Detection of Distinct Changes in Gene-expression Profiles in Specimens of Tumors and Transition Zones of Tenascin-positive/-negative Head and Neck Squamous Cell Carcinoma

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Abstract. Background/Aim: Having previously initiated genome-wide expression profiling in head and neck squamous cell carcinoma (HNSCC) for regions of the tumor, the margin of surgical resectate (MSR) and normal mucosa (NM), we here proceed with respective analysis of cases after stratification according to the expression status of tenascin (Ten). Materials and Methods: Tissue specimens of each anatomical site were analyzed by immunofluorescent detection of Ten, fibronectin (Fn) and galectin-1 (Gal-1) as well as by microarrays. Results: Histopathological examination demonstrated that Ten+Fn+Gal-1+ co-expression occurs more frequently in samples of HNSCC (55%) than in NM (9%; p<0.01). Contrary, the Ten−Fn+Gal-1− (45%) and Ten−Fn−Gal-1− (39%) status occurred with significantly (p<0.01) higher frequency than in HNSCC (3% and 4%, respectively). In MSRs, different immunophenotypes were distributed rather equally (Ten+Fn+Gal-1+ =24%; Ten−Fn+Gal-1− =36%; Ten−Fn−Gal-1− =33%), differing to the results in tumors (p<0.05). Absence/presence of Ten was used for stratification of patients into cohorts without a difference in prognosis, to comparatively examine gene-activity signatures. Microarray analysis revealed i) expression of several tumor progression-associated genes in Ten+ HNSCC tumors and ii) a strong up-regulation of gene expression assigned to lipid metabolism in MSRs of Ten− tumors, while NM profiles remained similar. Conclusion: The presented data reveal marked and specific changes in tumors and MSR specimens of HNSCC without a separation based on prognosis. Faced with the enormous quantity of details on cell features, it is tempting to relate cases of differential expression to clinically-relevant properties. Conceptually, however, it could be possible that dysregulation occurs in such RNA
profiles without any association to, most importantly, prognosis. Thus, it is an open question to what extent these profiles are similar or different in tissue specimens of tumors classified according to a certain immunophenotypical parameter. In our recent pilot study, we initiated a comparison of tumor, margin and normal tissue specimen in head and neck squamous cell carcinoma (HNSCC) (1). Building on the previously detected relationship of presence of a member of the family of adhesion/growth-regulatory galectins, a reader of the sugar code (2, 3), i.e. galectin-1 (Gal-1), with tumor invasiveness (4) and its activity to promote fibronectin (Fn) expression and fibroblast conversion to α-smooth muscle actin (α-SMA) expressing myofibroblasts (5) as well as the significance of tenasin (Ten) expression (6), we here examine a panel of HNSCC cases always with specimen of the margin of surgical resectate (MSR) and normal mucosa (NM) according to presence of these three extracellular matrix (ECM) effectors.

Of fundamental importance, tumors of diverse metastatic potential and progression status differ in the composition of both tumor- and stroma-derived ECM components (7, 8). In this study, we first ask the question whether detection of the three proteins, expressed individually and/or in combination, provides prognostic information in the studied cohort of HNSCC patients. Following the immunohistochemical part that results in stratification according to the status of Ten expression, microarray analysis between Ten⁺/Ten⁻ cases was performed in order to answer the question on occurrence of differences on the level of RNA presence. In addition to tumor tissues from specimens stratified according to Ten presence, we also ran array-based RNA profiling of MSR and NM of the two patient groups differing in Ten expression.

**Materials and Methods**

**Tissue samples.** The set of tissue specimen of i) HNSCC (n=80; for details on classification, see Table I; for details on anatomical localization of analyzed tumors, see Table II), of ii) normal oral mucosa (NM) contralaterally to the primary tumor (NE – normal epithelium; n=47), and of iii) margin of surgical resectate (MSR) and macroscopically healthy mucosa up to 1 cm to the tumor margin; n=45) was obtained from the Charles University, 1st Faculty of Medicine, Department of Otorhinolaryngology, Head and Neck Surgery. All tissue samples were collected with informed consent of patients and approval of local ethical committee according to the Helsinki Declaration.

**Tissue processing – immunohistochemistry.** Tissue specimen of NE, HNSCC and MSR were cryoprotected by Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Frozen sections, 7 μm thick, were prepared by a Cryocut-E microtome (Reichert-Jung, Vienna, Austria).

**Immunofluorescent detection of Ten, Fn and Gal-1.** Frozen sections were carefully washed with phosphate-buffered saline (PBS, pH 7.2) and fixed by exposure to 2% (w/v) buffered paraformaldehyde in PBS for five minutes. After extensive washing in PBS (three times for 10 minutes), the specimens were treated with PBS containing 0.2% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and then washed in PBS. Antigen-independent binding of antibody via the Fc part was precluded by preincubation of specimens with porcine serum (DAKO, Glostrup, Denmark) diluted as recommended by the supplier. A murine monoclonal antibody against Ten-C and a rabbit polyclonal antibody against Fib (both from Sigma-Aldrich St. Louis, MO, USA) were applied as recommended by the supplier. Home-made rabbit polyclonal antibody against Gal-1 (9) that had thoroughly been tested for any cross-reactivity against other members of the galectin family, was used at a dilution of 1:50 (v/v). The specificity of immunodetection was ascertained by replacing the first-step antibodies by an irrelevant antibody of the same isotype (in the case of monoclonal antibody) or by omitting the first-step polyclonal antibody from processing. DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) (Vector-Laboratories, Burlingame, CA, USA). All preparations were analyzed by a fluorescence microscope Eclipse 90i (Nikon, Tokyo, Japan) equipped with filter blocks for FITC, TRITC and DAPI and a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany). Data were processed using the LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic).

**Microarray analysis.** Material from a subset of 26 patients was processed using microarray techniques. Anatomical sites of specimens used for gene profiling are given in Table I. Briefly, total RNA was isolated using RNAeasy Micro Kit reagents (QIAGEN, Germantown, MD, USA) from cryostat sections, following the procedure optimized for animal cells. The quantity and quality of RNA were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, Germantown, MD, USA). The quantity and quality of RNA were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA). The quantity and quality of RNA were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies LLC, Wilmington, DE, USA).

**Table I. TNM classification.**

<table>
<thead>
<tr>
<th>G</th>
<th>T</th>
<th>N</th>
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<td>0</td>
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G: Grade; T: size of the primary tumor; N: degree of spread to regional lymph nodes; M: presence of distant metastasis; S: stage.

**Table II. Anatomical sites of the primary tumors analyzed in the present study.**

<table>
<thead>
<tr>
<th>Primary site</th>
<th>Number of patients/microarray</th>
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<tr>
<td>Oral cavity</td>
<td>11/4</td>
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<tr>
<td>Oropharynx</td>
<td>51/13</td>
</tr>
<tr>
<td>Hypopharynx</td>
<td>5/1</td>
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<tr>
<td>Larynx</td>
<td>13/8</td>
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USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Illumina HumanWG-6 V3 chips (Illumina, San Diego, CA, USA) were used for microarray analysis following a standard protocol: total RNA (150 ng) was amplified using an Illumina TotalPrep RNA Amplification Kit (Ambion™; Thermo Fisher Scientific, Waltham, MA, USA), and 1,500 ng of the amplified RNA was hybridized to oligonucleotides presented on the chips according to the manufacturer’s protocol. Including several technical replicates, 26 samples of tumor tissue (four Ten– tumors; 22 Ten+ tumors), 22 samples of stromal tissue (four samples of Ten– tumors and 18 samples of Ten+ tumors), and 25 specimen of normal tissue (four samples of Ten– tumors and 21 of Ten+ tumors) were processed. To control the quality of the microarray analysis, we analyzed several samples in technical replicates.

The raw data were pre-processed using Genome Studio software (version 1.9.0.24624; Illumina) and further analyzed using the R packages oligo (10) and limma (11) of the Bioconductor (12) within the R environment (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). In brief, transcription profiles were background corrected using a normal-exponential model, quantile normalized and variance stabilized using base 2 logarithmic transformation. A moderated t-test was used to detect differentially expressed transcripts after fitting the linear model I ~ Tissue * Presence of Ten in Tumor Stroma. Storey’s q-value less than 0.25 (13) and a minimally 1.5-fold change in expression intensity were required to consider genes as being differentially transcribed. The MIAME compliant data was deposited to the Array Express database (E-MTAB-5852 and E-MTAB-6364).

Gene set enrichment analysis (GSEA) was performed using Fisher’s exact test on KEGG pathways (14) and Gene Ontology (15). To consider the gene set significantly enriched by differentially expressed genes and to account for possible multiple testing issues, statistical significance of the test was set to p<0.005, enrichment odds ratio to at least two and at least three genes shared by the gene set and the set of differentially transcribed genes.

Results

Histology. Immunohistochemical detection of presence of the ECM proteins in sections of HNSCC, MSR and NM is exemplarily illustrated in Figures 1 and 2. There is an apparent similarity between the frequency of Ten and Gal-1 presence. These two proteins were consistently absent in NM and in MSR, but strongly expressed in HNSCC (NM or MSR vs. HNSCC; p<0.01). Fn expression in NM showed almost equal distribution between positive and negative samples. In MSR and HNSCC, a shift to positive cases was seen (NM vs. MSR or HNSCC; p<0.01).

Co-expression of markers to establish the Ten+Fn+Gal-1+ status occurred more frequently in samples of HNSCC (n=42; 55%) than in NM (n=3; 9%; p<0.01). The most common combinations of marker parameters observed in NM were Ten−Fn−Gal− (n=15; 45%) and Ten−Fn−Gal− (13; 39%). Of note, they were rather rare in HNSCC (3% and 4%, respectively; p<0.01). Interestingly, all three combinations occurred with rather similar frequency in MSR, i.e. Ten+Fn+Gal− (n=8; 24%), Ten−Fn+Gal− (n=12; 36%) and Ten+Fn+Gal− (11; 33%) (p=0.568). Of note, the difference of frequency values between HNSCC and MSR samples was statistically significant (p<0.05) (Figure 3).

Considering association of positivity for Ten, FN or Gal-1 with clinical characteristics, no significant correlation between immunopositivity and nodal stage of tumors (Ten: p=0.0715; Fn: p=0.906; Gal-1: p=0.963) was found in the present study, Figure 4 illustrating data for Ten. The data on the other histopathological parameters, too, revealed no correlations to the status of expression of these three proteins.
Prognostic correlations. Ten: No tendency for improved prognosis in short-term 2-year follow-up for patients with Ten+ tumor stroma in both overall survival (OS) and disease-free survival (DFS) was observed. The difference was not statistically significant (Figure 5; OS: \( p=0.245 \); DFS: \( p=0.369 \)). After 5-year follow-up, no difference was found between Ten+ and Ten− samples (in both OS and DFS). Positivity in MSR was likewise related to prognosis (2- and 5-year OS and DFS). The obtained data did not reach the level of statistical significance (2-year OS: \( p=0.168 \); 5-year OS: \( p=0.218 \); 2y DFS: \( p=0.0742 \); 5y DFS: \( p=0.122 \)). Respective data on 5-year OS and DFS were significantly higher in patients with Ten+ NE (\( p<0.05 \)) (Figure 5 and Figure 6).

Figure 2. Immunofluorescent detection of pairs of galectin-1 (green signal) and tenascin (red signal) in the top part and of fibronectin (green signal) and tenascin (red signal) in the bottom part in frozen sections of normal mucosa (NM) and squamous cell carcinoma (SCC). Nuclei were counterstained with DAPI (scale bar 50 μm).
**Fib:** No correlation between Fn expression and patients’ prognosis was delineated.

**Gal-1:** Although 2-year OS indicated that Gal-1+ tumors may have a relatively unfavorable prognosis, this correlation did not reach the threshold for statistical significance. Similarly, no association between Gal-1 expression and prognosis was found in peritumoral and normal tissues.

These immunohistochemical data thus document differences between HNSCC and NM but did not uncover a prognostically relevant association of Ten presence/absence. In the concept of our study design, we proceeded to map gene-expression profiles to answer the questions whether and which profile changes can occur.

**Microarray analysis: a) RNA profiles differ between Ten− and Ten+ tumors.** When comparing RNA preparations from Ten+ and Ten− tumors, changes in 115 genes were detected. They are compiled in the Table III and Supplementary Table I (available at http://www.physiolchem.vetmed.uni-muenchen.de/summary/anticancer_research/index.html) for the cases of most significant differences. The GSEA analysis of the Biological Process GO ontology terms (Table IV and Supplementary Table II available at http://www.physiolchem.vetmed.uni-muenchen.de/summary/anticancer_research/index.html) revealed an association of differentially transcribed genes with the JAK-STAT signaling cascade (GO:0046427), with expression of several genes down-regulated in Ten− tumors (JAK2, LIF, and CYP1B1) and that of the NOTCH1 gene down-regulated in Ten+ tumors. Also, a strong up-regulation of expression of genes involved in ncRNA processing (GO:0034470) in the Ten+ tumors was seen. A gene belonging to this section is argonaute 2 (AGO2), whose transcriptions appear to be significantly up-regulated in Ten+ tumors compared to Ten− tumors, MSR, and NE, as also seen for the gene for pseudouridylate synthase 7 (PUS7), while gene expression for integrator complex subunit 1 (INTS1) is significantly down-regulated in Ten− tumors in comparison to Ten+ tumors, MSR, and NE. GSEA of the Cellular Compartment GO ontology resulted in strong enrichment of the genes associated with components of the ECM and microenvironment (GO:0044421). Among them are lysyl oxidase-like 1 (LOXL1), whose expression is up-regulated in Ten− tumors, C1q and TNF related 1 (C1QTNF1), up-regulation seen in Ten− tumors and related MSR, and basal cell adhesion molecule (BCAM), up-regulation detected in Ten− tumors-derived MSR and normal tissues. There were no Molecular Function GO ontology terms found among the genes differentially expressed between Ten+ and Ten− tumors (Figure 7).

**Cases of gene dysregulation between Ten+ vs. Ten− tumors** furthermore include PRAME (preferentially expressed antigen in melanoma) strongly up-regulated in Ten+ tumors, G6PC3 (glucose-6-phosphatase catalytic subunit 3) and IDUA, α-L-
iduronidase, which are up-regulated in all Ten− samples, and also LIF, an interleukin 6 family cytokine, which is specifically up-regulated in Ten− tumors (for the most deregulated genes in this comparison, see Supplementary Table I).

**Microarray analysis: b) RNA profiles of MSR differ between Ten− and Ten+ tumors.** Comparing RNA preparations of MSR of patients with either Ten+ or Ten− tumors, dysregulation of 154 genes was found (Table V and Supplementary Table I). GSEA analyses revealed that major changes occurred in the metabolism of lipids (Table VI and Supplementary Table II). The most prominently enriched KEGG signaling pathway is the PPAR signaling pathway (hsa03320), with up-regulation of genes coding for peroxisome proliferator activated receptor gamma (PPARG), aquaporin 7 (AQP7), adiponectin ADIPOQ, perilipins 1 and 4 (PLIN1, PLIN4), lipoprotein lipase (LPL), and fatty acid desaturase 2 (FADS2) in MSR of patients with Ten− tumors. The glycerophosphatidyl metabolic pathway (hsa00561) also came up to be significantly enriched for up-regulated genes. Consistently, many GO terms associated with lipid metabolism are in the list of genes whose activity was enhanced in MSR of patients with Ten− tumors. These include cellular compartment lipid droplet (GO:0005811), lipid metabolic process (GO:0006629) and transferase activity (transferring acyl groups other than amino-acyl groups, GO:0016747). Other genes that showed significantly increased representation in the MSR of the patients with Ten− tumors are leptin (LEP) and galectin-12 (Gal-12; LGALS12) (for the most deregulated genes in this comparison, see Figure 8 and Supplementary Table I).

**Microarray analysis: c) RNA profiles of NM of the patients with Ten− and Ten+ tumors are similar.** The profiles of normal tissues of the cohorts of patients with Ten−/Ten+ tumors were very similar. In cases appearing to differ in expression activity such as genes for RAB11B, a member RAS oncogene family, and pancreatic progenitor cell differentiation and proliferation factor (PPDPF), no statistical significance (p=0.21) was reached. Obviously, MSR characteristics appeared to be more susceptible to an influence by the tumor than NM features, based on array-based RNA profiling.

**Discussion**

In the immunohistochemical part of our study, we revealed the possibility for a stratification according to the absence/presence of Ten. With relevance to prognosis, no
correlation between Ten expression and grade, TNM stage and primary site of examined tumors, respectively, was discerned. Of particular note, no significant prognostic correlations was disclosed in all three groups of patients. A lack of association between Ten expression and histopathological features has previously been reported in oral and pharyngeal cancers (16). Considering cell types other than tumor cells, Ten expression in cancer-associated fibroblasts was associated with patient age, tumor stage, lymph node metastasis, clinical stage, cancer recurrence and positively correlated with the presence of platelet-derived growth factor-α/β and α-SMA. Furthermore, its expression in cancer cells correlated with an increase in the population of tumor-associated macrophages, cancer recurrence and expression of hypoxia inducible factor-1α (17).

The following comparison of gene expression profiles of Ten+ and Ten− tumors by whole-genome transcriptome analysis led to detection of marked differences. The systematic comparison of profiles identified several genes that code for kinases and receptors relevant in tumor development that are transcriptionally dysregulated in Ten− tumor samples. Janus kinase 2 (JAK2), a case of down-regulation, is a non-receptor tyrosine kinase associated with cytokine receptors and involved in cell growth, development, differentiation or histone modifications and its overexpression predicts unfavorable prognosis for nasopharyngeal carcinoma (18). Notch1, the second prominent example of a down-regulated gene in Ten− tumors, controls cell-fate decisions including epithelial-to-mesenchymal transition and can hereby be involved a variety of developmental processes, mutations associated with several types of leukemia and HNSCC (19, 20). Leukemia inhibitory factor (LIF) can mediate pro-inflammatory activation of stromal fibroblasts in cancer (21).

Cytochrome P450 CYP1B1 is involved in an NADPH-
dependent electron transport pathway, oxidizing a variety of structurally unrelated compounds and promoting angiogenesis. Our data set on Ten⁺/Ten⁻ tumors furthermore revealed strong enrichment of factors of the extracellular region. Among these, BCAM was found to be up-regulated in Ten⁻ tumors, their stroma and matching normal tissues. Its expression is associated with immature states of human keratinocytes and it is induced in epithelial skin tumors and inflammatory epidermis (22, 23).

In this study, we next turned to the comparison of MSR specimens separated according to Ten expression of the tumor. Intriguingly, monitoring the MSR surrounding the
Ten− tumors found a strong up-regulation of transcription of genes within the lipid metabolism. A prominent gene on this list is LEP. It is a key player in the regulation of energy balance and body weight (24). Moreover, LEP has also been described as a tumor-promoting gene for example in breast (25) and liver cancers (26). In addition, the multifunctional LEP is involved in the regulation of the ERK signaling (27) and can be apoptotic via the JAK2-STAT3 pathway and up-regulation of BIRC5 expression, as well as regulates presence of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (28, 29).

Multifunctionality, too, holds true for the adhesion/growth-regulatory galectins (2, 30-32), Gal-12 expression exhibiting a similar increase as LEP does (see Supplementary Figure 1).
This galectin’s impact on growth control accounts for its current status as candidate tumor suppressor (33), in line with epigenetic gene silencing by promoter methylation and induction by butyrate in colorectal carcinoma lines (34, 35).

The herein reported up-regulation thus gives incentive to extend the immunohistochemical analysis of the galectin network in cancer, as described for example for colon cancer (36), and its surrounding tissue to this so far not studied...
family member, flanked by monitoring galectin binding using the labeled tissue lectin, an approach complementary to immunohistochemical monitoring (37-39). In this sense, determining this protein’s presence, prompted by the array data presented herein, is likely to add to the evidence for galectin involvement in processes related to malignancy, as assumed following the detection of expression of tissue lectins in cancer more than 30 years ago (40).

In summary, our results reveal marked disparities of gene-expression signatures between i) tumor specimens stratified according to matrix glycoprotein without prognostic relevance and ii) MSR specimens in the formal category of lipid metabolism. These data point to plasticity of gene-expression profiles without necessarily bearing prognostic relevance and are relevant in principle for considerations of relating differences detected on this level to clinical parameters.

Conflicts of Interest

The Authors declare no potential conflicts of interest.

Acknowledgements

This work was supported by Progres Q28, GAUK 165015, and the Ministry of Health of the Czech Republic (Grant no. 15-28933A). The present study was also supported in part by the Agency for Science and Research under the contract no. APVV-0408-12, APVV-14-0731, APVV-16-0446, and APVV-16-0207). Support was also provided by Ministry of Education, Youth and Sports of CR within the National Sustainability Program II (project BIOCEV-FAR: registration no. LQ1604) and project BIOCEV (grant no. CZ.1.05/1.1.00/02.0109). The present study used the equipment for metabolomics and cell analyses (grant no. CZ.1.05/2.1.00/19.0400) supported by the Research and Development for Innovations Operational Program, co-financed by the European regional development fund and the state budget of the Czech Republic. Inspiring discussions with Drs. B. Friday and A. Leddo are gratefully acknowledged.

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