Microsystems) and dropped immediately into a microcentrifuge tube cap filled with 30 μl of ATL buffer (DNeasy Tissue Kit; Qiagen), and then DNA was extracted by overnight incubation with proteinase K at 55°C according to the manufacturer’s instructions.

Methylation-Specific PCR for E-cadherin. Methylation-specific PCR (MSP) primers spanning the transcription start site of E-cadherin were reported previously as island 3 by Graff et al. (12). The sets of primers were E-cadherin M-sense (5’-TTAGGTTAGGTTATCCTACGAC-3’ and E-cadherin M-antisense (5’-TAATTTAGGTTATCCTACGAC-3’) for the methylated sequence, and E-cadherin U-sense (5’-TTTAGGTTATCCTACGAC-3’) and E-cadherin U-antisense (5’-CAACCAATCAACACACAC-3’) for the unmethylated sequence. DNA was isolated from cells by using the DNeasy kit (Qiagen). DNA was isolated from paraffin block sections of OSCCs as described above. DNA was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (Sigma) at pH 5.0, both freshly prepared, were added and mixed, and samples were incubated at 50°C for 16 h. Modified DNA was purified using Wizard DNA purification resin (Promega) and eluted into 50 μl of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. Modified DNAs were amplified in a total volume of 20 μl of 1X GeneAmp PCR Gold Buffer (PE Applied Biosystems) containing 1.0 mM MgCl2, 1 mM each primer, 0.2 mM deoxynucleoside triphosphates, and 1 unit of Taq polymerase (AmpliTaq Gold DNA polymerase; PE Applied Biosystems). After activation of the Taq polymerase at 95°C for 10 min, PCR was performed in a thermal cycler for 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 57°C (E-cadherin M-primer sets) or 53°C (E-cadherin U-primer sets) for 30 s, and extension at 72°C for 30 s, followed by a final 10-min extension at 72°C. PCR products were then loaded onto nondenaturing 12% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Silencing by Small Interfering RNA. Logarithmically growing HeLa cells were seeded at a density of 10^5 cells/6-cm dish and transfected twice with oligonucleotides (at 24 and 48 h after replating) using Oligofectamine (Invitrogen) as described previously (23). Forty-eight hours after the last transfection, lysates were prepared and analyzed by SDS-PAGE and immunoblotting. The small interfering RNA (siRNA) oligonucleotides used for both β-Trcp1 and β-Trcp2 silencing were 21-bp synthetic molecules (Dharmacon Research) corresponding to nucleotides 407–427 of human β-Trcp1 and nucleotides 161–181 of human β-Trcp2 (AB033279; Ref. 24). A 21-nucleotide siRNA duplex corresponding to a nonrelevant F-box protein (Fbp) gene was used as control.

RESULTS
Isolation of Highly Invasive Clones from MSCC-1 Cells by Using an Invasion Assay Device. We previously established an OSCC cell line, MSCC-1, from lymph node metastasis of a patient with OSCC of gingiva (20). MSCC-1 cells have a high invasive ability in comparison with other OSCC cell lines. In the present study, we isolated highly invasive clones from MSCC-1 cells by using an in vitro invasion assay device. We collected cells that migrated onto the lower side of the filter of the cell culture insert, and two clones, MSCC-Inv1 and MSCC-Inv2, were obtained. Although most of the parent MSCC-1 cells were polygonal in shape, MSCC-Inv1 and MSCC-Inv2 cells were rather spindle-shaped (Fig. 1A). Cell growth of these clones was the same as that of the parent cells (data not shown), but the number of MSCC-Inv1 and MSCC-Inv2 cells that migrated through the filter at 6 h was significantly higher than that of parent cells (P < 0.01; Fig. 1B). Moreover, we demonstrated that MSCC-Inv1 and MSCC-Inv2 cells had higher invasiveness than parent cells by using three-dimensional culture analysis (Fig. 1C). To determine the mechanism responsible for the higher invasiveness in MSCC-Inv1 and MSCC-Inv2 cells, we examined the expression of cell adhesion molecule E-cadherin and β-catenin in these cells. MSCC-Inv1 and MSCC-Inv2 cells showed reduced expression of E-cadherin and β-catenin in comparison with parent cells (Fig. 1D). A greater reduction of E-cadherin and β-catenin protein was seen in MSCC-Inv1 cells than in MSCC-Inv2 cells.

Loss of E-cadherin Protein Plays an Important Role for Invasion in MSCC-Inv1 and MSCC-Inv2 Cells. To confirm the correlation between invasiveness and reduction of E-cadherin protein, we examined whether an adhesion-blocking anti-E-cadherin antibody, HECD-1, could enhance the invasiveness of these cells. We used anti-E-cadherin antibody, which recognizes the cytoplasmic domain of E-cadherin, as a negative control. After 8 h of incubation, the E-cadherin antibody (HECD-1) significantly enhanced the invasiveness of parent cells (P = 0.026), but not of MSCC-Inv1 and MSCC-Inv2 cells (Fig. 2A). Control antibody did not enhance the invasiveness of any of the cells. Next, we transfected the E-cadherin gene into parent MSCC-1 cells, and MSCC-Inv1 and MSCC-Inv2 cells and examined the effects on invasion. E-cadherin transfection suppressed the invasiveness of these cells (Fig. 2B). The effect on suppression of invasion was greater in MSCC-Inv1 cells than in parent cells. Original MSCC-Inv1 cells did not attach to each other, but E-cadherin-transfected cells showed cell-to-cell adhesion (Fig. 2C). These data indicate that loss of E-cadherin plays an important role in invasion through loss of cell-to-cell adhesion in OSCC cells.

Methylation of E-cadherin Induces Reduction of E-cadherin Protein in Invasive and Metastatic OSCC Cells. Next we examined the mechanism of E-cadherin reduction in MSCC-Inv1 and MSCC-Inv2 cells. Loss of E-cadherin function during tumor progression can be caused by a variety of genetic or epigenetic mechanisms including mutational inactivation, chromosomal aberrations, transcriptional repression of the E-cadherin gene (e.g., by the repressors Snail and Sip-1), subsequent promoter hypermethylation, and chromatin rearrangement (11, 25–28). Because MSCC-Inv1 and MSCC-Inv2 cells were isolated from MSCC-1 cells, we thought that the reduction of E-cadherin found in MSCC-Inv1 and MSCC-Inv2 cells might be caused by a reversible mechanism such as transcriptional repression of the E-cadherin gene or hypermethylation of its promoter lesion. Therefore, at first, we examined the methyla-
tion of E-cadherin in MSCC-Inv1 and MSCC-Inv2 cells by MSP. Aberrant promoter methylation of E-cadherin was detected in MSCC-Inv1 and MSCC-Inv2 cells, but not in parent MSCC-1 cells (Fig. 3A). In MSCC-Inv1 cells, an obvious methylated band was detected in comparison with MSCC-Inv2 cells.

Then, we examined E-cadherin promoter methylation in OSCCs by MSP. Two highly invasive OSCCs and 18 lymph node metastases of OSCC were examined. Highly invasive cases (tumors were surgically resected from tongue) showed that cancer cells invaded the underlying muscle. We defined a mucosal or submucosal area without invasion of the underlying muscle as a "noninvasive area." In contrast, "invasive area" was defined as a submucosal area with invasion of the underlying muscle. All metastatic OSCCs were surgically resected from the cervical or submandibular lymph node. At first, we examined the immunoxpression of E-cadherin in paraffin-embedded tissues from these cases. In the two highly invasive OSCCs, OSCC cells in invasive areas showed reduced expression of E-cadherin, whereas OSCC cells in noninvasive areas showed high expression of E-cadherin in their cell membrane (Fig. 3B). All 18 metastatic OSCCs showed reduced expression of E-cadherin in nodal lesions, but expression of E-cadherin was heterogeneous (Fig. 3C). In these tumors, we asked whether the reduced expression of E-cadherin was caused by methylation or not.

**Fig. 1** Reduced expression of E-cadherin and β-catenin in highly invasive clones isolated from parent cells by in vitro invasion assay. A, morphology of parent MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells under phase-contrast microscopy. B, invasiveness of MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells. The number of cells that migrated through pores onto the lower side of the filter was counted. We compared the number of MSCC-Inv1 and MSCC-Inv2 cells that migrated with the number of MSCC-1 cells that migrated. The Y axis represents fold change of invasiveness. C, three-dimensional culture invasion assay detecting stromal invasion of MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells. Cells were cultured on reorganized stroma by the three-dimensional culture method described in "Materials and Methods." D, Western blot analysis of E-cadherin and β-catenin in MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells. β-Actin was used for normalization of Western blot analysis.

**Fig. 2** Loss of E-cadherin was involved in invasion of OSCC cells. A, effect of treatment with E-cadherin antibody on invasion of MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells. We used an antihuman E-cadherin antibody (HECD-1) at a final concentration of 100 μg/ml. As a negative control, we used an anti-E-cadherin antibody that recognizes the cytoplasmic domain of E-cadherin at a final concentration of 100 μg/ml. In vitro invasion of cells treated with E-cadherin antibody was compared with that of nontreated cells and cells treated with control antibody. Bars and error bars, mean value and SD of three different experiments. Statistical analysis was done by unpaired Student’s t test: *, P < 0.05. B, E-cadherin transfection inhibited the invasiveness of MSCC-Inv1 cells. After transfection of E-cadherin, the number of cells that migrated was counted. We compared the invasiveness of transfected cells with that of control cells. C, morphology of MSCC-Inv1 and E-cadherin-transfected MSCC-Inv1 cells under phase-contrast microscopy.
Because the pattern of E-cadherin expression in OSCC cases exhibited striking heterogeneity, we examined the methylation of E-cadherin only in microdissected OSCC cells that showed reduced expression of E-cadherin by using the laser capture microdissection technique. In the highly invasive OSCC cases, methylation of OSCC cells was detected in the invasive areas, but not in the noninvasive areas (Fig. 3B; Table 1). Interestingly, methylation was seen in 13 of 18 metastatic OSCCs that showed reduced E-cadherin expression (Fig. 3C; Table 1). Moreover, methylation was not detected in metastatic OSCC cells that showed E-cadherin expression (Fig. 3C). These results indicate that E-cadherin expression and methylation are heterogeneous in metastatic areas.

**Reduced Expression of β-Catenin in MSCC-Inv1 Cells Is Caused by Ubiquitin-Proteasome-Mediated Degradation.**

Because β-catenin is ubiquitinated by the E3 ubiquitin ligase complex SCFβ-Trcp and degraded by proteasome (15), we hypothesized that the reduction of β-catenin found in MSCC-Inv1 and MSCC-Inv2 cells may be caused by ubiquitin-mediated proteolysis. Therefore, we treated MSCC-Inv1 and MSCC-Inv2 cells with a proteasome inhibitor, ZLLL, and examined the expression of β-catenin by immunohistochemistry and Western blot. ZLLL treatment induced immunoprecipitation of β-catenin in the cell membrane (Fig. 4A) and accumulation of β-catenin protein by Western blot (Fig. 4B). E-cadherin protein levels did not change after ZLLL treatment. Because accumulation of p27 after ZLLL treatment was also observed, ZLLL could inhibit proteasome-mediated proteolysis. β-Catenin protein was not observed in the membrane fraction of MSCC-Inv1 and MSCC-Inv2 cells, but accumulation of membranous β-catenin was detected after ZLLL treatment (Fig. 4B). In Fig. 4A, the expression level of β-catenin was very low in the cytoplasm of both parent MSCC-1 cells and MSCC-Inv1 cells. After ZLLL treatment, accumulation of β-catenin was observed in the cytoplasm of both types of cells. This result indicates that cytoplasmic β-catenin is constitutionally degraded in both MSCC-1 parent cells and MSCC-Inv1 cells. Therefore, we used whole lysates in the following study (Fig. 4, C and D).

Accumulation of membranous β-catenin found in MSCC-Inv1 and MSCC-Inv2 cells correlated with a stabilization of the protein as shown by its half-life (Fig. 4C). These findings indicate that membranous β-catenin may be degraded by ubiquitin-mediated proteolysis in invasive and metastatic OSCC cells. These findings prompted us to test whether β-Trcp was involved in regulating membranous β-catenin stability. β-Trcp1 and β-Trcp2 are known to be involved in cytoplasmic β-catenin stability (24). To test this, we used the siRNA technique to reduce expression of β-Trcp1 and β-Trcp2 in MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells. We used an oligonucleotide that efficiently targets both β-Trcp1 and β-Trcp2, as shown previously by Northern blot analysis (24). We used IκBα protein expression as a control that showed the effect of β-Trcp siRNA. After β-Trcp siRNA treatment, phosphorylated IκBα was observed in all cells, indicating that β-Trcp was silenced. When compared with these cells transfected with a control double-stranded RNA (dsRNA) oligonucleotide, cells transfected with a dsRNA oligonucleotide corresponding to both β-Trcp1 and β-Trcp2 showed no dramatic increase in the levels of β-catenin (Fig. 4D).
E-Cadherin Methylation and β-Catenin Degradation

Next, to evaluate whether reduced expression of membranous β-catenin is observed in OSCC cases, we examined the expression of β-catenin in 10 normal oral mucosa samples, 7 primary OSCCs (tumors were surgically resected from tongue), and 17 metastatic OSCC cases (tumors were surgically resected from the cervical or submandibular lymph node). In all cases of normal oral epithelium, high expression of β-catenin was observed in the cell membrane (Fig. 4E; Table 2). Four of seven cases showed high expression of β-catenin in the noninvasive area. Interestingly, all cases showed low expression of β-catenin in the invasive area (Fig. 4F; Table 2). Moreover, most of the metastatic OSCC cases (15 of 17 cases) showed reduced expression of membranous β-catenin (Fig. 4F; Table 2). Of 15 metastatic OSCC cases showed nuclear expression of β-catenin, and only three metastatic OSCC cases showed cytoplasmic expression of β-catenin (Table 2).

**DISCUSSION**

In the present study, we isolated highly invasive OSCC cell clones MSCC-Inv1 and MSCC-Inv2 by using an *in vitro* invasion assay. Kalebic *et al.* (21) isolated highly metastatic K1735 melanoma cells by using an *in vitro* invasion assay method. Chu *et al.* (29) found that sublines selected by *in vitro* invasion assay had high invasiveness and increased colony-forming ability on soft agar. Similar to these reports, MSCC-Inv1 and MSCC-Inv2 cells showed higher invasiveness and were spindle-shaped in comparison with parent cells. In the present study, we focused on abnormalities of E-cadherin and β-catenin. In the future, we would like to discover the genes involved in the invasion and metastasis of OSCC by comparing the gene expression profiles of parent cells and highly invasive clones. We think that the method for isolation of highly invasive clones used in this study may be useful for the study of invasion and metastasis.

MSCC-Inv1 and MSCC-Inv2 cells showed reduced expression of E-cadherin and β-catenin in comparison with parent cells. These highly invasive clones acquired down-regulation of E-cadherin (Fig. 1D). This finding is supported by the previous reports (7–10) that reduced expression of E-cadherin is well associated with poor differentiation and a higher metastatic potential in OSCC. We also found that adhesion blocking by an anti-E-cadherin antibody increased the invasiveness of the parental MSCC-1 cells, and E-cadherin transfection inhibited the invasion of MSCC-Inv1 cells (Fig. 2). We suggest that down-regulation of E-cadherin may be strongly engaged in the invasion of OSCC cells. In fact, down-regulation of E-cadherin in mouse skin carcinoma cells increased motility *in vitro* and metastatic potential *in vivo* (30). Furthermore, we found that down-regulation of E-cadherin was brought about by CpG methylation around the promoter region in these highly invasive clones (Fig. 3A). Interestingly, methylation of E-cadherin was observed in cancer cells of invaded areas and metastatic lesions that showed reduced expression of E-cadherin (Fig. 3, B and C). It has been reported that the histological diffuse invasion type of OSCC showed reduced expression of E-cadherin and methylation (31). We also found that reduced expression of E-cadherin was heterogeneous within a metastatic OSCCs and that methylation was observed in metastatic cells with reduced expression of E-cadherin (Fig. 3C). Chang *et al.* found that methylation was observed in 67% of nodal metastases of OSCC cases (32). From these points of view, we hypothesize that methylation of E-cadherin may be reversible. Therefore, cancer cells with E-cadherin methylation may be focally dissociated at the invading fronts and metastasize to lymph nodes or other organs, and then demethylation may occur in metastatic cancer cells (Fig. 5). We emphasize that E-cadherin methylation may play an important role in invasion and metastasis in OSCC progression.

### Table 1 E-cadherin methylation in invasive and metastatic OSCC

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Abbreviation: LN, lymph node.
Fig. 4   Degradation of membranous β-catenin in highly invasive and metastatic OSCC cells. A, accumulation of β-catenin in cell membrane of MSCC-Inv1 cells after proteasome inhibitor treatment. We treated cells with 25 μM ZLLL for 5 h, and then expression of β-catenin was examined by immunohistochemistry in MSCC-1 and MSCC-Inv1 cells. B, accumulation of β-catenin in MSCC-Inv1 and MSCC-Inv2 cells. After ZLLL treatment, expression of β-catenin and E-cadherin was examined by Western blot analysis. We used whole lysates and membrane fraction of cells with or without ZLLL treatment. The membrane fraction of protein was extracted as described in “Materials and Methods.” Accumulation of p27 was used as a control for the effect of ZLLL. β-Actin was used to normalize Western blot analysis. p27 was also used as a negative control for the membrane fraction. C, half-life of β-catenin protein in MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells. To measure the half-life of β-catenin protein in MSCC-1, MSCC-Inv1, and MSCC-Inv2 cells, we treated cells with cycloheximide (50 μg/ml) for 0, 1, 2, and 4 h and then examined the expression of β-catenin and E-cadherin by Western blot analysis. We used whole lysates in this study. D, β-catenin is not ubiquitinated by SCF-β-Trcp. We examined the effect of knockdown of β-Trcp1 and β-Trcp2 by using β-Trcp1 siRNA. The dsRNA oligonucleotide corresponding to both β-Trcp1 and β-Trcp2 was transfected. A 21-nucleotide duplex corresponding to a nonrelevant Fbp gene was used as a control. Expression of β-catenin was examined by Western blot analysis, and we used whole lysates in this study. E, reduced expression of β-catenin in highly invasive OSCCs. Highly invasive OSCCs were surgically resected from tongue. In highly invasive OSCCs, cancer cells invaded the underlying muscle. Noninvasive area was defined as a mucosal or submucosal area without invasion of the underlying muscle. In contrast, invasive area is a submucosal area with invasion of the underlying muscle. β-Catenin expression was examined by immunohistochemistry. β-Catenin expression is shown in normal epithelial cells and OSCC cells of noninvasive and invasive areas. F, reduced expression of β-catenin in nodal metastasis of OSCC cases. All metastatic OSCC cases were surgically resected from cervical or submandibular lymph node. β-Catenin expression was examined by Immunohistochemistry.
In adherens junctions, β-catenin links E-cadherin and actin cytoskeleton (6). In addition to its critical role in cellular adhesion, β-catenin functions in the Wnt signaling pathway (14). Deregulation of the Wnt pathway has been shown to occur by several different mutational mechanisms in human cancers (14). Numerous studies showed that accumulation of β-catenin in the nucleus is frequently found, and β-catenin accumulation has been implicated in tumorigenesis in various cancers such as colon, breast, and stomach cancers (14). In these tumors, cytoplasmic β-catenin protein cannot be degraded by the ubiquitin-proteasome pathway because of abnormalities such as mutation of β-catenin and APC. On the other hand, decreased expression of β-catenin is frequently found in oral cancer (19, 32, 33). We also found reduced expression of membranous β-catenin in highly invasive clones as well as in highly invasive and metastatic OSCC cases (Fig. 4, E and F; Table 2). We think that reduction of membranous β-catenin protein may be involved in invasion and metastasis through impaired cell-to-cell adhesion. In our study, nuclear accumulation of β-catenin was not observed in OSCC cases. Interestingly, the reduced expression of membranous β-catenin was caused by the protein degradation (Fig. 4, A–C). This finding is supported by a recent report (34) that showed no β-catenin and AXIN1 gene mutations and no methylation of the CpG island of β-catenin, AXIN1, and GSK3β genes in oral cancer cells. Suzui et al. (35) also demonstrated that no β-catenin gene mutations were found in rat oral tumors induced by 4-nitroquinoline 1-oxide treatment. Cytoplasmic β-catenin is known to be phosphorylated by a multimolecule complex consisting GSK3, APC, and AXIN, and then phosphorylated β-catenin is recognized and ubiquitinated by SCFβ-Trcp (15). However, degradation of membranous β-catenin may not be ubiquitinated by SCFβ-Trcp because β-Trcp siRNA did not induce accumulation of β-catenin in the highly invasive clones (Fig. 4D). We suggest that another E3 ubiquitin ligase may be concerned with the degradation of membranous β-catenin. Recently, it has been reported that during myogenesis, OZZ-E3, a novel SOCS protein, controls the ubiquitination and degradation of the specific pools of β-catenin located at the sarcolemma (36). Because OZZ-E3 is a muscle-specific protein, future studies should be required to clarify the mechanism of membranous β-catenin degradation in OSCCs and other malignancies. This is the first report regarding the degradation of membranous β-catenin in epithelial cells, but future studies should be required to clarify the mechanism of the membranous β-catenin degradation in OSCCs and other malignancies.

In conclusion, our findings in the present study suggest the following: (a) loss of E-cadherin and β-catenin may be involved in the invasion and metastasis of OSCC cells; (b) OSCC cells with E-cadherin methylation and/or β-catenin degradation may be able to invade and metastasize (Fig. 5); and (c) the method we used for isolation of highly invasive clones may be useful for a study aimed at discovering novel genes involved in invasion.

### Table 2: Reduced expression of β-catenin in invasive and metastatic OSCC

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<thead>
<tr>
<th></th>
<th>Membrane</th>
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<tr>
<td></td>
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<td>Low</td>
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<tr>
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<td>0</td>
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<tr>
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<td>0</td>
<td>7</td>
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<tr>
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</table>

**NOTE.** β-Catenin expression was classified as high (>30%) and low (<30%).
and metastasis. We believe that E-cadherin methylation and β-catenin degradation can be a novel target for inhibition of invasion and metastasis of OSCC as well as a marker for prediction of metastasis.

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Invasion and Metastasis of Oral Cancer Cells Require Methylation of E-Cadherin and/or Degradation of Membranous β-Catenin


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