RESEARCH ARTICLE

Proteomic analysis of human papillomavirus-related oral squamous cell carcinoma: Identification of thioredoxin and epidermal-fatty acid binding protein as upregulated protein markers in microdissected tumor tissue

Christian Melle¹, Günther Ernst¹, Robert Winkler²*, Bettina Schimmel¹, Jens Peter Klussmann³, Claus Wittekindt⁴, Orlando Guntinas-Lichius⁴ and Ferdinand von Eggeling¹

¹ Core Unit Chip Application (CUCA), Institute of Human Genetics and Anthropology, Medical Faculty, Friedrich Schiller University, Jena, Germany
² Department of Biomolecular Chemistry, Leibniz Institute for Natural Products Research and Infection Biology – Hans-Knöll-Institute, Jena, Germany
³ University Hospital, Department of Otorhinolaryngology, Head and Neck Surgery, Medical Faculty, University Cologne, Köln, Germany
⁴ Department of Otorhinolaryngology, Medical Faculty, Friedrich Schiller University, Jena, Germany

Human papillomavirus (HPV) infection has been identified as an etiologic agent for a subset of oral squamous cell carcinoma (OSCC) with increasing incidence. HPV DNA-positivity may confer better prognosis but the related oncogenic mechanisms are unknown. For the identification of HPV relevant proteins, we analyzed microdissected cells from HPV DNA-positive (n = 17) and HPV DNA-negative (n = 7) OSCC tissue samples. We identified 18 proteins from tumor tissues by peptide fingerprint mapping and SELDI MS that were separated using 2-DE. Among a number of signals that were detected as significantly different in the protein profiling analysis, we identified thioredoxin (TRX) and epidermal-fatty acid binding protein as upregulated in HPV related tumor tissue. This study, investigating for the first time proteomic changes in microdissected HPV infected tumor tissue, provides an indication on the oncogenic potential of viruses.

Keywords:
Biomarker / Head and neck cancer / HPV / Immunohistochemistry / SELDI-TOF MS

1 Introduction

Oral squamous cell carcinomas (OSCC) arise from the mucosa of the oral cavity and the oropharynx. There is increasing evidence that a subset of OSCC is associated with oncogenic human papillomavirus (HPV) infection, in particular HPV type 16 [1]. These HPV-positive OSCC differ from HPV-negative tumors in several biological and clinical aspects including molecular alterations and prognosis indicating that the former group represents a separate tumor entity [2, 3]. To date, the underlying oncogenic mechanisms that distinguish HPV-positive from HPV-negative head and neck tumors are still poorly understood [4]. SELDI is a pro-

* Present address: Tecnológico de Monterrey, Departamento de Biotecnología e Ing. de Alimentos, Ave. E. Garza Sada 2501-Sur Monterrey, N.L. Mexico, CP 64849.
teomic high-throughput technique that uses chromatographic surfaces to retain proteins depending on their physicochemical properties, followed by direct analysis via TOF MS [5]. This technique requires only a small amount of sample, making it ideal for small biopsies or microdissected tissue required to produce the homogeneous tissue samples typically used in cancer research. Microdissected tissue material, free of contaminating and unwanted tissue components, is extremely important for producing clean data for biomarker identification in cancer diagnostics and in elucidating clonal heterogeneity or other characteristics of tumors. We were able to show in a previous study that the detection of differentially expressed proteins was possible only in pure microdissected samples [6]. This separation can be done only with an extremely precise technique such as laser-based microdissection. Laser-based microdissection has been combined with ProteinChip technology to identify protein markers in several cancers [7–10].

In this study, we used ProteinChip technology to analyze pure microdissected tumor cells with and without HPV infection to detect discriminating protein profiles which elucidate the infection activated pathways.

2 Materials and methods

2.1 Laser microdissection of tissue sections

Seventeen samples derived from HPV-related OSCC as well as seven samples from uninfected OSCC were obtained after surgical resection at the Department of Otorhinolaryngology of the University Hospital Jena or at the Department of Otorhinolaryngology of the University of Cologne, respectively; these were collected fresh, snap frozen in liquid nitrogen, and stored at −80°C. All but three patients were male. The age varied from 50 to 78 years (mean: 64.4 years). Tumor specimens were categorized according to the AJCC classification from 2002 (sixth edition). All tumors were localized in the tonsil, base of the tongue, or in both sites. Primary tumors were classified pT2 to pT4. The neck status varied form pN0 to pN3. All patients were free of distant metastasis (M0). After stage grouping, all patients were classified as stage IV.

Immunohistochemical staining for p16 as surrogate marker for HPV-positivity in tissue section through the tumor from formalin-fixed and paraffin-embedded tumor samples was performed. Details of the staining procedure are published elsewhere [4]. Strong nuclear staining as well as strong cytoplasmic staining was considered positive for p16 expression. P16 immunostaining was regarded as overexpression if it was strong and diffuse and more than 60% of the tumor cells were p16-positive.

Laser microdissection was performed with a laser microdissection and pressure catapulting microscope (LMPC; Palm, Bernried, Germany) as previously described [8]. Therefore, we microdissected on native air-dried cryostat tissue sections ~3000–5000 cells each in a maximum of 20–30 min. Proteins were extracted by 10 μL lysis buffer (100 mM Na-phosphate (pH 7.5), 5 mM EDTA, 2 mM MgCl₂, 3 mM β-mercaptoethanol, 0.1% CHAPS, 500 μM leupeptin, and 0.1 mM PMSF) for 30 min on ice. After centrifugation (15 min; 15,000 rpm) the supernatant was immediately analyzed or frozen in liquid nitrogen for a maximum of 1 day.

2.2 Profiling of microdissected samples derived from tumor tissue

The protein lysates from microdissected tissues (OSCC-HPV and OSCC) were analyzed on both strong anion exchange arrays (Q10) as well as weak cation exchange arrays (CM10; BioRad) as described elsewhere [8]. In brief, array spots were preincubated by a washing/loading buffer containing 100 mM Tris-buffer, pH 8.5 with 0.02% Triton X-100 for Q10 arrays and 100 mM Tris-buffer, pH 4.5 with 0.02% Triton X-100 for CM10 arrays followed by application of 2 μL of sample extract on ProteinChip Arrays, which were incubated at room temperature for 90 min in a humidity chamber. After washing three times with the same buffers and two final washing steps with water, 2 × 0.5 μL sinapinic acid (saturated solution in 0.5% TFA/50% ACN) were applied. Mass analysis was performed in a ProteinChip Reader (Series 4000, Ciphergen Biosystems, Fremont, CA, USA) according to an automated data collection protocol. Spectra were normalized with TIC and cluster analyses of the detected signals and the determination of respective p-values for OSCC-HPV tissue and OSCC tissue were carried out with the CiphergenExpress Program (Version 3.0; Ciphergen Biosystems). For p-value calculation, normalized spectra with signals in the range between 2.5 and 200 kDa exhibiting a S/N of at least ten were selected and analyzed with the Mann–Whitney U-test for nonparametric data.

2.3 2-DE

Samples for 2-DE were prepared directly from surgical material of an OSCC which was infected by HPV and a uninfected OSCC both assessed by a pathologist. Proteins were isolated and 2-DE was performed as described [10]. Briefly, IEF was carried out on a Multiphor II (Amersham) using 7 cm IPG strips in a pH range of 3–10. Vertical SDS–PAGE was performed in a Novox Mini-Gel facility (Invitrogen) using 4–12% Bis-Tris Zoom™ gel (Invitrogen). The gels were stained with Simply Blue Safe Stain (Enhanced Coomassie, Invitrogen).

2.4 In-gel digestion

Protein patterns of the 2-DE gels from OSCC-HPV tissue and uninfected OSCC tissue were comparatively analyzed and protein spots with a molecular mass of nearly 10–130 kDa were excised. In-gel digestion of proteins was per-
formed as previously described [10]. Thereby, excised gel pieces were destained and dried. After rehydration and digestion with 10 μL of a trypsin solution (0.02 μg/μL; Roche) at 37°C over night supernatants were applied directly on a Au coated array (Ciphergen Biosystems). An empty gel piece underwent the same treatment as a control. After addition of the matrix (CHCA; BioRad), peptide fragment masses were analyzed using the ProteinChip Reader. The spectra for the peptide mapping experiments were externally calibrated using five standard proteins including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin β-chain (3495.94 Da). Proteins were identified using the fragment masses generated through trypsin digestion by searching in a publicly available database (profound; http://prowl.rogerfeller.edu/prowl-cgi/profound.exe).

2.5 MS/MS analysis

MALDI TOF/TOF spectra were generated with a Bruker ultraflex MALDI-TOF/TOF device (Bruker Daltonics, Bremen, Germany) as described elsewhere [10]. Briefly, samples were prepared in dried-droplet-preparation with CHCA. Peptide mass fingerprint and peptide fragmentation spectra of each sample were submitted for identification using MASCOT interface (MASCOT inhouse server 2.1.03, Matrix Science, London, UK) for search in the NCBI database. Hits were considered significant according to the MASCOT score (p = 0.05).

2.6 Immunodepletion assay

Five microliters of anti-thioredoxin (TRX)mAb (2G11/TRX; BD Pharmingen) was incubated with 10 μL protein A-agarose (Sigma) for 30 min on ice. A pellet was generated by centrifugation and the supernatant was discarded. The pellet BD Pharmingen) was incubated with 10 μL of an anti-TRX antibody (H-45; Santa Cruz) or, as a negative control, normal rabbit IgG (Peprotech; Hill, NJ, USA) were bound on protein A-agarose beads. Protein extract (10 μL) from microdissected OSCC-HPV tissue was incubated with the antibody loaded beads for 1 h on ice. Then the resins were washed two times with a buffer containing 20 mM HEPES (pH 7.8), 25 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, and 0.05% NP-40. Bound proteins were eluted from the beads by incubation with 10 μL 50% ACN/0.5% TFA for 10 min at 50°C. Eluted proteins were analyzed by NP20 ProteinChip arrays (BioRad) and SELDI-TOF MS.

2.8 Immunohistochemistry (IHC)

Eight micrometer cryostat sections of OSCC-HPV tissue and uninfected OSCC tissue were placed on slides, air dried for ~60 min at 20°C and fixed in paraformaldehyde as described [8]. After fixation, slides were treated in the microwave at 80 watts (3 × 3 min) in 10 mM citric acid pH 6.0 to inhibit endogenous peroxidatic activity. Subsequently, they were rinsed twice with TBS pH 7.4, and incubated overnight at 4°C in humidity chamber with the corresponding primary anti-TRX antibody or anti-E-FABP antibody, respectively. Slides were rinsed 3 × 10 min in TBS and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and the Jenzchrom pxbl-kit (MoBiTec, Göttingen, Germany) were used according to the manufacturer’s instructions to visualize the localization of the antibody. Negative controls were incubated with the labeled secondary antibody only. Sections cut in parallel to the IHC-treated sections were stained by HE for better identification of different tissue areas. IHC staining was evaluated by a pathologist.

3 Results

3.1 Protein profiling of HPV-related OSCC and unrelated OSCC

Tissue areas corresponding to about 3000–5000 cells per probe by use of laser microdissection and pressure catapulting microscope were excised by a pathologist. In this way, we successfully collected 17 samples derived from human papilloma virus-infected head and neck squamous cell carcinoma tissue (OSCC-HPV) and seven samples derived from uninfected OSCC in total. All protein lysates from the microdissected tissues were applied to both the Q10 arrays and the CM10 arrays and analyzed on a ProteinChip Reader Series 4000. The SELDI measurements of all tissue samples detected up to 373 peaks in the 2.5–200 kDa interval, with normalized intensities. After evaluation with the Ciphergen-Express Program, a number of these peaks were found to be significantly different between OSCC-HPV and OSCC tissue samples (Table 1).

3.2 Identification of significantly different signals

To separate protein lysates, we subjected histologically checked OSCC tissue to 2-DE (Fig. 1A). Thirty-four protein spots possessing visually different intensities in the interval...
Table 1. Significantly different signals which separate samples from OSCC-HPV and OSCC on strong anion exchanger (Q10) arrays and weak cation exchanger (CM10) arrays, respectively. Signals that are subsequently identified as TRX and E-FABP, respectively, are displayed bold.

<table>
<thead>
<tr>
<th>Molecular mass (kD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.704</td>
<td>4.54 × 10^{-2}</td>
</tr>
<tr>
<td>4.123</td>
<td>1.01 × 10^{-2}</td>
</tr>
<tr>
<td>6.892</td>
<td>4.55 × 10^{-2}</td>
</tr>
<tr>
<td>7.668</td>
<td>2.04 × 10^{-2}</td>
</tr>
<tr>
<td>9.249</td>
<td>3.90 × 10^{-2}</td>
</tr>
<tr>
<td>11.343</td>
<td>2.04 × 10^{-2}</td>
</tr>
<tr>
<td>11.988</td>
<td>3.90 × 10^{-2}</td>
</tr>
<tr>
<td>15.339</td>
<td>2.04 × 10^{-2}</td>
</tr>
<tr>
<td>26.633</td>
<td>2.41 × 10^{-2}</td>
</tr>
<tr>
<td>30.581</td>
<td>2.84 × 10^{-2}</td>
</tr>
<tr>
<td>41.733</td>
<td>3.33 × 10^{-2}</td>
</tr>
<tr>
<td>43.995</td>
<td>1.21 × 10^{-2}</td>
</tr>
<tr>
<td>53.622</td>
<td>4.55 × 10^{-2}</td>
</tr>
<tr>
<td>80.319</td>
<td>1.72 × 10^{-2}</td>
</tr>
<tr>
<td>91.502</td>
<td>3.14 × 10^{-3}</td>
</tr>
<tr>
<td>94.052</td>
<td>4.54 × 10^{-2}</td>
</tr>
</tbody>
</table>

of about 10–130 kDa were excised from the gels. Thereafter, we analyzed peptide fingerprints of the tryptic-digested spots by use of SELDI TOF MS. In this way, we were able to identify 18 proteins (Supporting Information Figure 1 and Table 1) by database searching (http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

One of these identified proteins, TRX (Fig. 1B), matched well in molecular mass with a significantly differentially expressed signal detected in prior protein profiling using SELDI. This signal of approximately 11 kDa was detected on Q10 arrays and showed an increased expression in samples derived from OSCC-HPV tissue ($p = 2.04 \times 10^{-2}$). Another significantly different signal possessing an m/z of nearly 15 kDa matched well to a protein identified as epidermal-fatty acid binding protein (E-FABP; also named FABP5) (Fig. 1B). This significantly different signal was up-regulated in OSCC-HPV tissue compared with OSCC tissue ($p = 2.04 \times 10^{-2}$) as detected on Q10 arrays in prior protein profiling. The distribution of the significantly differentially expressed signals possessing molecular masses of 11.343 and 15.339 kDa which correspond to TRX and E-FABP, respectively, in OSCC-HPV and OSCC tissue samples as well as representative SELDI-TOF MS spectra of these tissues are shown in Figure 2.

These results were further confirmed by tandem MS analysis. The peptides generated by tryptic in-gel digestion of TRX and E-FABP were selected and their PSD peptide fragments were analyzed by TOF/TOF (Figure 2 of Supporting Information). Sequences of the peptides are given in Table 2.

Figure 1. 2-DE of tissue extracts derived from head and neck squamous cell carcinoma (OSCC). (A) 2-D analyses of OSCC (left panel) and OSCC-HPV (right panel). (B) Enlargements of the framed areas displayed in (A). Arrows indicate spots later identified as TRX and E-FABP.
Proteomics 2009, 9, 2193–2201

Figure 2. Significantly different signals in different OSCC tissues. (A) Distribution of the intensities of the significantly differentially expressed signal possessing a molecular mass of 11.343 or 15.339 kDa, respectively, in OSCC-HPV and OSCC. (B) Representative examples of SELDI-TOF MS spectra of OSCC-HPV and OSCC. The peaks of interest at 11.343 and 15.339 kDa are marked with frames. Data are obtained using Q10 arrays.

We double-checked that TRX and E-FABP match the differentially expressed peaks at 11.3 and 15.3 kDa by use of ProteinChip analysis with immunodeplete assays and immunocapturing assays, respectively, using microdissected OSCC-HPV tissue as starting material. Analysis showed that the peak corresponding to TRX was reduced compared to the negative control without the specific antibody, in which this peak was clearly detectable (Fig. 3). In an immunocapturing assay, a specific antibody was able to capture E-FABP from the starting protein lysate. In the negative control without the specific antibody, this peak was absent (Fig. 4). Very dominant signals corresponding to the molecular masses of the alpha chain and the beta chain of hemoglobin were captured unspecifically by both used antibodies. As the assumed hemoglobin chain alpha possessed a much higher intensity than the E-FABP signal, the peak corresponding to E-FABP was only detectable as a shoulder in the tailing of the very dominant signal which corresponds to the molecular mass of the alpha chain of hemoglobin.
Table 2. Protein identification by peptide mapping and PSD MS/MS

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide [m/z]</th>
<th>Sequence</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioredoxin</td>
<td>1205.318</td>
<td>CMPTFQFFK</td>
<td>73–81</td>
</tr>
<tr>
<td>E-FABP</td>
<td>2433.838</td>
<td>TQTVCNFTDGALVHQEGWDGK</td>
<td>83–103</td>
</tr>
</tbody>
</table>

Figure 3. Immunodepletion assay using microdissected OSCC-HPV tissue. The signal of 11.357 kDa corresponding to TRX was detectable in the negative control using an unspecific antibody for depletion. In the depletion assay using a specific anti-TRX antibody, the signal corresponding to TRX is depleted and not detectable. Reference peaks that were not influenced by immunodepletion are labeled by asterisks.

Figure 4. Immunocapturing assay using microdissected OSCC-HPV tissue. An antibody which recognized E-FABP captured the specific antigen from microdissected tissue extract as analyzed by SELDI-TOF MS (labeled by arrow). In the negative control using an unspecific antibody a specific signal is absent. The two dominant peaks in the figure corresponding to the molecular masses of hemoglobin chain alpha and chain beta (labeled by asterisks) are unspecifically captured by both used antibodies.

3.3 Characterization of thioredoxin and E-FABP by immunohistochemistry

To characterize the identified markers and to localize TRX and E-FABP in tissue sections, we examined their expression in several OSCC-HPV tissue samples by immunohis-
Figure 6. Re-analysis of IHC-treated tissue sections by ProteinChip arrays. The signal with a molecular mass of approx. 11.36 kDa representing TRX was detectable in IHC positive tissue areas derived from OSCC-HPV. In IHC negative tissue this signal is absent (left panel). A signal with a MW of nearly 15.32 kDa which corresponds to E-FABP is detectable in IHC positive OSCC-HPV tissue in contrast to the IHC negative tissue where this signal is absent (right panel).

tochemistry using specific antibodies. Intensities of both proteins as assessed by IHC were detected to be different between several tumor areas of the same tumor as well as between the investigated tumors (Fig. 5). To further confirm unequivocally that the localized TRX and E-FABP are identical to the significantly different peaks found by ProteinChip analyses, areas from OSCC-HPV tissue that were positively analyzed in IHC were obtained by tissue laser microdissection. In protein lysates of microdissected tissue areas possessing a positive IHC reaction, the signal identical in mass to the significantly different peaks obtained in the initial SELDI-MS analysis was detected on Q10 arrays. In the protein lysates derived from IHC negative tissue these peaks were absent (Fig. 6).

4 Discussion

In the present study we analyzed tissue derived from both HPV-related and nonrelated OSCC regarding the identification of differentially expressed proteins using a technical triade comprising tissue microdissection, MS, and immunohistochemistry. Hereby, we detected and identified TRX and E-FABP as protein markers which were significantly up-regulated in OSCC-HPV.

The incidence of oral tumors is increasing. In the United States 34 360 new cases of oral cancer were expected in 2007 [12]. Tobacco and alcohol are the strongest etiologic factors, but high-risk HPV, commonly HPV16, is an emerging etiologic factor for oral cancer [13]. Genomic DNA of oncogenic HPV is detected in approximately 26% of all squamous-cell carcinomas of the head and neck worldwide [14]. In contrast, persistent infection by certain oncogenic HPV types is firmly established as the necessary cause of most malignant epithelial lesions of the cervix: HPV 16 and 18, the two most common oncogenic types, cause approximately 70% of all cervical cancers worldwide [15]. Therefore, comparison studies between HPV-related and nonrelated head and neck cancer seem to be a better qualified model to study the effect of HPV in oncogenesis than studies in cervical cancer, even when the role of HPV is more relevant at this tumor site.

TRX is an important redox mediator in biochemical pathways which promotes cell survival under adverse conditions including hypoxia and oxidative stress [16]. Elevated TRX is also associated with resistance to certain forms of cancer treatment [17]. An up-regulation of TRX mRNA as well as protein is linked to several cancer types including lung cancer, gastric carcinoma, pancreas cancer, and squamous cell carcinoma of the cervix [18–21]. A more aggressive tumor phenotype associated with bad prognostic features and a poorer outcome was concluded from over-expressed TRX in non-small cell lung carcinomas [22]. Additionally, the expression of TRX in skin tumors seems related to epithelial cell differentiation as no immunoreactivity was detectable in tissues derived from malignant skin tumors such as basal cell carcinoma and poorly differentiated squamous cell carcinoma by a specific antibody. In contrast, well-differentiated squamous cell carcinoma was immunostained by the TRX antibody [23]. Recently, Stacy et al. showed an elevated expression of TRX in 75% of 47 OSCCs in response to up-regulation of NRF2 but no data available about the status regarding a possible HPV infection of this analyzed head and neck tumors [24].

E-FABP belongs to a group of various types of FABP depending upon the tissue from which they were originally isolated. Originally, the family of FABP was regarded as
building blocks for membranes and as metabolic fuels, but recently they are recognized to be involved in cellular signaling affecting differentiation, regulation of growth, and gene expression [25]. It is reported that the several forms of FABP are found with both down- or up-regulated expression dependent on investigated tumor entity [26–29]. In OSCC, there are a number of studies showing an up-regulated expression of E-FABP [30–32]. In contrast, we and others were not able to detect significantly different E-FABP levels in OSCC tissue compared to normal squamous cell mucosa [33–37]. Here, only samples derived from microdissected tissue were used.

Currently, there are only a small number of studies which investigated HPV-infected tumors regarding differences in the proteome [38, 39]. To our knowledge, we present here for the first time a proteomic study which investigates (the study) HPV-infected OSCC and we identify/the study identifies differential expressed marker proteins. In conclusion, we could show that a proteomic procedure composing tissue microdissection, protein profiling by ProteinChip technology, separation, and identification of interesting proteins by 2-DE, PMF by SELDI MS and tandem-MS as well as confirmation of these proteins using immunological techniques is able to identify and characterize differentially expressed proteins. If it is possible to detect and confirm similar discriminating rates in further studies in an extended set of samples, then TRX and E-FABP might be marker proteins for HPV-infected OSCC.

We thank Juliane Förste for excellent technical assistance. The CUCA is supported by the German Federal Ministry of Education and Research (BMBF) and the Interdisciplinary Center of Clinical Research (IZKF), Jena.

The authors have declared no conflict of interest.

5 References

[21] Hedley, D., Pintilie, M., Woo, J., Nicklee, T. et al., Up-regulation of the redox mediators thioredoxin and apurinic/apyr-


