

# Comparative expressed sequence hybridization detects recurrent patterns of altered sequence expression in oral squamous cell carcinoma

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Received February 24, 2010; Accepted April 28, 2010

DOI: 10.3892/or\_00000869

**Abstract.** Despite its common histology and presentation, oral squamous cell carcinoma (OSCC) is associated with widely varying clinical behaviour and response to therapy. To further elucidate the molecular basis of OSCC, an approach for gene expression analysis termed comparative expressed sequence hybridization (CESH) was used in the present study. This straightforward approach allows the rapid delineation of pathophysiologically interesting candidate chromosome regions by a direct detection of aberrant transcriptional activation. CESH profiling of OSCC specimens led to the identification of several novel chromosomal regions. Increased expression compared to a set of control mucosa specimens was found on 1q22-q23, 3q26.3-qter, 4q31.1-q32, 11q12-q13.2, 14q32, 18q12, 19q13.2-q13.3 and 22q13.1-q13.2. Decreased expression was found on 8p22-p23, 16p12 and 16q23-q24. Using CESH, common patterns of altered sequence expression in different OSCC samples were obtained. While some of these regions overlap with those known to be frequently altered in OSCC on the genomic level, this screen revealed novel chromosome subregions with increased trans-

criptional activity, which are probably independent of the genomic status of the tumor cells.

## Introduction

Oral squamous cell carcinoma (OSCC) is the 10th most common human malignancy worldwide affecting more than 500,000 individuals per year. The 5-year survival rate for oral squamous cell carcinoma does not currently exceed 55%, which is mainly caused by locally aggressive tumor phenotypes and early loco-regional metastases (1). In general, clinicopathological parameters such as the TNM system, which are used as basis for therapeutic decisions, do not adequately predict the biological behaviour of the tumors. To improve the clinical management of individual patients, there is a strong requirement for a better understanding of molecular events involved in OSCC formation. Genetic changes associated with malignant transformation and tumor progression critically affect the expression of key genes. To further elucidate the molecular basis of these critical events, an approach termed comparative expressed sequence hybridization (CESH) was recently introduced in molecular analyses of tumor specimen (2). The principles of CESH are similar to chromosomal comparative genomic hybridization (cCGH), but instead of differently labeled genomic DNA, cDNA sequences derived from cellular RNA are used as hybridization probe for test and control specimen. This allows the analysis of global expression patterns of both coding and non-coding genes in tumor specimens. Since its first publication, the CESH method was established and applied on different subtypes of rhabdomyosarcoma (RMS), Wilms' tumors, hairy cell leukemia, acute lymphoblastic leukemia and breast cancer (3-5). It was shown that individual tumor subtypes within these tumor entities could be distinguished by their expression profiles obtained by CESH. Moreover, combining cCGH and CESH experiments allowed the correlation of chromosome copy number changes and transcriptional activities within the same chromosome subregions (3,6) further narrowing the alterations, which might cause effects in tumor development. In this study we applied CESH to a series of 28 cases of advanced OSCC in order to obtain an overview of global gene expression. Comparing the CESH data to existing cCGH data,

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**Key words:** oral squamous cell carcinoma, comparative expressed sequence hybridization, mRNA

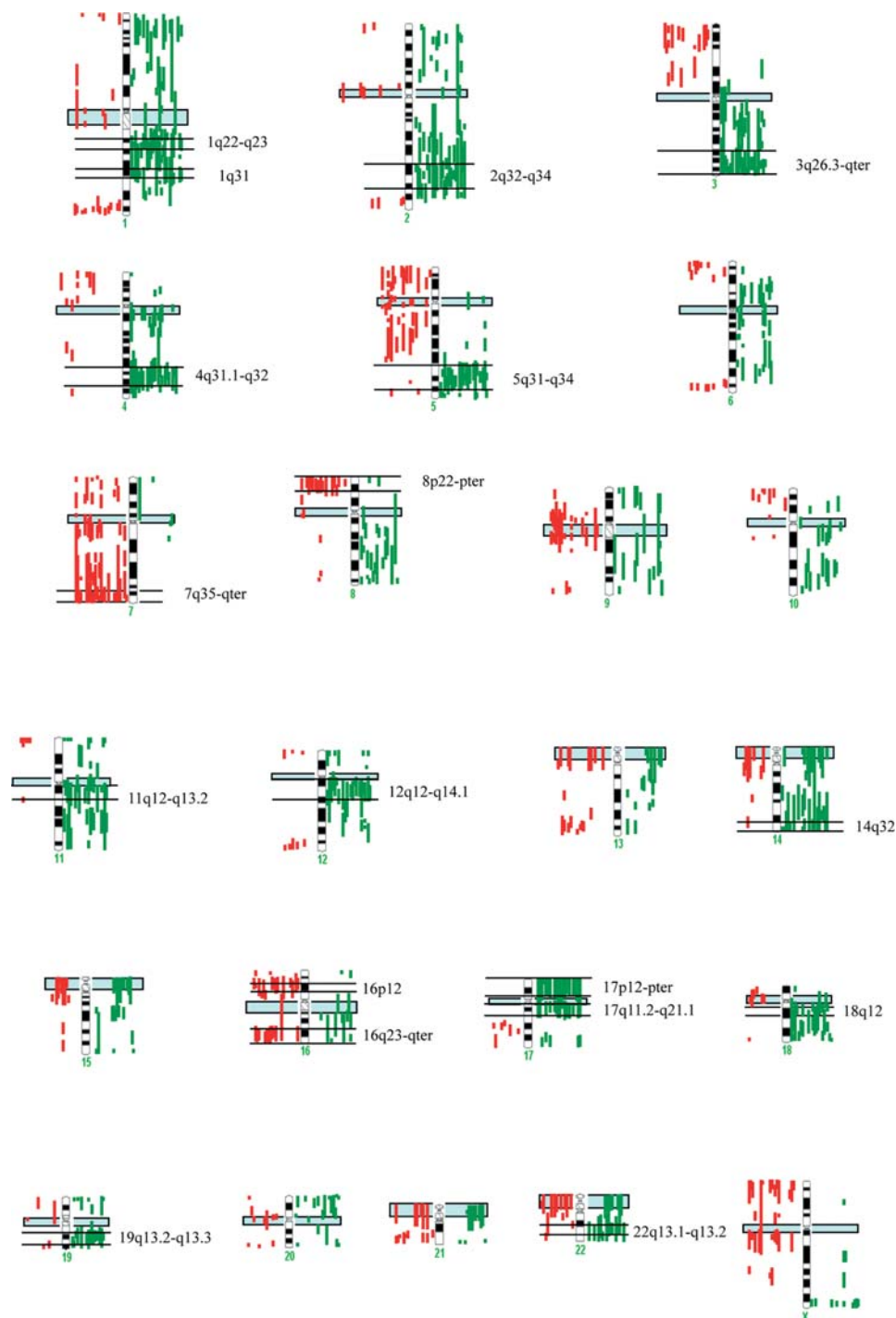


Figure 1. Schematic overview of changes in relative mRNA expression in 28 cases of OSCC detected by CESH. Red and green bars on the left and right side of chromosomal ideograms indicate relative low or high average expression levels, respectively. Blue boxes represent centromeric regions of the chromosomes. The chromosome regions most commonly altered in expression are indicated by the horizontal lines.

we aimed to identify the relationship between copy number changes and expression profile at the corresponding chromosome regions.

## Materials and methods

**Tumor material.** Twenty-eight frozen-tissue tumor samples were collected from patients with histological confirmed OSCC after approval by the institutional review board of the

University Hospital Heidelberg and informed consent. As control tissue, oral mucosa from 4 healthy donors was used. As reference, human reference RNA from a commercially available pool of ten different cell lines was used (Stratagene, Amsterdam, The Netherlands).

**RNA preparation and reverse transcription.** DNA and RNA extraction from OSCC samples was carried out by ultracentrifugation in cesium chloride as previously described



A quality and concentration were tested with the 100 bioanalyzer (Agilent Technologies, Palo Alto, USA). RNA was reversely transcribed using random hexamer primers and Superscript II (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions.

**Complementary DNA labeling.** Complementary DNAs (100 ng) reverse transcribed from tumor, control and reference RNA were differently labeled with Cy3-dCTP (Amersham Biosciences) and biotin-dCTP (Loxo-Life Sciences) using BioPrime DNA Labeling Kit (Invitrogen). Unincorporated nucleotides, random primers and dyes were removed by use of the QIAquick PCR Purification kit (Qiagen, Hilden) according to the manufacturer's instructions. Incorporation of dyes into the labeled DNA was determined by UV spectrophotometry.

**Hybridization to metaphase chromosomes.** Five micrograms of each differently labeled probes (tumor vs. reference, and control vs. reference) were denatured together with 5  $\mu$ g of unlabeled Cot-1 DNA in 5  $\mu$ l of deionized formamide and 5  $\mu$ l of hybridization mixture (20% dextranulphate/4X SSC) at 75°C for 7 min followed by pre-annealing at 37°C for 20 min. Human metaphase slides (Abbott, Wiesbaden, Germany) were pretreated with 70% acetic acid for 10 min, washed with PBS, treated with RNase A for 30 min (0.1  $\mu$ g/ml, diluted in PBS) in a humidified chamber at 37°C and rinsed in 2X SSC. After dehydration, denaturation step was performed in 70% formamide (49 ml formamide, 7 ml 2X SSC, 14 ml 0.5 M NaPO<sub>4</sub>) for 2 min at 70°C. After dehydration (70, 90 and 100% ethanol), 10  $\mu$ l of hybridization mixture with denatured probe was applied to the denatured chromosomes on the slide. Hybridization was carried out in a humidified chamber at 37°C for 72 h. After hybridization, slides were washed in 50% formamide, 20X SSC (pH 7.0) and 0.5X SSC (pH 7.0) at 42°C. Incubation with avidin-FITC antibody (2  $\mu$ g/ml) was performed for 30 min at 37°C in humidified chamber, followed by incubation with 2  $\mu$ g of anti-avidin-FITC antibody (Vector-Burlingame, Canada). Finally, the slides were washed with 4X SSC, 1% Tween (pH 7.3) at 42°C, and mounted in an antifade solution containing 4',6-diamidino-2-phenylindole to visualize cell nuclei.

**Image acquisition and the analysis.** Images were acquired through a Zeiss Axioplan fluorescence microscope equipped with filter sets appropriate for DAPI, FITC and Cy3 by using CCD camera. For quantitative analysis of CESH experiments, commercially available analysis software, CytoVision 3.6 (Applied Imaging, Thunderland, UK) was used. For each metaphase spread, three digital images were acquired, one image for each fluorochrome. At least 15 metaphase spreads were analyzed for each tumor sample examined.

**Definition of cut-off values for CESH experiments using standard reference intervals (SRIs).** As thresholds for determination of a relative increase or decrease of expression, dynamic standard reference intervals (SRIs) were used (5). SRIs were created in order to reduce the amount of false positive and false negative results and to detect the relative expression profile by CESH with higher specificity and

sensitivity (9-11). They were generated by the analysis of 100 metaphase spreads after hybridization of the same but differently labelled cDNA. The confidence intervals of 95% were used in this analysis.

## Results

**CESH profile of oral squamous cell carcinoma specimens.** Initially, to define tissue specific changes in expression compared to the reference RNA used, four oral mucosa specimen derived from healthy individuals were analyzed by CESH comparing them to human reference RNA chromosomal regions showing high mRNA expression were 1q36.2-p35, 1q31.2-q32.1, 2q23-q32.2, 5q31, 6q24, 10p13-p12, 10q22.3-q25, 12q13.12-q13.3, 15q22.1-q24, 17p12 and 17q12-q21.1. Low mRNA expression was seen in the chromosomal regions 1q34 and 7q35. Consecutively, 28 OSCC specimens were analyzed by CESH in order to define chromosomal regions with altered average expression levels. Chromosomal regions exhibiting high mRNA expression in >40% of cases examined are 1q22-q23, 1q31, 2q32-q34, 3q26.3-qter, 4q31.1-q32, 5q31-q34, 11q12-q13.2, 12q12-q14.1, 14q32, 17p12-pter, 17q11.2-q21.1, 18q12, 19q13.2-q13.3 and 22q13.1-q13.2. Low mRNA expression was found on chromosomal regions 7q35-q36, 8p22-p23, 16p12 and 16q23-q24. A summary of the results is presented in Fig. 1. A detailed summary of all chromosomal regions exhibiting changes in expression in >40% of OSCC cases analysed is presented in Table I. On chromosomal regions 1q31, 2q32-q34, 5q31, 12q13, 17p12 and 17q12-q21.1, low mRNA expression was found in OSCC specimens and oral mucosa specimens as well, while decreased expression in both types of tissue was found on 7q35-qter. These findings most likely indicate tissue specific expression patterns. Conclusively, high mRNA expression of chromosomal regions 1q22-q23, 3q26.3-qter, 4q31.1-q32, 11q12-q13.2, 14q32, 18q12, 19q13.2-q13.3 and 22q13.1-q13.2 and low mRNA expression of chromosomal regions 8p22-p23, 16p12 and 16q23-q24 were considered to be specific for OSCC specimen.

## Discussion

Genetic changes associated with malignant transformation and tumor progression critically affect the expression of key genes. Therefore, expression profiling approaches including microarray-based technologies are potential tools in cancer research. Global expression profiling has been successfully used for the molecular classification of tumors, identification of genetic markers, determination of the clinical behaviour of a tumor and prediction of oncogenic pathways (12-14). In this study, the method termed comparative expressed sequence hybridization (CESH) was used in order to search for new chromosome regions that may play a role in OSCC development. CESH is a relatively simple and rapid approach, which gives a genome-wide overview of relative expression patterns within tissues according to the chromosomal location (2,15). The aim of the present study was to identify common chromosome regions, which are affected by differential mRNA expression profiles in OSCC specimens. Among others, such an aberrant high mRNA expression was

Table I. Chromosome bands with high and low expression, respectively, comparing OSCC specimens to reference.

Chromosome bands with high expression	No. of cases (%)	Chromosome bands with high expression	No. of cases (%)
1q22	60.7 (17)	11q12	60.7 (17)
1q23	75 (21)	11q13	39.28 (11)
1q31	57 (16)	12q12	57 (16)
2q32.1-q32.2	57 (16)	12q13	60.7 (17)
2q32.3-q33	71.4 (20)	14q32.1	42.86 (12)
2q24	53.6 (15)	14q32.2-q32.3	53.57 (15)
3q26.3-q27	75 (21)	17p12	67.85 (19)
3q28-qter	71.4 (20)	17p13	67.85 (19)
4q31.1	60.7 (17)	17p11.2	46.42 (13)
4q31.2-q31.3	75 (21)	17q11.2-q12	53.6 (15)
4q32	64 (18)	17q21.1-q21.2	46.42 (13)
5q31	67.85 (19)	18q12	50 (14)
5q32	71.4 (20)	19q13.1-q13.2	50 (14)
5q33	60.7 (17)	22q13.1-q13.2	46.43 (13)
Chromosome bands with low expression	No. of cases (%)	Chromosome bands with low expression	No. of cases (%)
7q35	75 (21)	16p12	53.57 (15)
7q36	78.75 (22)	16q23	42.86 (12)
		16q24	39.28 (11)
8p22	57 (16)		
8p23	53.57 (15)		

frequently found for chromosomal bands 3q26.3-qter and 11q12-q13. Previous cCGH analyses suggested that DNA copy number gains of 3q26.3-qter and 11q12-q13 are driving genetic events in HNSCC progression, which are associated with adverse patient outcome (16). On the distal part of chromosome arm 3q, several candidate proto-oncogenes have been identified in HNSCC and OSCC including *PIK3CA* (17), *CCNLI* (18) and *ACK1* (19). For 11q12-q13, we described a distinct amplicon of recurrent DNA amplification in OSCC, which contains the proto-oncogenes *CCND1* and *CTTN* (20). The *CCND1* encoded protein cyclin D1 promotes the progression through the G1-S phase of the cell cycle by phosphorylation of the retinoblastoma protein. In addition, it is involved in the regulation of cellular growth, metabolism, and cellular differentiation (21). *CTTN* encodes cortactin, an actin-binding protein involved in cytoskeleton organization and cell adhesion structures. An increase of motility and invasiveness was described for HNSCC cell lines with *CTTN* amplification and cortactin overexpression, which decreased after selective cortactin knock-down (22). Retrospective studies found an association of amplification and adverse patient outcome in HNSCC (23). In this respect, the increase of transcriptional activity on chromosomal bands 3q26.3-qter and 11q12-q13 detected by CESH in our analysis is probably the result of distinct DNA copy number gains in these chromosome

regions underlining the validity and reliability of the present experimental approach. Furthermore, it further points to the presence of candidate proto-oncogenes in 3q26.3-qter and 11q12-q13 in OSCC.

A novel critical chromosome region, which exhibited an increase in mRNA expression in OSCC, was chromosome 19q. In chromosomal comparative genomic (cCGH) analysis, the evaluation of chromosome 19 hybridization signals was considered to be critical due to a large amount of GC-rich DNA sequences (24). In recent array-CGH analyses, however, distinct regions of frequent DNA copy number gain were found on chromosome 19, including 19q12 and 19q13.12 (25). The proto-oncogene *CCNE1* is located at 19q12, it is a member of the cyclin family, which has been shown to play an important role in G1/S transition of the cell cycle. Gene copy number gain of the *CCNE1* locus and high cyclin E1 protein expression was found in about 25% of OSCC cases analyzed suggesting an activation of *CCNE1* by gene copy number gain (25). According to our CESH data, the most frequent high mRNA expression is found on the distal chromosome bands 19q13.1-19q13.2. These findings are in concordance with a region of common DNA copy number gain in array-CGH analysis. While no candidate proto-oncogene is currently known in this chromosome region, the finding of recurrent high transcriptional activity of 19q DNA





SPANDIDOS<sup>3</sup> encourages further research activities to define the proto-oncogenes.

Further chromosome regions of recurrent high mRNA expression detected in the present study are 14q32 and 22q13, for which DNA copy number gain has been found in previous studies in OSCC (25-27). These findings underline the assumption that high mRNA expression levels detected by CESH reflect an increase in gene transcription, which is frequently the result of distinct DNA copy number gain.

A relative decrease of mRNA expression was found on 8p22-p23, which is in concordance with frequent DNA copy number loss on chromosome arm 8p in cCGH analysis in HNSCC (16,28). For chromosome 18, however, a relative increase of transcriptional activity was found in up to 50% of tumors analyzed in the present CESH analysis with the minimal overlapping region on 18q12. This was surprising, since monoallelic chromosome 18 deletions with several minimal overlapping regions have been previously described in OSCC and HNSCC, which were suggested to indicate a down-regulation of relevant tumor suppressor genes (29,30). Furthermore, a loss of heterozygosity (LOH) affecting 18q was found associated with decreased patient survival (31). Subsequent studies identified candidate tumor suppressor genes inside these deleted regions including *DCC* (32) and *GALRI* (33), but failed to define a relevant impact of a loss of gene function on the clinical course of the disease. Due to the limited resolution of chromosomal CESH, the data do not allow to exactly delineate, which DNA sequences are responsible for the signal of increased expression in CESH experiments. However, the current findings do not provide evidence that segmental DNA copy number losses on chromosome 18 result in a reduced expression of tumor suppressor genes, which are critically involved in OSCC formation. To further investigate this question, the application of CESH to DNA arrays with high resolution might be helpful to define, which chromosome 18 regions and corresponding genes are actually subject of increased and decreased mRNA expression, respectively.

A major advantage of CESH analysis is the potential to define novel chromosome regions, which are subject of increased transcriptional activity, but were not yet investigated in detail, since no respective DNA copy number aberrations have been found. In the present analysis, a relative decrease of mRNA expression was detected on chromosome regions 16p12 and 16q23-q24, for which no DNA copy losses have been described in previous CGH analyses (25,26,34). Candidate tumor suppressor genes in 16p12 are *TNRC6A*, which is involved in the regulation of mRNA silencing (35) and *JMJD5*, which may function in DNA mismatch repair (36). On 16q23-q24, the tumor suppressor gene *WWOX* is located, which was found inactivated in esophageal carcinoma (37). Further expression and functional studies are required to elucidate a possible role of these candidate genes in OSCC pathophysiology.

In conclusion, our study demonstrates that CESH is a reliable and potential technique for a genome-wide profiling of aberrant expressed chromosomal regions in OSCC. It allows the definition of consistent expression patterns characteristic for a tumor entity. Identification of targeted genes present in changed regions warrants further molecular investigation.

## Acknowledgements

This study was supported in part by the National Genome Research Network (NGFN2/No. 01 GR 0417), the Manfred-Lautenschläger-Stiftung and the Medical Faculty of the University Heidelberg.

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