Gene expression profiling of head and neck squamous cell carcinoma using cDNA microarrays

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Abstract. The molecular mechanisms causing the development of squamous cell carcinomas in the head and neck region are mostly unknown. Identification of molecular changes which are involved in carcinogenesis might play a key role in improving the diagnosis, therapy and prognosis of patients with carcinomas in the head and neck. The purpose of the study was to identify transcriptional alterations of apoptosis associated genes between normal mucosa and tumor tissue. We measured the mRNA expression of 408 apoptosis associated genes by microarray-technique in normal upper aerodigestive tract mucosa (n=4), and in cancer tissue (n=8) of squamous cell carcinomas of the upper aerodigestive tract. RT-PCR was performed to confirm the microarray results. A hierarchial cluster analysis, based on 22 selected marker-genes showing a separation of the two tissue types supports the hypothesis of a specific expression pattern associated with tumor development. Additionally, we found 11 genes associated with anti-apoptotic processes to be upregulated while 12 genes associated with proapoptotic functions as well as 5 DNA-replication and chromosome cycle associated genes were found to be downregulated in the tumor tissue. Furthermore 6 of 8 genes which are known to be associated with lymphocyte activation were upregulated in tumor edge tissue. These results represent a first step for the diagnostic use of microarrays in squamous cell carcinomas of the head and neck region and might improve the understanding of the molecular mechanisms of carcinogenesis in the head and neck region.

Introduction

Head and neck malignancies account for approximately 6% of all malignancies, being mainly squamous cell carcinomas. Worldwide, an estimated 333,000 patients with head and neck cancer die per year (1).

Tumor development and tumor progression on a molecular basis in head and neck squamous cell carcinoma (HNSCC) are complex processes and so far little understood. Effective treatment of this harmful disorder requires a broad understanding of the underlying mechanisms. In cancer research molecular profiling is a promising tool. Molecular features of tumors have been investigated by DNA microarray to achieve a specific molecular classification and to formulate molecular predictors for survival (2,3). Recent studies investigated gene expression signatures to subclassify HNSCC and to predict patients at high risk for local recurrence or metastasis. Furthermore new genes were discovered which might be helpful markers to discover premalignant lesions and act as targets for novel pharmacological intervention (4,5). One study of the gene expression levels of normal mucosa, premalignant lesions and malignant lesions of the upper aerodigestive tract described a transcription progression model differentiating clearly between these groups (6).

The purpose of our study was to determine a so-called molecular fingerprint of apoptosis associated genes that allows the differentiation of cancer tissue and healthy mucosa of the upper aerodigestive tract. Additionally, the apoptosis related microarray was used to find specific gene expression differences, that allow the identification of new oncogenes and to guide insight into the process of tumor genesis and tumor invasion on molecular basis. Finally, investigation of tumor edge and tumor center tissue differences between these tissue types were monitored with this highly sensitive method.

Materials and methods

cDNA microarray

Tissue samples. Twelve tissue samples were taken from 6 patients with HNSCC. The tumor biopsies of one female and 5 male patients between 45 and 69 years were investigated (mean age, 59). Three tumors were localized in the hypopharynx and 3 in the larynx. All cancers were squamous cell carcinomas. Samples were collected from each patient: 6 from the tumor center tissue, 2 from the tumor edge tissue and 4 from healthy mucosal tissue. The biopsies were snap-frozen immediately after surgery in liquid nitrogen and stored at -80˚C until further usage.

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Informed consent and approval by the Ethics Committee. All patients in this study consented to biopsies being used for research purposes. The study setup was approved by the Ethics Committee of the Medical Faculty of the Christian-Albrechts-University prior to the start of the study.

RNA extraction. For microarray analysis and RT-PCR the rapid and reproducible preparation of total RNA with the Rneasy kit (Quiagen, Hilden, Germany) was used following the manufacturer's instruction.

For RT-PCR a determination of the RNA content using the UVICON-931 UV spectralphotometer (Kontron, Hamburg, Germany) was applied and samples of total RNA were adjusted to 1.0 μg for following first-strand cDNA synthesis. Quantitative analysis of the RNA content was carried out by means of RT-PCR of GAPDH as control.

Sample preparation, microarray hybridization and analysis. Expression profiles were generated using superamine coated glass-based cDNA microarrays, containing 1344 features representing 408 different apoptosis associated genes (Scienion AG, Berlin, Germany). All cDNA features (PCR-products) were designed to be specific to the selected genes and sequence verified prior to spotting. Each feature was spotted in duplicates. Sample preparation and microarray hybridization was performed according to the manufacturer's guidelines. Briefly, 20 μg of total RNA were labeled in a reverse transcription reaction (Cyscribe, Amersham Biosciences, Freiburg, Germany) by incorporating either Cyanine5-dUTP or Cyanine3-dUTP (Amersham Biosciences). External controls, consisting of 10 different mRNA sequences (spikes) from *A. thaliana* were added to each labeling reaction at a final amount of 400 pg per spike and labeling reaction (Spot-report 10, Stratagene, La Jolla, CA). Microarrays were hybridized with combined Cy3 and Cy5 labeled targets at 42˚C overnight in a hybridization buffer containing 15 μl HybBuffer (Amersham Biotech, Freiburg, Germany), 50% formamide, 1 μg humanCot DNA (Stratagene) and 5 μg Yeast tRNA (total volume: 60 μl). After hybridization, the slides were washed at room temperature in 1X SSC (Ambion Inc., Huntington, UK), 0.03% SDS for 2 min, in 0.2X SSC for 2 min and in 0.1X SSC for 2 min. After centrifugation in a Spectrafuge Mini (Abimed, Düsseldorf, Germany) for 1 min, the slides were scanned in a GT-LSIV Laserscanner (Genomic Solutions, Huntington, UK). Microarray image processing was performed using the AIDA 3.2-gridding software (Raytest, Straubenhardt, Germany). Normalization of the data was performed using a non-linear curve fitting method (7). Subsequent data analysis was carried out using Spotfire 8.0 for functional genomics (Spotfire, Summerville, MA). Fold changes for the individual genes were obtained by calculating the ratio of the medians of independent measurements.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney U test. Functional groups were assigned to the transcripts according to the Gene Ontology classifications for biological processes (www.geneontology.org). The Euclidisan distance (UPMGA, unweighted average) was used to determine differences between expression patterns in the cluster analysis.

RT-PCR

Tissue samples. Tissue samples (8) were taken from 6 different patients with HNSCC. Patients were 45-68 years-old (mean age: 57 years). One hypopharyngeal squamous cell carcinoma, 2 laryngeal squamous cell carcinomas and 3 oropharyngeal squamous cell carcinomas were diagnosed. Normal control mucosa was taken during routine surgery from patients without any malignancy of the upper aerodigestive tract. The patients whose tissue samples were used for RT-PCR represent a verification-group that was independent from the patients examined during the microarray analysis.

Reverse transcription. Total RNA (1.0 μg) was heat-denatured (65˚C, 10 min), chilled on ice and subjected to random hexadeoxynucleotide primed reverse transcription using the first-strand cDNA synthesis kit (Pharmacia, Freiburg, Germany). Reverse transcription (final volume, 15 μl) was conducted at 37˚C for 60 min in the presence of 0.2 μM of random hexanucleotide primer [pd(N)6] and 40 U RNAse inhibitor (RNAsin, Gibco, Karlsruhe, Germany). Following synthesis of the completed first-strand cDNA the resulting double-stranded RNA:cDNA heterodublex was heat-denatured to 95˚C, for 5 min to provide cDNA as a template for polymerization. The following oligonucleotides were used: GAPDH; sense 5'-CCA GCC GAG CCA CAT CGC TC-3'; antisense: 5'-ATG AGC CCC AGC CTT CTC CAT-3'; ETS2 sense: 5'-CTG TGG AGT GAG CAA CAG G-3'; antisense: 5'-GAG CAG TAG GTG ACG CTG-3'; RBP1 sense: GAG ATC GTG CAG GAC GGT G-3'; antisense: 5'-CAG ACC ACA CCT TCC ACT C-3'; STAT3 sense: 5'-GAT GTC CGG AAG AGA GTG C-3'; antisense: 5'-GTT GAG ATT CTG CTA ATG ACG-3'.

Polymerase chain reaction. Reverse transcription cDNA product (0.25 μl of the 15 μl final volume) was incubated in 50 μl reaction mixture containing 0.2 μM 5'-3' sequence specific sense oligonucleotide primers, 0.2 μM of the 3'-5' corresponding sequence specific antisense oligonucleotide primers, 200 μM dNTP's, 1.5 mM $MgCl_2$, 5.0 μl 10X PCR-buffer (Gibco) and 2.5 U Taq-polymerase (Gibco). Following initial denaturation at 93˚C for 3 min high stringency PCR was run for 35 cycles of 93˚C for 90 sec, 58˚C for 90 sec and 72˚C for 2 min. The amplicons were purified using Quiaquick spin (Quiagen) and 4.0 μl of the 50 μl purifying product were resuspended in 3.0 μl TE-buffer containing 0.02% (v/v) bromphenolblue and run on a 1% agarose gel containing 0.01% ethidiumbromide.

Results

Expression analysis using cDNA microarrays resulted in 722 of 892 signals meeting the criteria for further analysis (signal intensity above background cut off, duplicate difference ≤15%). A set of 22 genes was identified that allowed a differentiation between tumor tissue and healthy mucosal tissue using cluster analysis. Additionally, the gene expression pattern showed an increasing diversity (between individuals) in transition from healthy mucosal tissue to tumor center tissue (Fig. 1). The gene set represents genes which are significantly ($p \leq 0.05$) regulated in all cluster groups.

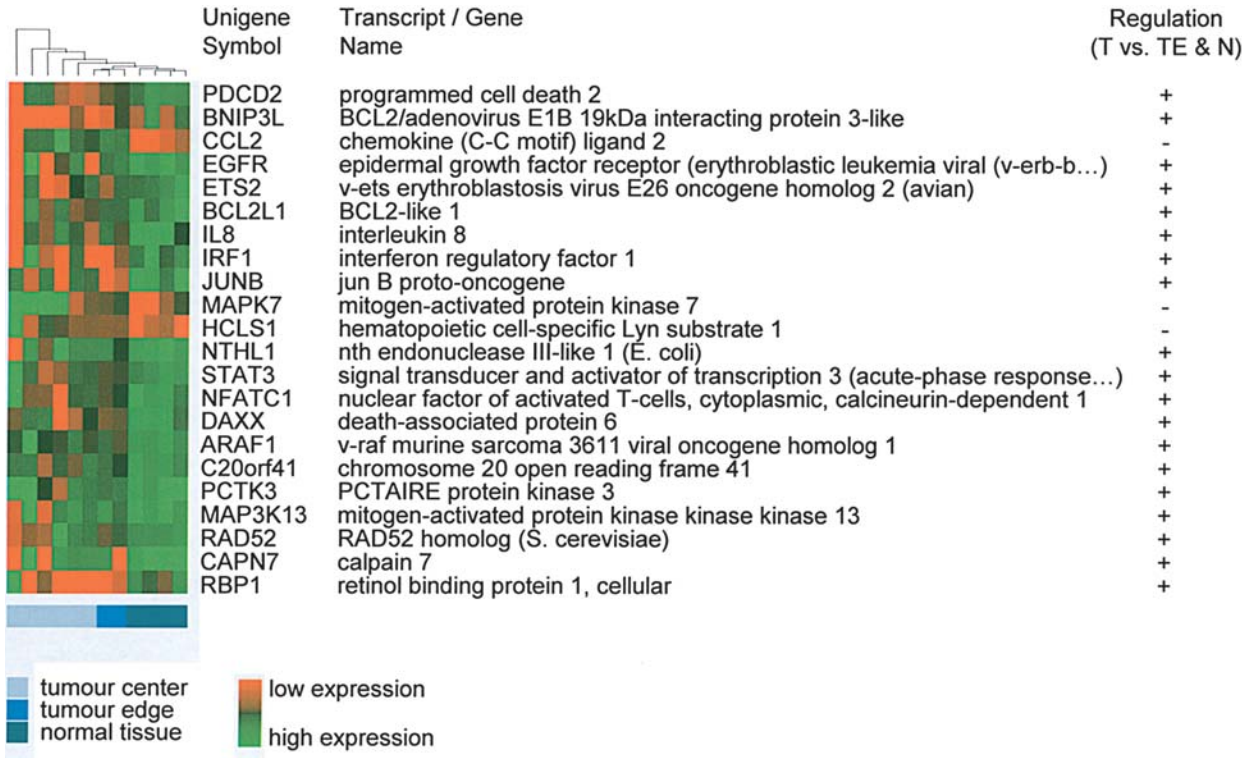

Figure 1. Classification by cluster analysis. Each line represents a gene signal, each column represents a single sample. Levels of significance of each gene is $p \leq 0.05$. The dentogram shows similarities of data sets. Vertical order is based on similarities of the genes (not shown). Regulation shows the gene expression from tumour tissue to healthy mucosa (+, increased expression in tumour tissue; -, decreased expression).

Tumor tissue to healthy mucosa. The analysis of the transcriptional differences between tumor tissue and healthy mucosal tissue revealed several gene-transcripts to be significantly differentially expressed ($p \leq 0.05$). 19 genes ARAF1, BCL2L1, BNIP3L, C20orf41, CAPN7, DAXX, EGFR, ETS2, IL8, IRF1, JUNB, MAP3K13, NFATC1, NTHL1, PCTK3, PDCD2, RAD52, RBP1 and STAT3 had increased expression levels in tumor tissue, while 3 genes CCL2, HCLS1 and MAPK7 showed decreased expression levels (Fig. 1). All genes are functionally characterized and related to apoptosis since only such genes were represented on the array (Table I).

These genes are involved in multiple pathways. The most significant ones are the MAPK signalling pathway (DAXX, EGFR, MAPK7, MAP3K13), apoptosis (CAPN7, BCL2L1), integrin-mediated cell adhesion (CAPN7, ARAF1, MAPK7), JAK/STAT signalling pathway (BCL2L1, STAT3), Toll-like receptor signalling pathway (IL8), Wnt signalling pathway (NFATC1) and some Cytokine-cytokine receptor interactions (EGFR, IL8, CCL2) (Kyoto Encyclopedia of Genes and Genomes; www.genome.jp/kegg/).

Tumor center tissue to tumor edge tissue. Analysing tumor center tissue and tumor edge tissue the gene expression differences were not that manifold but the two genes DAP and TLR6 showed significantly increased expression levels in the tumor center tissue (Table II).

Anti-apoptotic processes. In the tumor center tissue 11 genes which are associated with anti-apoptotic processes were upregulated (Fig. 2). Upregulation refers to significantly stronger transcript signals in the tumor center than in the tumor edge and stronger transcript signals in the tumor edge than in healthy mucosal tissue. These genes were AATF, BAX, BCL2L1, BCL2L10, BCL2L2, BIRC5, BNIP3L, BRAF, GG2-1, IER3 and TNFRSF18.

Proapoptotic processes. In contrast to the upregulation of 11 anti-apoptotic genes, 12 genes that are associated with proapoptotic processes were downregulated in tumor center tissue, which means that the lowest gene expression was seen in the tumor center, stronger gene expression in the tumor edge and the strongest gene expression was found in healthy mucosal tissue (Fig. 3). Those genes were ABL1, ASC, BCL2L11, CASP6, CASP8AP2, CRADD, DAP3, IKBKG, TIA1, TNFSF10, TRADD and TRAF3.

Genes associated with lymphocyte activation. Six of 8 genes from the used array associated with lymphocyte activation were upregulated in tumor edge tissue (Fig. 4). They follow the model strongest gene expression in the tumor edge, while showing lower gene expression in the tumor center and healthy mucosal tissue. Those genes are CD28, CD3D, CD4, IL18, IL2 and TNFRSF7.

Two genes associated with lymphocyte activation do not follow this model. CD28 shows the gene expression pattern of tumor center < tumor edge < healthy mucosal tissue and IL10 follows the pattern of tumor center > tumor edge < healthy mucosal tissue.

Genes associated with DNA replication and the chromosomal cycle. In total, 8 genes associated with DNA replication and

Table I. Classification of genes for cluster analysis.[a]

Symbol	P-value	Regulation	Classification
ARAF1	0.048	+	8151 cell growth and/or maintenance
BCL2L1	0.016	+	6915 apoptosis; 6916 anti-apoptosis; 8637 apoptotic mitochondrial changes
BNIP3L	0.048	+	6915 apoptosis; 6917 induction of apoptosis
C20orf41	0.004	+	(Unknown)
CAPN7	0.048	+	6508 proteolysis and peptidolysis
CCL2	0.048	-	6468 protein amino acid phosphorylation; 6935 chemotaxis; 6959 humoral immune response
DAXX	0.016	+	6915 apoptosis; 6355 regulation of transcription, DNA-dependent
EGFR	0.048	+	6468 protein amino acid phosphorylation; 7173 epidermal growth factor receptor signaling pathway
ETS2	0.016	+	1501 skeletal development; 8151 cell growth and/or maintenance
HCLS1	0.016	-	6355 regulation of transcription, DNA-dependent
IL8	0.048	+	1525 angiogenesis; 6935 chemotaxis; 8285 negative regulation of cell proliferation; 42119 neutrophil activation
IRF1	0.048	+	6355 regulation of transcription, DNA-dependent; 6955 immune response
JUNB	0.028	+	6357 regulation of transcription from Pol II promoter
MAP3K13	0.048	+	6468 protein amino acid phosphorylation
MAPK7	0.048	-	6468 protein amino acid phosphorylation; 7049 cell cycle
NFATC1	0.048	+	6355 regulation of transcription, DNA-dependent; 6357 regulation of transcription from Pol II promoter
NTHL1	0.028	+	5975 carbohydrate metabolism; 6296 nucleotide-excision repair, DNA incision, 5'-to lesion
PCTK3	0.048	+	6468 protein amino acid phosphorylation
PDCD2	0.016	+	6915 apoptosis
RAD52	0.048	+	6302 double-strand break repair
RBP1	0.048	+	6776 vitamin A metabolism
STAT3	0.048	+	122 negative regulation of transcription from Pol II promoter; 6357 regulation of transcription from Pol II promoter; 6953 acute-phase response

[a]Selection of genes according to p-value by Mann-Whitney U test (tumour tissue to healthy mucosa), criteria $p \leq 0.05$. Regulation shows the gene expression from tumour tissue to healthy mucosa (+, increased expression in tumour tissue; -, decreased expression). Classification performed by Gene-Ontology, number according to Ontology term. Genes were only classified into 'biological process'. Since some genes show a wide variation of classifications we only present a selection of them.

Table II. Classification of genes for cluster analysis.[a]

Symbol	P-value	Regulation	Classification
DAP	0.02	+	6915 apoptosis; 8624 induction of apoptosis by extracellular signals
TLR6	0.02	+	42116 macrophage activation; 6955 immune response

[a]Selection of genes according to p-value by Mann-Whitney U test (tumour centre tissue to tumour edge tissue), criteria $p \leq 0.02$. Regulation shows the gene expression from tumour centre tissue to tumour edge tissue (+, increased expression in tumour centre tissue). Classification performed by Gene-Ontology, number according to Ontology term. Genes were only classified into 'biological process'. Since some genes show a wide variation of classifications we only present a selection of them.

chromosomal cycle were represented on the array. Five genes from this group showed increasing expression from tumor center over tumor edge to normal mucosa: ABL1, BLM, CDC34, LIG1 and RBBP4 (Fig. 5).

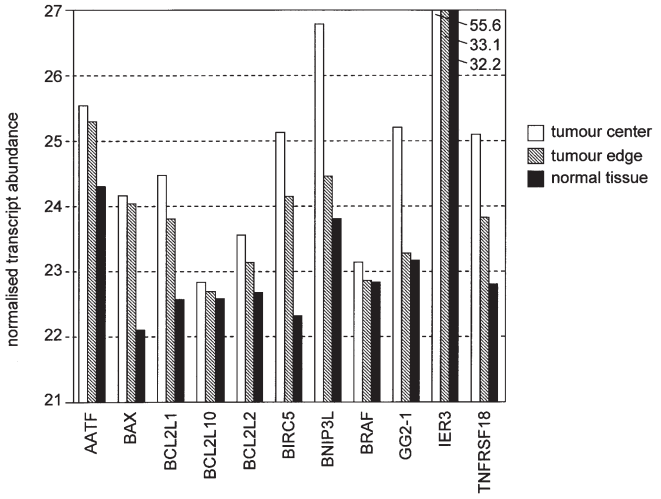

Figure 2. Regulation of genes associated with the anti-apoptotic program in HNSSC tissue types. For each gene, several biological replicates were measured (n=4 normal tissue; n=2 tumor edge; n=6 tumor center). The individual samples were measured in 2 technical replicates. The bars represent normalised transcript amount, to make measurements comparable. In total, 27 genes from this category were analysed: eleven genes from this category showed an expression pattern of T>TE>N (presented in the graph), while 6 showed a pattern of T<TE<N (not shown).

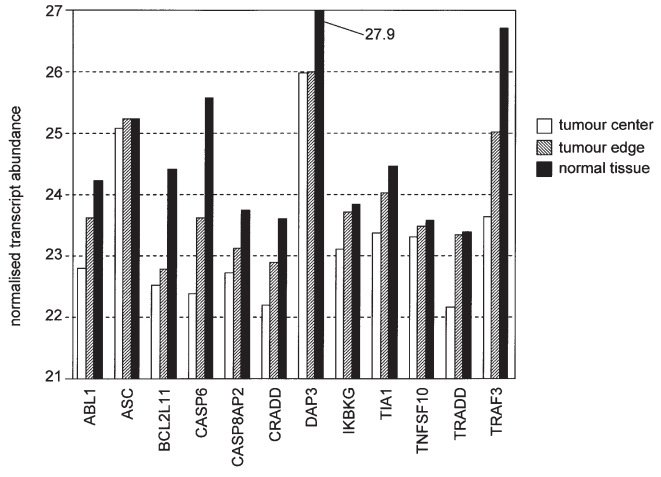

Figure 3. Regulation of genes associated with positive regulation of apoptosis in HNSSC tissue types. For each gene, several biological replicates were measured (n=4 normal tissue; n=2 tumor edge; n=6 tumor center). The individual samples were measured in 2 technical replicates. The bars represent normalised transcript amount, to make measurements comparable. In total, 41 genes from this category were analysed: twelve genes from this category showed an expression pattern of T<TE<N (presented in the graph), while 11 showed a pattern of T>TE>N (not shown).

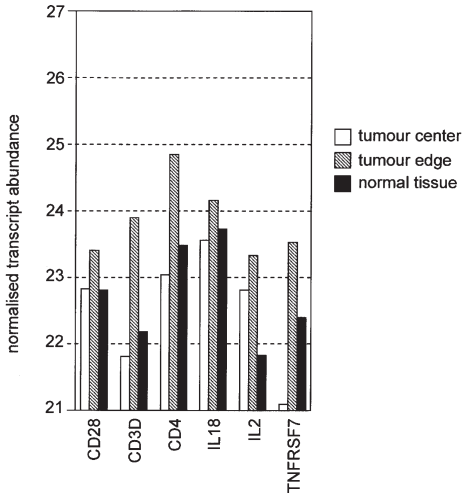

Figure 4. Regulation of genes associated with regulation of lymphocyte activation in HNSSC tissue types. For each gene, several biological replicates were measured (n=4 normal tissue; n=2 tumor edge; n=6 tumor center). The individual samples were measured in 2 technical replicates. The bars represent normalised transcript amount, to make measurements comparable. In total, 8 genes from this category were analysed: six genes from this category showed an expression pattern of T<TE>N (presented in the graph), while 2 showed other patterns (not shown).

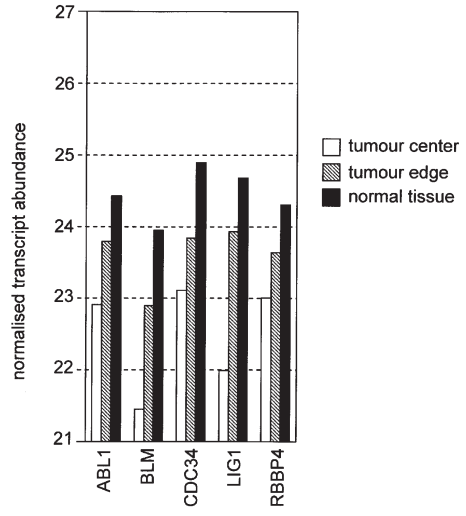

Figure 5. Regulation of genes associated with DNA replication and chromosome cycle in different HNSSC tissue types. For each gene, several biological replicates were measured (n=4 normal tissue; n=2 tumor edge; n=6 tumor center). The individual samples were measured in 2 technical replicates. The bars represent normalised transcript amount, to make measurements comparable. In total, 8 genes from this category were analysed: five genes from this category showed an expression pattern of T<TE<N (presented in the graph), while 3 showed other patterns (not shown).

Statistical analysis. Statistical analysis comparing gene expression levels between tumor center tissue and healthy mucosal were significant ($p \leq 0.05$) for 22 genes. When comparing tumor center tissue to tumor edge tissue only two of the above mentioned differentially expressed genes met the p-value cut-off ($p \leq 0.05$).

RT-PCR. The RT-PCR for GAPDH (Fig. 6) showed good quality RNA and a sufficient and comparable amount of RNA from each source for the following specific RT-PCR. The RT-PCR with the sequence specific oligonucleotide primers for RBP1, ETS2 and STAT3 showed the following results. STAT3 expression in tumor tissue was high, while in healthy mucosal tissue there was no STAT3 expression. This confirmed the result from the microarray analysis (Fig. 7). ETS2 expression in tumor tissue was strong, while in healthy mucosal tissue there was a low ETS2 expression (Fig. 8). This also confirmed the result from the microarray analysis. RBP1 expression in the RT-PCR was inhomogeneous in the tumor tissue. Some cancer biopsies showed a comparable

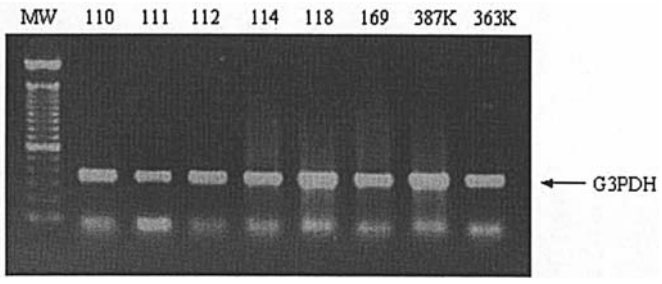

Figure 6. Confirmation of RNA quality and quantity for microarray analysis by RT-PCR for G3PDH gene expression (110-112, 114, 118, 169, carcinomas; 387K and 363K, normal mucosa; MW, molecular weight marker).

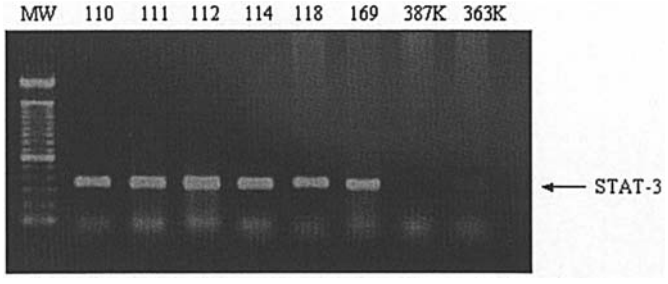

Figure 7. Confirmation of microarray analysis by RT-PCR. STAT3 gene-expression (110-112, 114, 118, 169, carcinomas; 387K and 363K, normal mucosa; MW, molecular weight marker).

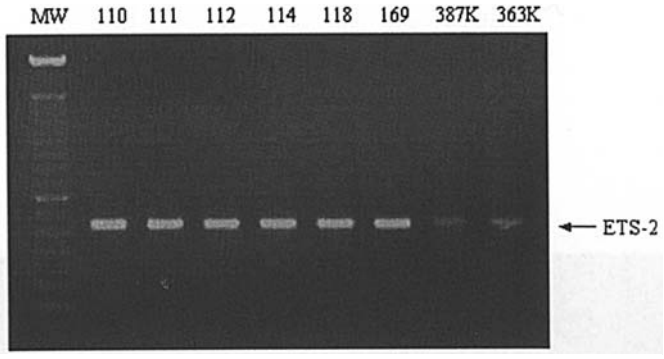

Figure 8. Confirmation of microarray analysis by RT-PCR. ETS2 gene-expression (110-112, 114, 118, 169, carcinomas; 387K and 363K, normal mucosa; MW, molecular weight marker).

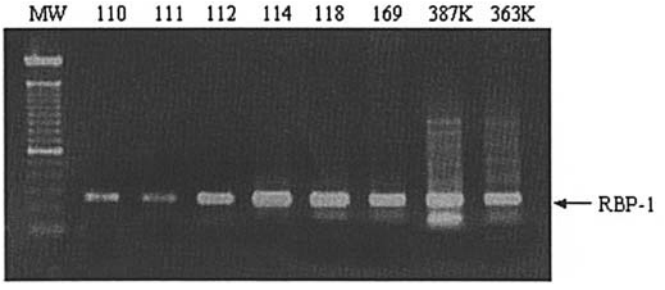

Figure 9. No confirmation of microarray analysis by RT-PCR. RBP1 gene-expression (110-112, 114, 118,169, carcinomas; 387K and 363K, normal mucosa; MW, molecular weight marker).

RBP1 expression to healthy mucosal tissue, others showed a decreased or rather increased expression (Fig. 9) and this was not a confirmation of the microarray analysis results.

Discussion

In this study we first describe a gene expression profile of apoptosis-associated genes for HNSCC. We identified 22 significantly differentially expressed genes representing an expression pattern, that can be used to differentiate between healthy mucosal tissue and cancer tissue of the upper aero-digestive tract. Additionally, two genes were found showing differences between tumor edge tissue and tumor center tissue.

Although of increasing number, only a few studies assessing expression patterns of HNSCC have been published. These studies used cDNA microarrays to investigate differences between normal mucosa and HNSCC. Tumors of 3-16 patients were compared with matched normal mucosal tissue and 985-12530 genes were screened. In these studies 9-227 differentially expressed genes were detected (8-12). Two recently published studies used larger groups of patients. Ginos *et al* investigated 41 HNSCC by screening 14500 genes finding 2890 genes that were differentially expressed (4). Chung *et al* investigated tumors of 60 patients with HNSCC looking at 12814 genes (13). The study setup of comparing expression pattern of normal versus tumor tissue enabled them to distinguish four different subtypes discriminating for recurrence-free survival. Two previously published studies described a pattern for tumor progression in HNSCC. Ha *et al* investigated the tumor progression by investigating the gene expression of more than 12000 genes in patients with HNSCC. Differences between carcinoma and normal tissue were significantly greater than differences between carcinoma to premalignant lesion and premalignant lesion to normal tissue (6).

Based on our results we describe a pattern for tumor progression and a gene expression profile in HNSCC. Only two genes could be found showing differences comparing tumor edge tissue and tumor center tissue. The data indicates not only a tumor progression from healthy toward premalignant towards invasive carcinoma as shown in previous studies but also a slight trend for tumor progression from tumor center to tumor edge. Thereby tumor center might be more necrotic and less invasive, whereas tumor edge might be actively fighting immune response, more invasive and inducing metastasis, which is also supported by our findings of lymphocyte activation being a prominent pattern in tumor edge.

We described 22 differentially expressed genes building two gene expression profiles, which are able to differentiate between tumor tissue and healthy mucosal tissue. In our study the following 19 genes showed increased expression levels in tumor center tissue: ARAF1, BCL2L1, BNIP3L, C20orf41, CAPN7, DAXX, EGFR, ETS2, IL8, IRF1, JUNB, MAP3K13, NFATC1, NTHL1, PCTK3, PDCD2, RAD52, RBP1 and STAT3.

Some members of this group, such as EGFR and IL8 represent well-known or potential key-role players in development and progression of HNSCC. For further verification, we selected one key-gene responsible for cell growth and

maintenance (ETS2), an expemplary gene from the JAK/STAT cascade (STAT3), and one gene involved in vitamin A metabolism (RBP1).

EGFR: Studies have shown that overexpression of EGFR, a well-known and well-examined apoptosis-associated gene is involved in the development and progression of HNSCC. It has been observed in 42-80% of HNSCC studied. There is evidence that EGFR expression adversely influences relapse and survival in HNSCC patients. Blocking this receptor in HNSCC cell lines and animal models inhibits tumor growth (14-16). In one of these studies the majority of 75 HNSCC tumors showed dramatic increased expression of EGFR. It was significantly increased in stage III and IV tumors compared to early stage cancers. These results indicate that increased expression of cell cycle regulatory proteins correlates with advanced tumor stage in HNSCC (17). Our result supports these findings by showing an increased expression of EGFR in tumor tissue and validates the microarray results, since the overexpression of EGFR in HNSCC as decribed above is a well-known fact in HNSCC.

IL8: The aggressive nature of metastatic human cancer has been shown to be related to numerous abnormalities in growth factors and their receptors. These perturbations confer a tremendous growth advantage to the malignant cells. IL8, originally discovered as a chemotactic factor for leukocytes (18), has recently been shown to contribute to human cancer progression through its potential functions as a mitogenic, angiogenic and motogenic factor. While it is constitutively detected in human cancer tissues and established cell lines, IL8 expression is regulated by various tumor microenvironment factors, such as hypoxia, acidosis, nitric oxide and cell density (19). In another study eight of 20 samples of epithelial ovarian cancer were positive for IL8 mRNA by RT-PCR, seven of them were in malignant group, with highest frequency in stages III and IV of the disease (50%). IL8 correlated with poor survival of the patients. These results indicate that IL8 is related to the malignant transformation process and can be considered as an indicator of poor prognosis in epithelial ovarian cancer patients (20). In 38 HNSCC patients one other study demonstrated that IL8 receptors are expressed by cancer cells and microvessel endothelial cells in HNSCC, suggesting that IL8 may act in an autocrine/paracrine fashion to stimulate cellular proliferation and angiogenesis (21). The results presented in our study confirm the reported role of IL8 in HNSCC and suggest a potential key role in the development of HNSCC.

ETS2: The ETS2 gene was translocated in the translocation t(8;21)(q22;q22), which is commonly found in patients with acute myloid leukemia (AML) (22,23). In a study of AML patients with complex karyotypes and abnormal chromosome 21 ETS2 ranked among the most highly expressed chromosome 21 genes compared to a control group of AML with normal cytogenetics. Overexpression of ETS2 correlated with genomic amplification. The authors hypothesized that the transcription factor ETS2 is altered by yet unknown molecular mechanisms involved in leukemogenesis (24). In another study expression levels of ETS2 protein were increased in 73% of oesophageal squamous cell carcinoma (ESCC) tissue samples contrary to their normal counterparts. These results suggested that ETS2 is overexpressed in paired human ESCC tissue samples at mRNA and protein levels and may be associated with the tumorigenesis of the oesophagus (25). Our results indicate that, in addition to the previously reported role of ETS2 for other tumors, this gene might play also an important role in the carcinogenesis of HNSCC.

STAT3: In many human cancers and transformed cell lines, STAT3 is persistently activated, and in cell culture active STAT3 is either required for transformation, enhances transformation or blocks apoptosis (26). STAT3 is considered an oncogene, since it accomplishes the activation of cyclin D1, c-Myc, and bcl-xl expression and is involved in promoting cell-cycle progression, cellular transformation, and in preventing apoptosis (27). In HNSCC STAT3 was up-regulated as well as in normal mucosa from these cancer patients compared with control normal mucosa from healthy patients. By STAT3 antisense plasmid activation of STAT3 was efficiently inhibited, increasing tumor cell apoptosis, and decreasing bcl-xl expression in a head and neck xenograft model. These findings provide evidence that activated STAT3 is an early event in HNSCC carcinogenesis that contributes to the loss of growth control by an anti-apoptotic mechanism (28). STAT3 mRNA expression by RT-PCR analysis showed high expression in T1 and T2, moderate in T3 and T4, and no expression in normal samples. STAT3 activation seems to be an early event in head and neck carcinogenesis (29). Our result is consistent with previously reported findings by showing an increased expression of STAT3 in tumor tissue and underlines a potential key role through its deregulation in HNSCC.

RBP1: Vitamin A is needed for the differentiated state of adult epithelia. The vitamin A metabolite retinoic acid regulates multiple biologic processes, including cell proliferation and differentiation, by modulating the transcription of numerous target genes and therefore it is postulated that defects in the Vitamin A metabolism contribute to tumorigenesis. Retinol metabolism is thought to be regulated by retinol binding proteins (RBP) among them the first one described, RBP1. In several cancer entities (30-32) a reduced expression of RBP1 is reported and was interpreted as contributing to the loss of differentiation and to tumor progression. Also the overexpression of cellular RBP1 was observed in certain malignancies (33-35) and was seen as a potential factor in carcinogenesis. Nonetheless there seems to be no uniform pattern in the disregulation of RBP1 in malignancies and the specific role of RBP1 in carcinogenesis has still to be identified. The results in our head and neck cancer biopsies seem to reflect this, because the microarray results showed an overexpression of RBP1, but the RT-PCR that was carried out demonstrated a rather heterogeneous expression pattern in the cancer tissue compared to normal mucosa. Some biopsies seem to have a rather weak, others a more strong expression. Although genes with strong patient to patient variations might not be suitable as markergenes, this inhomogeneous deregulation of RBP1 in head and neck cancer and this deregulation may indicate a potential role of RBP1 in carcinogenesis in the head and neck region.

Comparing the results presented here with other microarray studies the expression of four genes out of the 22 significantly differentially expressed genes has been described previously. RAD52, ETS2 and IL8 were upregulated, whereas CAPN7

was downregulated in cancer tissue compared to healthy mucosal tissue (4,6). These results are supported by the results of our study, while CAPN7 was upregulated in our study as well. All other transcripts reported in this study, have not been described to show differences comparing HNSCC tumor tissue to healthy mucosal tissue by previous publications (4-12,36-38) and therefore represent new findings in the field of HNSCC research.

In our study we could identify 22 differentially expressed genes out of 408 genes investigated. This result of 5.4% is within limits and close to the mean value of other reported studies with a range from 0.3 to 37.8% differentially expressed genes (mean value 9.2%) (4-12,36-38). However, the meaning of this observation remains unclear, since the amount of differentially expressed genes depends on various factors, such as the array type used.

Furthermore we report in our study 11 genes associated with anti-apoptotic functions to be upregulated and 12 genes with proapoptotic function to be downregulated in tumor tissue. This supports the hypothesis that deregulation of apoptosis plays an important role in carcinogenesis in head and neck cancer. Friedlander showed that genomic instability led to a higher risk of developing HNSCC and especially deregulation of p53 as an important regulator of DNA repair and apoptosis may contribute to carcinogenesis (39).

Six of 8 genes involved in lymphocyte activation were downregulated in the investigated tumor tissue. A similar finding was published by Ellisen *et al* (40). The authors examined whether microarray analysis would accurately reflect the normal pattern of gene expression following human T-cell activation, and whether the complex expression patterns identified are able to reflect a functional profile of lymphocyte activation. They found that expression profile analysis revealed the sequential induction of groups of functionally similar genes, whose temporary coregulation underscores known cellular events during T cell activation (40).

Our results, combined with the previously published observations indicate that in tumor progression not only single genes but whole functional groups are deregulated. This phenomenon has been reported in other cancer entities before. Gutierrez *et al* showed that in different subclasses of acute myeloid leukemia genes associated with angiogenic factors (FGF13 and FGF1) as well as genes involved in cell adhesion showed altered gene expression in acute myeloid leukemia subclasses (41). Lahdesmaki *et al* showed that in breast cancer functional gene sets were able to separate primary tumors and their metastases. Genes involved in metabolism, signal transduction, cell cycle, and transcriptional factor and DNA binding molecules were able to separate both groups while apoptosis and cell adhesion and migration genes did not provide a clear separation of the two groups of samples (42). In HNSSC Nagata *et al* showed that in oral squamous cell carcinoma proteolytic enzymes and integrin-related molecules are involved in cervical lymph node metastasis (43).

These previous findings support the hypothesis that carcinogenesis of human HNSCC is not the result of individual genes being disregulated, moreover it is controlled by a network of functionally related genes.

Further studies, using larger patient groups in order to increase the statistical power will help to verify the observed results and additionally identify molecular patterns of tumor progression by comparing tumor centre to tumor edge. Furthermore using a whole genome array will expand the perspective, increase the number and widen the function of genes and deregulated functional groups to be found.

These results will lead to a better understanding of carcinogenesis and invasion of HNSCC on a molecular level. In addition the classification of HNSCC will improve and subgroups might be identified, which show differences toward clinical parameters such as survival or risk for metastasis and therapeutical response. The variety of the involved functional processes presented in this study underlines the complexity of HNSCC phenotype, but represents an important initial step in making the development and clinical outcome of premalignant lesions reliably predictable.

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