Truncated P-cadherin is produced in oral squamous cell carcinoma

Richard Bauer¹, Albert Dowejko¹, Oliver Driemel¹, A.-K. Boßerhoff² and T. E. Reichert¹

1 Department of Oral and Maxillofacial Surgery, University of Regensburg, Germany
2 Institute of Pathology, University of Regensburg, Germany

Oral squamous cell carcinoma (OSCC) is the most common cancer in the head and neck region [1]. Despite improved therapeutic intervention, the 5 year survival rate is still only 50% [2]. The poor prognosis is closely related to frequent lymph node metastasis involving migration and invasion of aberrant cells from the primary neoplasm to distant sites. Malignant alteration of cells involves various pathological steps, including changes in intercellular adhesion. Cadherins comprise an important family of adhesion molecules that form adhesive contacts between the cells of solid tissues by means of Ca²⁺-dependent homophilic interactions. They are single-pass transmembrane proteins whose extracellular sequence contains several distinctive, tandemly repeated, extracellular cadherin domains (ECs) [3]. Up to now, more than 80 members of the cadherin superfamily have been identified. Cadherin subfamilies can be divided into type I cadherins (classical cadherins containing an HAV amino acid sequence in EC1) and type II cadherins. Type I and type II cadherins are characterized by the presence of five extracellular cadherin repeats, EC1–EC5; intracellularly, they are linked to the actin cytoskeleton [4]. During embryonic development, cadherins control diverse morphogenetic processes determining tissue boundaries and separate or fuse different tissue layers, respectively. In pathological processes, they play a prominent role in tumor metastasis and cell migration [5].

Abbreviations
CK, cytokeratin; EC, extracellular cadherin domain; HOK, human oral keratinocyte; HRP, horseradish peroxidase; NHEK, normal human keratinocyte; OSCC, oral squamous cell carcinoma; Pcad50, truncated N-terminal fragment of P-cadherin with a molecular mass of 50 kDa; Pcad50biot, biotinylated truncated N-terminal fragment of P-cadherin with a molecular mass of 50 kDa; RTS, rapid transcription and translation system.
OSCC cells are malignantly transformed keratinocytes. They show a strong tendency to invade lymph nodes and spread to distant sites relatively quickly. This can be attributed to the early gain of migratory and invasive abilities of malignant cells during tumor progression [6]. One important step prior to migration and invasion is the loss of cell adhesion. Keratinocytes express two classical cadherins: E-cadherin and P-cadherin [7]. It is well known that loss of E-cadherin expression is one important step in the development of OSCC [8]. Reduction of E-cadherin correlates with reduced differentiation, and is frequently observed in undifferentiated OSCC cells [9]. In our previous work, we have found a soluble secreted 50 kDa form of P-cadherin (Pcad50) that plays a role in the progression of malignant melanoma [10,11]. We found that truncated P-cadherin is strongly involved in migration and invasion of malignant melanoma and can be considered as a diagnostic marker [11,12].

It has been shown in the literature that truncated cadherins positively or negatively influence tumor progression. Soluble E-cadherin has been shown to disrupt cell–cell adhesion in cultured epithelial cells [13]. Transfection of E-cadherin cDNA into invasive carcinoma cells leads to a significant reduction of their invasive capability in vitro [14,15], and activation of E-cadherin expression results in growth inhibition of tumor cell lines [16]. Also, T-cadherin (cadherin-13/H-cadherin), a special form of truncated cadherin anchored in the cell membrane with a glycosyl phosphatidylinositol moiety, is involved in tumor growth [17,18]. Moreover, truncated VE-cadherin has been shown to induce breast cancer cell apoptosis and growth inhibition [19].

In this study, we investigated whether soluble truncated P-cadherin produced in OSCC has any influence on cellular behavior. P-cadherin is known to be expressed in keratinocytes. However, its role in the progression of OSCC is still elusive.

Results

It is now known that several variants of cadherin play a role in the progression of various types of cancer [20]. Recent studies revealed that P-cadherin is expressed in keratinocytes and human OSCC, but most studies were based on immunohistochemical studies. Recently, Pcad50 was shown to play a role in the progression of malignant melanoma [10,11]. In this study, we concentrated on the expression of P-cadherin variants, especially Pcad50, in OSCC of the head and neck region.

Aberrantly expressed P-cadherin in vivo

An aberrantly expressed P-cadherin was detected in vivo when P-cadherin expression from normal oral mucosa was compared with that from OSCC by immunohistochemical staining. Figure 1A shows that P-cadherin is specifically located in the membrane of the basal cell layer in normal oral mucosa. In contrast, OSCC exhibits strong overall staining in the cytoplasmic and extracellular regions of malignant cells, whereas there is an increasing loss of P-cadherin in the cell membrane with progression of OSCC (Fig. 1B, arrows). Furthermore, cell lysates gained from brush biopsies of patients with OSCC were...
analyzed by western blot. In patients suffering from OSCC, among other fragments, Pcad50 was revealed (Fig. 2).

Influence of cellular differentiation on the truncation of P-cadherin

To examine P-cadherin expression in OSCC cell lines, western blot analysis was performed from cell lysates of five OSCC cell lines, normal human keratinocytes (NHEKs), and human oral keratinocytes (HOKs). Figure 3A shows the expression of full length P-cadherin (molecular mass 120 kDa) in cell lysates of all controls and three OSCC cell lines (PCI 13, PCI 68, and PCI 1). Additionally, several truncated versions of P-cadherin, including Pcad50, were detected in all OSCC cell lines. Figure 3B shows that Pcad50 was secreted, as the supernatants of PCI 13 and PCI 68 produced an abundant amount of Pcad50 as compared to the control NHEKs. Up to now, Pcad50 has only been detected in malignant melanoma [10]. In RT-PCR analysis, the correct lengths of exon-spanning coding sequences of P-cadherin exons 2–3, 5–8, 8–10, 10–11, 11–12 and 15–16 could be detected in all OSCC cell lines (exemplified by PCI 13 in Fig. 3C), meaning that mRNA splicing can be ruled out as a potential mechanism behind the production of Pcad50 in OSCC. Interestingly, Pcad50 showed up in the cell lysates and in the supernatants of HOKs (Fig. 3A,B). Because HOKs were cultured from embryos, we assumed that Pcad50 could originate from undifferentiated cells. To
confirm this notion, we analyzed the expression level of cytokeratin (CK) markers usually described for undifferentiated/proliferating and differentiated/differentiating cells.

Figure 4A shows the expression of CK markers for both differentiated cells and undifferentiated cells in four out of six examined cell lines (HOKs, PCI 13, PCI 68, and PCI 1), meaning that these cell lines consist of cell populations still capable of differentiating. In two cell lines (PCI 4 and PCI 52), only markers for undifferentiated or proliferating cells could be detected; these cell lines can obviously not differentiate at all. Interestingly, the latter largely generated P-cad50 (Fig. 3). To further corroborate this result, P-cadherin immunodetection was performed by western blot analysis with cell lysates from sparsely grown and 100% confluent cells. Additionally, terminal differentiation was induced by raising the Ca2+ concentration in the media from 0.07 mM to 1.5 mM for 48 h [according to the manufacturer’s instructions (ScienCell, Carlsbad, CA, USA)] [21]. Figure 4A shows an increase in Pcad50 in cell lysates from sparsely grown cell culture as compared to confluent cell culture or terminally differentiated cells, respectively. In cells still expressing full-length P-cadherin and capable of differentiation, Pcad50 disappeared when the cells were grown to 100% confluence; in contrast, the cell line PCI 52, although grown to 100% confluence, still produced Pcad50.

Functional influence of Pcad50 on OSCC cells

To investigate the functional influence of Pcad50 on OSCC cells, we generated a biotinylated version of Pcad50 (Pcad50biot) by cell-free recombinant expression via rapid transcription and translation system (RTS) (Fig. 5A). Biotinylation was used to enable detection of the protein. Subsequently, we treated the cells with the recombinant protein and analyzed their behavior in terms of migration, cell aggregation, and proliferation. To demonstrate that the recombinant fragment has biological activity, i.e. is able to directly interact with full-length P-cadherin, an immunoprecipitation experiment was performed using the cell lysates from OSCC cell lines PCI 13 and PCI 52. Figure 5B shows direct interaction with full-length P-cadherin from the OSCC cell line PCI 13, whereas there is no detectable 120 kDa band for full-length-deficient PCI 52.

The wound healing assay in Fig. 6A demonstrates that OSCC cells expressing full-length P-cadherin (PCI 13) migrate 20–50% faster under the influence of Pcad50biot at dilutions of 1 : 100 and 1 : 1000 as compared to the control without Pcad50biot. However, Pcad50biot did not show any effect on OSCC cells that exhibited low or no expression of full-length P-cadherin (PCI 52), meaning that Pcad50 could interfere with normal homophilic cell–cell adhesion, disrupt cellular integrity, and thus lead to a more migratory phenotype (Fig. 6B). To corroborate the results of the positive effect of truncated P-cadherin on the migration of tumor cells, a Boyden chamber migration assay was performed. Figure 6C shows a significant increase of 150–270% in the migration of two different squamous cell carcinoma cell lines, PCI 13 and PCI 68 (both still expressing full-length P-cadherin), when treated with Pcad50. Figure 6C also shows a significant influence of Pcad50 on normal cells (NHEKs). When they were treated with Pcad50biot at dilutions of 1 : 1000 and 1 : 100, there was an increase in cell migration of 200–235% as compared to control cells without Pcad50biot treatment.
When taken into 3D cell culture, OSCC cells typically form tight spheroids within 2 days. To investigate whether Pcad50biot exerted any influence on the formation and compaction of spheroids, cells were treated with the truncated protein in different dilutions and pelleted in concave 96-well plates. Figure 7A shows a significant increase in cell diameter in treated 3D cell pellets as compared to untreated cell pellets, meaning that Pcad50biot managed to diminish cell compaction in 3D cell culture. Figure 7B shows electron microscope images of a PCI 13 pellet treated with Pcad50biot and an untreated control. The overall appearance of the Pcad50biot-treated cell line shows wider intercellular gaps with disrupted adhesion complexes as compared to the untreated control cell line without treatment, supporting the notion that truncated P-cadherin is able to weaken cell–cell contacts by competing with the homophilic interaction of full-length cadherin. To confirm that the increase in diameter was not due to Pcad50biot-induced cell proliferation, we performed 2D and 3D cell proliferation assays [based on 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and picogreen measurement, respectively]. Figure 8 shows that there is no influence of Pcad50biot on cell proliferation in 2D (Fig. 8A) or 3D (Fig. 8B) cell culture. Moreover, to proof that cell adhesion can be abrogated by truncated P-cadherin, 2 × 10^5 OSCC cells were incubated with Pcad50biot, and flow cytometric analysis was performed over a period of 4 h (Fig. 9A). Statistical analysis of 2 × 10^4 cells revealed only a 3.4% increase in cell aggregation with a dilution of 1 : 100 Pcad50biot. In contrast, there was a 10.7% increase in cell aggregation with a dilution of 1 : 1000 Pcad50biot and a 12% increase in cell aggregation in the untreated control. In summary, relating the data to the untreated control, the experiment revealed 11–72% diminished cell aggregation after 4 h in probes treated with dilutions of 1 : 100 and 1 : 100 Pcad50biot.

Discussion

In this study, we investigated the expression of P-cadherin in OSCC cell lines and cells from patients suffering from OSCC. We detected truncated P-cadherin in samples of brush biopsies. One patient (patient 38) showed abundant expression of Pcad50. Interestingly, this patient suffered from a recurrent OSCC, meaning that Pcad50 could serve as potential marker for this disease. Among other fragments, Pcad50 was found in dedifferentiating OSCC cells. We recently found Pcad50 in malignant melanoma [10]. We recombinantly expressed Pcad50 and found that it had a significant functional influence on cell aggregation and migration of OSCC cell lines. Here we found full-length (120 kDa) P-cadherin and Pcad50 in OSCC cell lines and their lysates. Recently, it has been shown that truncated variants of cadherins natively generated by mutations, splicing or shedding, respectively, are important determinants in developmental remodeling and differentiation events; furthermore, has become apparent that truncation of proteins can be important factors during the progression of diseases [22–27]. Pcad50 was found abundantly in the supernatants of the cell lines. In recent studies, we have also shown
that Pcad50 plays a role in the progression of malignant melanoma [10,11].

Interestingly, together with the full-length protein, Pcad50 was also expressed and secreted in HOKs, in contrast to NHEKs. Closer examination revealed that the primary cell line HOK is derived from embryonic cells (ScienCell, personal communication). This result indicates that Pcad50 might play a role in undifferentiated cell populations and is utilized to maintain a dynamic epithelial architecture for tissue remodeling during development. In malignantly transformed cells, however, dedifferentiation is closely linked to tumor progression [28]. The observation of a loss of full-length P-cadherin and an increase in Pcad50 during the dedifferentiation process of OSCC cell lines suggests a link between P-cadherin expression and cellular differentiation. To corroborate this hypothesis, the OSCC cell lines were characterized by analyzing the expression of CKs by RT-PCR, thus determining the state of differentiation or dedifferentiation. For this purpose, CK5, CK14 and CK19 were used as markers for proliferating or poorly differentiated cells [29–32]. CK10 and involucrin were used as markers for differentiating and terminally differentiated cells [32,33]. According to the cytokeratin expression data, most of the OSCC cell lines comprised cell populations of both differentiating and dedifferentiated cells. Our results show that cells capable of terminal differentiation initiated either by confluency or increasing Ca²⁺ concentration express full length P-cadherin. In contrast, the cell lines not capable of progressing to a terminal differentiation state (i.e. PCI 52) hardly express any full length P-cadherin. As described in the literature, cadherins are involved in differentiation. Wertz et al. reported cdh-16 to be responsible for the differentiation of kidney, lung and sex duct epithelia [34]. Moreover, E-cadherin expression inversely correlates with tumor dedifferentiation in OSCC [35]. Our results suggest that the full-length version of P-cadherin is also involved in the regulation of differentiation in OSCC cells. The suggestion that P-cadherin is engaged in this event is undermined by the knockout phenotype of P-cadherin-deficient mice. Loss of P-cadherin in myoepithelial cells of knockout mice leads to precocious alveolar differentiation of their mammary glands. Furthermore, histological examination of the tissue revealed focal hyperplasia and ductal dysplasia in the mutant mice [36,37]. The cell line PCI 52 is not able to differentiate by means of confluency, and contains only dedifferentiated cell populations with a highly expressed marker, CK19, for poor differentiation [31]. PCI 52 does not express full-length P-cadherin and constitutively generates Pcad50 under conditions of

---

**Fig. 6.** Influence of Pcad50biot on cell migration. (A) Wound healing assay of OSCC cell line PCI 13 treated with Pcad50biot. OSCC cells containing full-length P-cadherin (i.e. PCI 13) migrate significantly faster (25–40%) when treated with different dilutions of Pcad50biot. (B) Different dilutions of P-cad50biot did not have any effect (5–10%) on OSCC cells without full-length P-cadherin (PCI 52). The migration of cells was measured over a period of 24 h. One hundred per cent represents full closure of the wound. (C) Boyden chamber migration assay. A significant influence can be seen of 1 : 100 and 1 : 1000 dilutions of Pcad50biot on the migratory behavior of OSCC cell lines PCI 13 and PCI 68 and NHEKs.
both sparse growth and confluent growth. This corroborates the result that without full-length P-cadherin, the cells are not able to differentiate.

To investigate the functional influence of Pcad50 on OSCC cell lines, cells were treated with Pcad50biot. We found an interaction between Pcad50biot and full-length P-cadherin. Both wound healing assays and Boyden chamber assays revealed that recombinant Pcad50biot significantly enhanced cell migration in OSCC cell lines that contained full-length P-cadherin (i.e. PCI 13 and PCI 68), and was even able to trigger migration in NHEKs. However, Pcad50biot did not exert any influence on the migration of the full-length-deficient cell line PCI 52, meaning that Pcad50 might competitively interact with the adhesion complexes of full-length P-cadherin and thus facilitate migration. It has been shown by Chappuis-Flament et al. [38] that homophilic interactions of cadherins are mediated not only by EC1, but also by multiple extracellular repeats; although our recombinant Pcad50biot is N-terminally biotinylated, it might be capable of interacting laterally with EC2 and EC3, and may even disturb the homodimerization of cadherins, abrogating cell–cell contacts. The fact that Pcad50 needs full-length P-cadherin to exert an effect shows that Pcad50 might play an important role in cell migration, especially at the early stages of OSCC tumor progression, when full-length P-cadherin is still expressed on the cell surface and Pcad50 is being
secreted from cells. There is evidence that soluble and truncated forms of E-cadherin play an important role in the development of cancer. Increased soluble E-cadherin has been shown to contribute to melanoma progression [39]. Furthermore, an impact on cell adhesion and migration of truncated E-cadherin has been shown by Maretzky et al., who reported that ADAM-10-regulated shedding of this protein is associated with epithelial cell–cell adhesion, migration and β-catenin translocation in fibroblasts and keratinocytes [40]. Proteolytic cleavage of E-cadherin has also been reported in prostate and mammary epithelial cells [41]. In the context of OSCC, aberrant cells might be able to produce proteases capable of processing full-length P-cadherin intracellularly, leading to a truncated 50 kDa form that is secreted and thus might be able to trigger the abrogation of intact tissue architecture. In contrast to malignant melanoma in OSCC, a spliced mRNA variant can be ruled out as potential mechanism for the production of truncated P-cadherin, as our RT-PCR experiments revealed exon-spanning coding sequences for all relevant exons in the cell lines. Pcad50 is also expressed and secreted in normal undifferentiated oral embryonic keratinocytes.

As a conclusion, the generation of Pcad50 during embryonic development could be a controlled event that leads to a more migratory phenotype capable of accommodating epithelial growth until the cells are in contact which each other or start to differentiate. However, as a consequence of cellular dedifferentiation at the onset of OSCC progression, Pcad50 could be generated and facilitate disaggregation and cell migration. This hypothesis is also supported by our cell aggregation assays and electron microscopic images of Pcad50biot-treated cell lines showing that Pcad50biot was able to attenuate the formation of tight aggregates by causing disruption of cell–cell adhesion. Taken together, our results confirm the hypothesis that during dedifferentiation of aberrant cells, Pcad50 might competitively interfere with the interaction of membrane-bound full-length P-cadherin of adjacent cells, weakening tissue architecture and thus facilitating migration in OSCC. How the interference takes place is still elusive. Further investigations are needed to determine whether trans-interaction or cis-interaction takes place to abrogate cell–cell contacts.

In summary, our results suggest a role for Pcad50 in the progression of OSCC in vitro and in vivo, facilitating migration and weakening cellular aggregation; thus, Pcad50 could be considered as a diagnostic marker.

**Experimental procedures**

**Protein analysis in vitro (western blotting)**

Prior to lysis, cells were scraped off with a cell scraper. No trypsinization was carried out. For protein isolation, 2 x 10^6 cells were washed with 1x NaCl/Pi, lysed in 200 μL of RIPA buffer (Roche Applied Science, Mannheim, Germany), and incubated for 15 min at 4 °C. RIPA buffer with a cocktail of protease inhibitors was used. Insoluble material was removed by centrifugation at 15 000 g for 10 min, and the cell lysate was immediately shock frozen and stored at −80 °C. Furthermore, cell culture supernatant
was analyzed by western blotting. Here, 2 mL of cell culture supernatant was concentrated to 150 μL with a SpeedVac. The protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA). Balanced amounts of cell proteins (40 μg) were denatured at 70 °C for 10 min after addition of Roti-load-buffer (Roth, Karlsruhe, Germany), and subsequently separated on NuPAGE-SDS gels (Invitrogen, Karlsruhe, Germany). After transfer of the proteins onto poly(vinylidene difluoride) membranes (Bio-Rad, Munich, Germany), the membranes were blocked in 3% BSA/NaCl/Pi with Tween (150 mM NaCl, 100 mM Tris, 0.1% Tween-20) for 1.5 h and incubated with a 1 : 10 000 dilution of primary monoclonal mouse antibody to P-cadherin (P-cadherin N-terminal; BD Transduction Laboratory, Heidelberg, Germany) or β-actin (1 : 5000; Sigma, Hamburg, Germany) overnight at 4 °C. A 1 : 3000 dilution of antibody to mouse horseradish peroxidase (HRP) (Pierce) was used as a secondary antibody. Staining was performed using ECL Substrate (Pierce). All of the experiments were repeated at least three times, with similar results.

Cell lines and culture conditions

PCI 13-1: this cell line was established from a male patient who suffered from low-grade OSCC of the retromolar triangle. PCI 1-1: the origin of this cell line was a larynx carcinoma of the glottis; it was harvested from a male patient. PCI 52: this tumor originated from the aryepiglottic fold of a male patient; it was a primary carcinoma.

PCI 68: this cell line was established from a primary tongue carcinoma of a male patient. PCI 4: this cell line was established from male patient with a primary carcinoma at the root of the tongue.

NHEKs

The adult NHEK cell line was obtained from PromoCell GmbH (Heidelberg, Germany). The cell line was established using adult keratinocytes. Cell culturing was carried out according to the manufacturer’s instructions.

HOKs

This cell line was obtained from Sciencell (San Diego, CA, USA) and was delivered by PromoCell GmbH. The cell line is of fetal origin. Cell culturing was carried out according to the manufacturer’s instructions.

Expression of Pcad50biot

A prokaryotic expression vector with the sequence for Pcad50 and a 15 amino acid Avi-tag peptide sequence was constructed by overlap extension PCR. Primers were used with the following sequences: forward primer 5’-GCTAC CAT ATG GAG GGT TTA AAC GAT ATT TTC GAG GCT CAG AAA ATC GAA TGG CAC GAA GAT TGG GTG GGT CTA-3’, comprising an NdeI restriction
site and the coding sequence for an Avi-tag; and reverse primer 5′-GAC GGA TCC TCA GTA GAC ACA CAC AGG CTC-3′, with a BamHI restriction site. The coding sequence contained the immunogenic N-terminal region for the monoclonal P-cadherin antibody (BD Transduction Laboratories) and did not contain the P-cadherin transmembrane domain and the C-terminal intracellular domain. The length of the construct was calculated such that the resulting peptide had a molecular mass of 50 kDa without the signal peptide sequence. The Pcad50biot cDNA construct was cloned into the vector pIVEX2.3-MCS (Roche Applied Science, Mannheim, Germany). By addition of biotin, ATP, and the vector was used in the rapid translation system, a cell-free Escherichia coli-based protein transcription/translation system (Roche Applied Science). By addition of biotin, ATP, and the E. coli biotin protein ligase BirA during the procedure, the protein was biotinylated at the introduced Avi-tag at the N-terminus. The correct function and folding of the protein was tested by performing functional assays.

**Coimmunoprecipitation with Pcad50biot**

For coimmunoprecipitation, 150 µg cell lysates dissolved in binding buffer (20 mM NaPO₄, 150 mM NaCl, pH 7.5) were precleared with 25 µL of protein streptavidin-coupled Sepharose (GE Healthcare, Munich, Germany) at 4°C overnight. After centrifugation at 250 g, the supernatant was transferred into a fresh vial and incubated with Pcad50biot with shaking at 4°C overnight. Fifty microliters of protein streptavidin-coupled Sepharose was added for 1 h, pelleted, washed three times with binding buffer, resuspended in 20 µL of Laemmli buffer, heated at 95°C for 5 min, and subjected to western blot analysis on 10% SDS/PAGE gels. Detection was performed as described above. The first antibody was monoclonal antibody to P-cadherin (BD Transduction Laboratories).

**RNA isolation and RT-PCR**

Expression of mRNA was detected by RT-PCR. Total RNA from the tumor cell lines examined was extracted using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The isolated RNA was stored at −20°C until reverse transcription. First-strand cDNA was synthesized from 2 µg of total RNA using dN6 random primers (Roche Pharma AG, Munich, Germany) and reverse transcription with Superscript II (Invitrogen). cDNA was incubated with 1 µL of RNaseA (Roche Pharma AG) for 60 min at 37°C. The cDNA was stored at −20°C until RT-PCR analysis. RNA integrity was tested by RT-PCR of the housekeeping gene β-actin. Specific RT-PCR detection of P-cadherin, CK5, CK14, CK19, CK10, involucrin and β-actin was performed with the primers listed in Table 1. The primers were obtained from TibMolBiol (Berlin, Germany). The ideal annealing temperature of primers was defined by a gradient RT-PCR (52–72°C in 12 steps). The following program was used for primers: initial denaturation at 94 °C for 5 min, 33 cycles of amplification with denaturation at 94 °C for 1 min, primer annealing for 1 min and elongation at 72 °C for 1 min, and a final elongation at 72°C for 10 min. The synthesized RT-PCR products were separated by electrophoresis in an agarose gel, stained with ethidium bromide, and visualized with UV light.

**Acquisition and analysis of flow cytometry data**

Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with 488 nm blue and 633 nm red diode lasers. Data analysis was carried out using FACSDIVA software and winMDI 2.9. OSCC cells were dissociated with Accutase (PAA Laboratories GmbH, Cölbe, Germany) and washed in NaCl/P₁. As analyzed by western blotting, Accutase did not exert any effect on P-cadherin in normal epidermal keratinocytes (Fig. 9B). Cells (2 × 10⁶) were seeded in FACS vials (BD Falcon, Heidelberg, Germany) and gently resuspended in DMEM. Single cells were generated, and 2 × 10⁶ cells were treated with dilutions of 1 : 100 and 1 : 1000 Pcad50biot and analyzed directly (Tₒ) and after 1, 2, 3 and 4 h. Immediately prior to the analysis, cells were incubated with fresh propidium iodide. For calculating statistics, only living cells were used, gating propidium iodide-negative cells. As a measure of cell aggregation, forward scatter was used on the y-axis. Quadrant markers were used to distinguish single from aggregated cells.

**Immunohistochemistry**

Paraffin-embedded preparations of normal mucosa and OSCC were stained for P-cadherin protein expression with the Envision/HRP system (DAKO, Carpinteria, CA, USA). The tissues were deparaffinated, rehydrated, and subsequently incubated with primary monoclonal P-cadherin antibody (1 : 100; BD Transduction Laboratories) overnight at 4°C. The secondary antibody attached to a dextran backbone carrying the HRP was incubated for 30 min at room temperature. Antibody binding was visualized using dextran/HRP solution. Finally, the tissues were counterstained with hematoxylin.

**Brush biopsies**

Lesions from patients suffering from OSCC were scraped with a brush (Cytophrough Plus GT non-sterile; Medscand Medical AB, Malmö, Sweden), applying pressure and rotation. The cells harvested were transferred to a tube containing NaCl/P₁ and pulse-vortexed. The brush was
Table 1. Primers for P-cadherin, differentiated and undifferentiated cell lines. For better legibility some letters are lower case.

| Primer name | Sequence (5’-3’) | Annealing temperature (°C) |
|-------------|-----------------|--------------------------|
| Forward primer |                 |                          |
| Ck10        | GGATGAGCTGACCTGACCAA | 60                       |
| Involucrin   | TGGCTCTCATCCALEDGAGTC | 58                       |
| Ck5         | TTCTTTTATAGGGAAGAATGCA | 60                       |
| Ck19        | AGGTCTGATCCGCTCAGGCA | 61                       |
| p-cad2–3    | TCAAGGGAGCTGAAAGCTAGC | 59                       |
| p-cad5–8    | GAGGAGTGGGCTGTTGCTC | 60                       |
| p-cad9–10   | CCAGGCCACAGCATGATC | 59                       |
| p-cad10–11  | TCCCAAGTGCTTGATC | 60                       |
| p-cad11–12  | AgAAGGTTGAGGAGAAGACA | 60                       |
| p-cad15–16  | TGCAATACACGAGTCCTCA | 59                       |
| Reverse primer |               |                          |
| Ck10        | GCGACATTCATTCCAGTTTAC | 60                       |
| Involucrin   | ATTTTCTCAAGCCTTGGTCTC | 58                       |
| Ck5         | CCAGCGATCCGTCTGCTTGCAC | 60                       |
| Ck19        | ATCTTTCTGCTCCCTAGTCAC | 61                       |
| p-cad2–3    | gCCATTCCgCACAgTgAAg | 59                       |
| p-cad5–8    | GCCTGGATGGTCAGTGTGTA | 60                       |
| p-cad9–10   | AGGTCTGATCCGAGTCCTCA | 59                       |
| p-cad10–11  | AgAAGGTTGAGGAGAAGACA | 60                       |
| p-cad11–12  | AgAAGGTTGAGGAGAAGACA | 60                       |
| p-cad15–16  | CCCACCTGCTCCAGATACAG | 59                       |

removal, and the cells were centrifuged for 5 min at 1500 g. Cells were lysed in 50 μL of RIPA buffer (Roche Pharma AG), and the protein concentration was measured with the bicinchoninic acid assay.

Wound healing assay (scratch assay)

Cells were cultured to confluence (> 90%) in six-well dishes. On the bottom of each dish, a horizontal line was drawn with a marker. Perpendicular to this line, two separate wounds were scratched with a sterile 1 mL pipette tip. The cells were rinsed with NaCl/Po, which was replaced by DMEM containing 10% fetal bovine serum and dilutions of recombinant Pcad50biot, depending on the experimental procedure. Using phase contrast microscopy with ×10 magnification, images were taken at time 0 (T0) and after 12 h and 24 h, and the gaps were measured. After each measurement, the old medium was replaced with fresh medium. All experiments were repeated three times. Statistical analysis was carried out by one-way ANOVA and Dunnett’s test.

Migration assay (Boyd en chamber)

The migration assays were performed using Boyden chambers containing polycarbonate filters coated with gelatine, as previously described [42]. The lower compartment was filled with fibroblast-conditioned medium, used as a chemoattractant. OSCC cells were harvested by trypsinization for 5 min, resuspended in DMEM without fetal bovine serum at a density of 30 000 cells·mL⁻¹ with dilutions of recombinant Pcad50 according to the experimental procedure, and placed in the upper compartment of the chamber. After incubation at 37 °C for 4 h, the filters were collected, and the cells adhering to the lower surface were fixed, stained, and counted. Results were repeated three times with similar results.

Electron microscopy

OSCC cells (1 × 10⁶) were seeded in a 96-well plate coated with 1% agarose. A 3D culture was made by centrifuging the plates at 50 g for 1 min and incubating the cells for 2 days at 37 °C in a 5% CO2 atmosphere. Electron microscopy was performed by the Central Laboratory for Electron Microscopy, at the institute of Pathology, University of Regensburg, Germany, essentially as described previously [43].

Cell aggregation assay

After dissociation of the cells with Accutase (PAA Laboratories GmbH) and one washing step, 8000 OSCC-cells/well were seeded in a 96-well culture plate in a volume of 200 μL. Cells were treated with different dilutions, 1 : 10 to 1 : 10 000, of the recombinant protein Pcad50biot. Plates were centrifuged at 50 g for 1 min. After day 1, day 2 and day 3, images were taken, and the area of the aggregates was measured under a light microscope at ×4 magnification.

Cell proliferation assays

Double-stranded DNA measurement was performed with a PicoGreen dsDNA assay kit (Invitrogen) according to the manufacturer’s instructions. Cell proliferation measurement was performed with a CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI, USA).

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (DFG, Grant BA 3696/1-1).

References

1. Lutz BS (2002) Aesthetic and functional advantages of the anterolateral thigh flap in reconstruction of tumor-related scalp defects. Microsurgery 22, 258–264.
Truncated P-cadherin in oral squamous cell carcinoma

2 Bray F, Sankila R, Ferlay J & Parkin DM (2002) Estimates of cancer incidence and mortality in Europe in 1995. *Eur J Cancer* **38**, 99–166.

3 Gumbiner BM (2005) Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol* **6**, 622–634.

4 Hirano S, Nose A, Hatta K, Kawakami A & Takeichi M (1987) Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *J Cell Biol* **105**, 2501–2510.

5 Wheelock MJ & Johnson KR (2003) Cadherin-mediated cellular signaling. *Curr Opin Cell Biol* **15**, 509–514.

6 Baatenburg de Jong RJ, Hermans J, Molenaar J, Briëtte JJ & le Cesse S (2001) Prediction of survival in patients with head and neck cancer. *Head Neck* **23**, 718–724.

7 Takeichi M (1995) Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol* **7**, 619–627.

8 Sakaki T, Wato M, Tamura I, Nakajima M, Morita S, Kakudo K, Shirasu R, Tanaka A & Sakaki T (1999) Correlation of E-cadherin and alpha-catenin expression with differentiation of oral squamous cell carcinoma. *J Osaka Dent Univ* **33**, 75–81.

9 Mattijsen V, Peters HM, Schalkwijk L, Manni JJ, van’t Hof-Grootenboer B, de Mulder PH & Ruiter DJ (1993) E-cadherin expression in head and neck squamous-cell carcinoma is associated with clinical outcome. *Int J Cancer* **55**, 580–585.

10 Bauer R, Hein R & Bosserhoff AK (2005) A secreted form of P-cadherin is expressed in malignant melanoma. *Exp Cell Res* **305**, 418–426.

11 Bauer R & Bosserhoff AK (2006) Functional implication of truncated P-cadherin expression in malignant melanoma. *Exp Mol Pathol* **81**, 224–230.

12 Bauer R, Wild PJ, Meyer S, Bataille F, Pauer A, Klinkhammer-Schalke M, Hofstaedter F & Bosserhoff AK (2006) Prognostic relevance of P-cadherin expression in melanocytic skin tumours analysed by high-throughput tissue microarrays. *J Clin Pathol* **59**, 699–705.

13 Ranscht B & Dours-Zimmermann MT (1991) T-cadherin, a novel cadherin cell adhesion molecule in the nervous system lacks the conserved cytoplasmic region. *Neuron* **7**, 391–402.

14 Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D & Birchmeier W (1991) E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* **113**, 173–185.

15 Vleminckx K, Vakael L Jr, Mareel M, Fiers W & van Roy F (1991) Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* **66**, 107–119.

16 Watabe M, Nagafuchi A, Tsukita S & Takeichi M (1994) Induction of polarized cell–cell association and retardation of growth by activation of the E-cadherin–catenin adhesion system in a dispersed carcinoma line. *J Cell Biol* **127**, 247–256.

17 Hebbard LW, Garlatti M, Young LJ, Cardiff RD, Oshima RG & Ranscht B (2008) T-cadherin supports angiogenesis and adiponectin association with the vasculature in a mouse mammary tumor model. *Cancer Res* **68**, 1407–1416.

18 Lee SW (1996) H-cadherin, a novel cadherin with growth inhibitory functions and diminished expression in human breast cancer. *Nat Med* **2**, 776–782.

19 Shi XY, Lu H, Li WL, Tang HL, Xiong JJ, Zhang JQ, Opolon P, Legrand C, Perricaudet M & Li H (2006) A soluble truncated cadherin induces breast cancer cell apoptosis and growth inhibition. *J Cancer Res Clin Oncol* **132**, 561–571.

20 Masterson J & O’Dea S (2007) Posttranslational truncation of E-cadherin and significance for tumour progression. *Cells Tissues Organs* **185**, 175–179.

21 Darmstadt GL, Fleckman P, Jonas M, Chi E & Rubens CE (1998) Differentiation of cultured keratinocytes promotes the adherence of *Streptococcus pyogenes*. *J Clin Invest* **101**, 128–136.

22 Wada T, Wakabayashi Y, Takahashi S, Ushiki T, Kikkawa Y, Yonekawa H & Kominami R (2001) A point mutation in a cadherin gene, Cdh23, causes deafness in a novel mutant, Waltzer mouse niga. *Biochem Biophys Res Commun* **283**, 113–117.

23 Kawaguchi J, Takeda S, Kashima T, Imai T, Machi-nami R & Kudo A (1999) Expression and function of the splice variant of the human cadherin-11 gene in sub-ordination to intact cadherin-11. *J Bone Miner Res* **14**, 764–775.

24 Dussaux-Garin L, Blayau M, Pagenuault M, Le Berre-Heresbach N, Raouf JL, Campion JP, David V & Bretagne JF (2001) A new mutation of E-cadherin gene in familial gastric linitis plastica cancer with extra-digestive dissemination. *Eur J Gastroenterol Hepatol* **13**, 711–715.

25 Tschau MP, Fischer KM, Fung VS, Piri nia F, Borner MM, Fey MF, Tobler A & Torbett BE (2003) Alternative splicing of the human cyclin D-binding Myb-like protein (hDMP1) yields a truncated protein isoform that alters macrophage differentiation patterns. *J Biol Chem* **278**, 42750–42760.

26 Davies ML, Roberts GT, Spiller DG & Wakeman JA (2004) Density-dependent location and interactions of truncated APC and beta-catenin. *Oncogene* **23**, 1412–1419.

27 Fei Q, Boden SD, Sangadala S, Vigg eswarapu M, Liu Y & Titus L (2007) Truncated human LMP-1 triggers differentiation of C2C12 cells to an osteoblastic phenotype in vitro. *Acta Biochim Biophys Sin (Shanghai)* **39**, 693–700.

28 Fusenig NE, B€urkrechtz D, Boukamp P, Tomakidi P & Stark HJ (1995) Differentiation and tumor progression. *Recent Results Cancer Res* **139**, 1–19.
29 Fillies T, Werkmeister R, Packeisen J, Brandt B, Morin P, Weingart D, Joos U & Buerger H (2006) Cytokeratin 8/18 expression indicates a poor prognosis in squamous cell carcinomas of the oral cavity. BMC Cancer 13, 6–10.

30 Hsieh CB, Chen CJ, Yu JC, Chang TM, Gao HW & Liu YC (2005) Primary squamous cell carcinoma of the liver arising from a complex liver cyst: report of a case. Surg Today 35, 328–331.

31 Ram Prasad VV, Nirmala NR & Kotian MS (2005) Immunohistochemical evaluation of expression of cytokeratin 19 in different histological grades of leukoplakia and oral squamous cell carcinoma. Indian J Dent Res 16, 6–11.

32 Gosselin F, Magloire H, Joffre A & Portier MM (1990) Cytokeratins as molecular markers in the evaluation of the precise differentiation stage of human gingival epithelium reconstituted in vitro. Arch Oral Biol 35(Suppl), 217S–221S.

33 Kannan S, Balaram P, Chandran GJ, Pillai MR, Mathew B, Nalinakumari KR & Nair MK (1994) Alterations in expression of terminal differentiation markers of keratinocytes during oral carcinogenesis. Pathobiology 62, 127–133.

34 Wertz K & Herrmann BG (1999) Kidney-specific cadherin (cdh16) is expressed in embryonic kidney, lung, and sex ducts. Mech Dev 84, 185–188.

35 Frixen UH & Nagamine Y (1993) Stimulation of urokinase-type plasminogen activator expression by blockage of E-cadherin-dependent cell-cell adhesion. Cancer Res 53, 3618–3623.

36 Radice GL, Ferreira-Cornwell MC, Robinson SD, Rayburn H, Chodosh LA, Takeichi M & Hynes RO (1997) Precocious mammary gland development in P-cadherin-deficient mice. J Cell Biol 139, 1025–1032.

37 Cardiff RD & Muller WJ (1993) Transgenic mouse models of mammary tumorigenesis. Cancer Surv 16, 97–113.

38 Chappuis-Flament S, Wong E, Hicks LD, Kay CM & Gumbiner BM (2001) Multiple cadherin extracellular repeats mediate homophilic binding and adhesion. J Cell Biol 154, 231–243.

39 Billion K, Ibrahim H, Mauch C & Niessen CM (2006) Increased soluble E-cadherin in melanoma patients. Skin Pharmacol Physiol 19, 65–70.

40 Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D & Saftig P (2005) ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. Proc Natl Acad Sci USA 102, 9182–9187.

41 Rios-Doria J, Day KC, Kuefer R, Rashid MG, Chinnaiyam AM, Rubin MA & Day ML (2003) The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. J Biol Chem 278, 1372–1379.

42 Kawano Y, Yoshimura T & Kaibuchi K (2002) Involvement of small GTPase Rho in cardiovascular diseases. Nippon Yakurigaku Zasshi 120, 149–158.

43 Kunz-Schughart LA, Schroeder JA, Wondrak M, van Rey F, Lehle K, Hofstaedter F & Wheatley DN (2006) Potential of fibroblasts to regulate the formation of three-dimensional vessel-like structures from endothelial cells in vitro. Am J Physiol Cell Physiol 290, C1385–C1398.