

# Oncogenes and tumor suppressor genes in squamous cell carcinoma of the tongue in young patients

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## ABSTRACT

### Objectives

The occurrence of squamous cell carcinoma of the tongue (SCCT) of young patients increased. There are still controversies about patient prognosis. The underlying molecular mechanisms remain unclear.

### Methods

276 patients (66 ≤45, 210 >45 years) with SCCT were included. Clinical parameters and survival data were assessed. Oncogenes and tumor suppressors were analyzed via immunohistochemistry (p53, CXCR4, p16, EGFR) and qPCR (*CDK4*, *CDKN2A*, *TP53*, *MDM2*, *AKT1*, *PIK3CA*, *NRAS*, *HRAS*, *KRAS*, *HGF*, *MET*, *EGF*, *ATM*, *BRCA1*, *E2F1*, *FHIT*, *RUNX3*, *STK11*, *BCL2*, *CTNNB1*).

### Results

The median overall survival was 142 (≤45 years) and 34 months (>45 years) ( $p < 0.0001$ ; HR [95%CI]: 0.37 [0.30–0.58]). Disease specific survival in patients ≤45 years was with 181 months significantly higher than in patients >45 years ( $p < 0.0001$ ; HR [95%CI]: 0.33 [0.26–0.57]). Immunohistochemistry visualized a comparable expression of analyzed proteins. QPCR demonstrated in patients ≤45 years a higher expression of genes that are associated with carcinogenesis (*CTNNB1*, *STK11*, *CDKN2A*, *HGF*, *MET*) as well as tumor suppressors that constitute an enhanced radio-sensitivity (*ATM*, *BRCA1E2F1*, *FHIT*).

### Conclusion

Derogation of the *WNT-CTNNB1-STK11* and *CDKN2A-HGF-MET* pathway can constitute the carcinogenesis, while the higher expression of radio-sensitizers *ATM*, *BRCA1E2F1* and *FHIT* can explain the better OS/DSS in young patients.

## INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide with an annual incidence of more than 270,000 cases [1]. The 5-year survival rate is estimated to be about 50% [2]. While the overall incidence rate in HNSCC slightly reduced over the past decade, the occurrence of oral HNSCC increased [3–8]. Particularly, the incidence rate of young (<45 years) non-smokers with squamous cell carcinoma of the tongue (SCCT) increased significantly [8–11]. These patients show a highly aggressive phenotype with a high proportion of lymph node positivity at the time of diagnosis, a high recurrence rate after therapy, and therefore a poor prognosis [8, 12, 13]. The molecular mechanisms underlying the clinical behavior remain unclear. In common oral HNSCC alcohol and nicotine abuse represent the most important risk factors [7, 14–16]. Recently, the association between human papilloma virus (HPV) and tongue cancer (SCCT) was demonstrated with a HPV-positivity in 25–60% of SCCT [7]. Saito et al. observed an increased incidence of HPV-positive SCCT in Japan [15]. Recent studies failed to demonstrate an HPV-association in SCCT of young patients [3, 10]. Genomic aberrations apart from alcohol, nicotine, or virus induced carcinogenesis might constitute the malignant phenotype [17–19]. The activation of oncogenes (*EGFR*, *CCND1*, *MYC*, *PIK3CA*, *RAS*) and an abrogated pathway in tumour suppressors (*TP53*, *TP73*, *RB*, *CDKN2A*, *CDKN1A*) could be widely demonstrated in HNSCC [20–28]. The purpose of this study is to give a detailed assessment of the oncogene and tumor suppressor profile in patients with common SCCT (>45 years) compared with SCCT in younger patients (≤45 years) using quantitative PCR (qPCR) and EGFR, p53, p16, and CXCR4 immunohistochemistry.

## RESULTS

### Epidemiology

From January 1983 to December 2013, a total of 276 patients with SCCT were treated in the Department of Otorhinolaryngology, Technical University Munich. There were 66 patients ≤45 years (mean: 38 years; range: 20–45 years) and 210 patients >45 years (mean: 60 years; range: 46–91 years). Both groups demonstrated the same gender distribution resulting in a moderate male predominance of 2.7 and 2.8 to 1. We did not see any differences in the nicotine and alcohol consumption ( $p = 0.41$ ;  $p = 0.22$ ). The cumulative consumption differed significantly. The prevalence of synchronous and metachronous malignancy was 24% in patients ≤45 years and 31% in patients >45 years ( $p = 0.3$ ). In synchronous and metachronous malignancy pharyngeal carcinoma occurred in 9%, bronchial carcinoma in 7%, urogenital

carcinoma in 5%, oesophageal carcinoma in 4%, oral cavity carcinoma (other than tongue) in 3%, gastrointestinal carcinoma 3%, laryngeal carcinoma in 2%, cutaneous carcinoma in 2%, and other malignancies in 3% respectively. With respect to the TNM-stage, patients ≤45 years demonstrated significant smaller tumour sizes ( $p = 0.011$ ), while the N- and M-status was comparable in both groups ( $p = 0.59$ ;  $p = 0.76$ ). The majority of patients were diagnosed with T1/2 tumours, solitary neck metastases (N1/2a), moderate histological differentiation (G2) and histologically complete resection (R0). Thirty-two patients (12%) underwent a primary radio-(chemo-) therapy. The majority of patients (68%) underwent radical surgical approaches comprising the trans-oral resection, mandible split with subsequent reconstruction, and neck dissection. An adjuvant radio-(chemo-) therapy was done in 111 patients (45%).

### Survival analysis

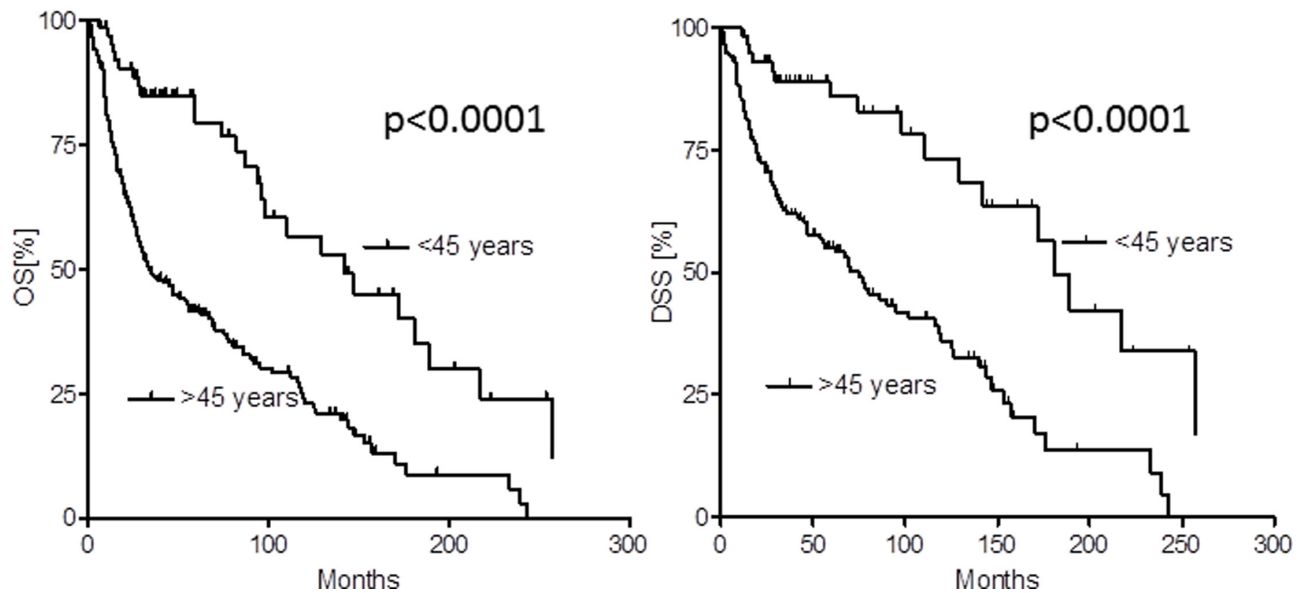
Recurrent disease was demonstrated in 20% of patients ≤45 years and 18% of patients >45 years ( $p = 0.3$ ). The 5-year recurrence free interval (RFI) in patients ≤45 years was 84%, in patients >45 years 75% respectively. After a mean follow-up of 54 months in both, patients ≤45 years and >45 years, there were significant differences in the OS and DSS. The median OS in patients ≤45 years was 142 months and 34 months in patients >45 years, respectively ( $p < 0.0001$ ; HR [95%CI]: 0.37 [0.30–0.58]). DSS in patients ≤45 years was with 181 months significantly higher than in patients >45 years ( $p < 0.0001$ ; HR [95%CI]: 0.33 [0.26–0.57]). The 5-year survival rate in patients ≤45 years was 79%, in patients >45 years 41% (Fig. 1).

### Immunohistochemistry

Immunohistochemistry did not reveal any differences between both groups. 73% of the patients ≤45 years and 87% of patients >45 years demonstrated p53-positivity (Fig. 2a, 2b). A positive p16 staining, as visualized in Fig. 2c, 2d, was seen in 13% of the patients ≤45 years and in 33% of patients >45 years. The vast majority of patients were p16 negative. All analyzed samples showed a strong EGFR and CXCR4 staining (Fig. 2e–h).

### Quantitative PCR of oncogenes and tumor suppressors

*RUNX3*, *AKT1*, *PIK3CA*, the *RAS* family, *TP53*, *MDM2*, *CDK4*, and *BCL2* demonstrated comparable expression levels or were slightly minor expressed in patients ≤45 years (Fig. 3). *CDKN2A*, *MET*, *HGF*, *ATM*, *BRCA1*, *E2F1*, *FHIT*, *CTNNB1*, and *STK11* were significantly higher expressed in patients ≤45 years demonstrating differences from 4- to 11-fold (Fig. 3).

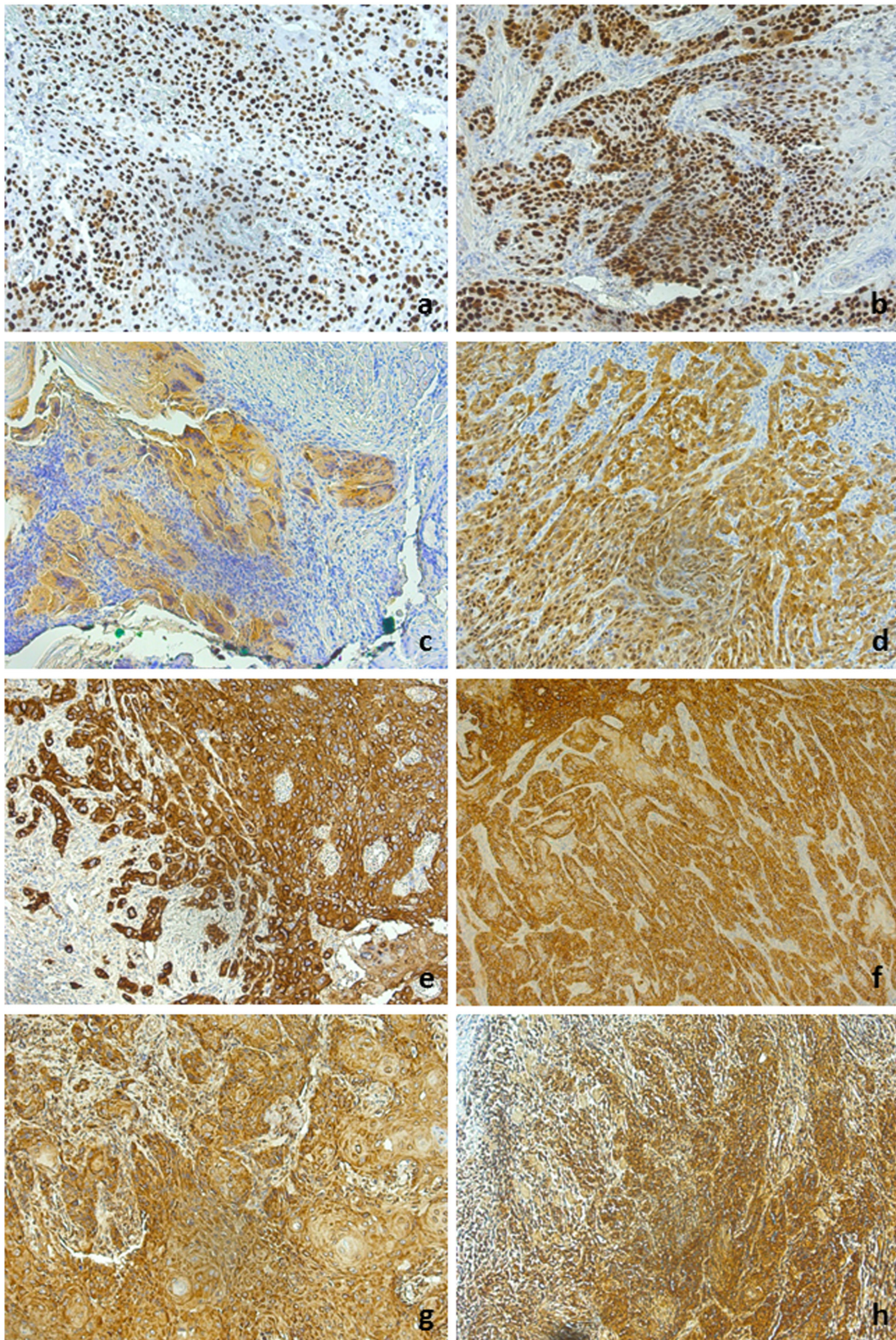


**Figure 1: Kaplan-Meier estimates of the overall (OS) and disease specific survival in patients  $\leq 45$  years and patients  $> 45$  years.**

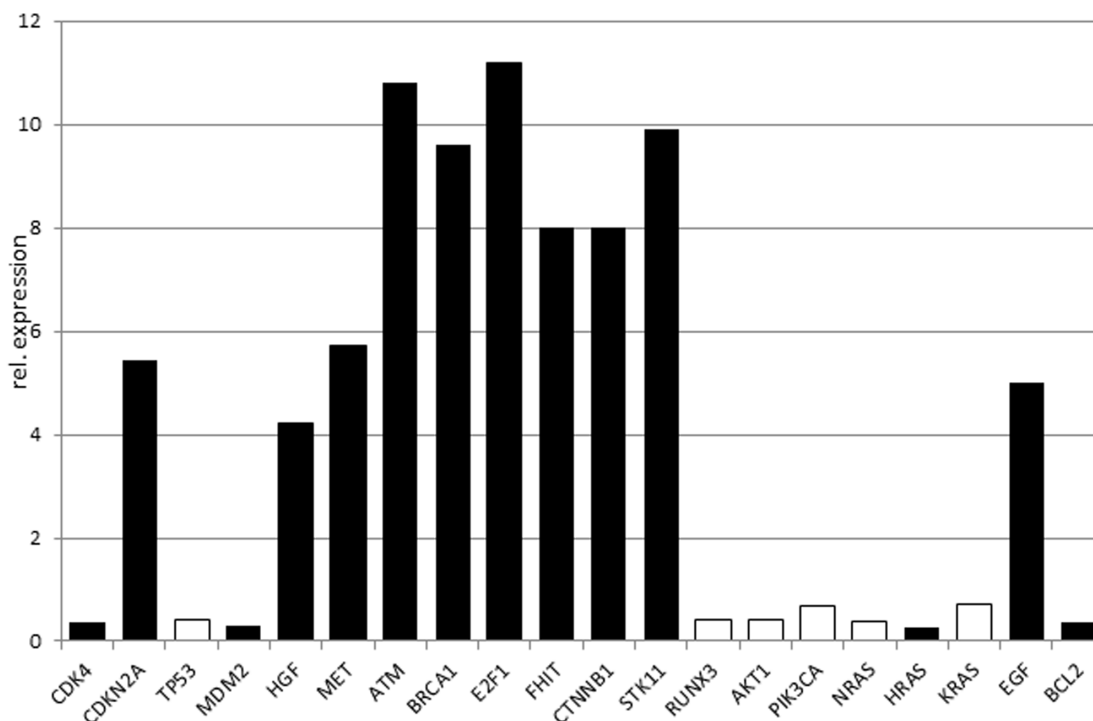
## DISCUSSION

Squamous cell carcinoma represents the most frequent histological differentiation in head and neck malignancy. While overall incidence rates decrease, several studies demonstrated an increased incidence of oral tongue squamous cell carcinomas (SCCT) in young adults [6, 10, 10, 11, 29–31]. Many authors attribute SCCT of young adults a more aggressive phenotype with a high proportion of lymph node positivity at the time of diagnosis and a high recurrence rate after therapy. But, there are still controversies about the patient prognosis [8, 12, 13, 32–40]. The molecular mechanisms underlying the aggressive behavior remain unclear. The heterogeneity of head and neck squamous cell carcinoma was demonstrated in whole-exome sequencing identifying a small number of activating mutations in oncogenes and highlighting the role of *p53*, *Rb/INK4/ARF* and *Notch* tumor suppressor pathways [18, 21, 22, 41]. *EGFR* represents an oncogene that is frequently over-expressed in HNSCC due to activating mutations. After binding of EGF to its receptor the dimerization of EGFR results in an auto-phosphorylation of several tyrosine residues that lead to cell proliferation by activating the *MAPK*, *AKT*, and *JNK* pathways. In the current study, the majority of samples stained positive for EGFR without differences between the subgroups. While qPCR of *AKT1* did not reveal any differences between the groups, *EGF* was significantly higher expressed in young adults. *In-vitro* analysis demonstrated a dose-dependent rise of BCL-x(L) and p21(CIP1/WAF1) protein after incubation with EGF, whereas no influence was seen on BCL-2 [42]. According with the recent literature, qPCR showed a slight minor-expression of *BCL-2* in patients  $\leq 45$  years, despite *EGF*

over-expression. Thus, a disruption of the *EGF-EGFR-AKT1* pathway that mediates the hypothesized aggressive phenotype of SCCT in young adults might be unlikely. *PIK3CA*, the *RAS*-family, *RUNX3*, or *CXCR4* that were associated with loco-regional metastases when hyper-methylated or over-expressed showed a comparable or minor expression in patients  $\leq 45$  years. In contrast with recently published data, in our cohort *CTNNB1* expression did not correlate with tumor differentiation or lymph node metastasis. Authors notice that *CTNNB1* expression was no independent prognostic factor for disease-specific survival [43]. The *WNT/CTNNB1* pathway inhibits mitochondria- and detachment-mediated apoptosis [44]. *In vitro* analysis demonstrated that *CTNNB1* silencing activates the *STK11/AMPK* pathway resulting in a G1 arrest by phosphorylating p53 and suppressing *mTOR* signaling [45]. In our cohort, *STK11* was significantly higher expressed in patients  $\leq 45$  years. Besides *STK11*, *TP53* represents a further tumor suppressor that is frequently mutated in SCCT [46]. The *TP53-CDKN2A-CCND1-CDK4-RB1* pathway represents a tumor suppressor pathway that is frequently abrogated in HNSCC. Immunohistochemistry and qPCR identified p53 positivity in the majority of samples without any differences between the subgroups. QPCR revealed a higher expression of *CDKN2A* in patients  $\leq 45$  years, while *CDK4* was down-regulated. Subsequent p16 protein was visualized in 13% of patients  $\leq 45$  years and 33% of patients  $> 45$  years, indicating a minor impact of HPV associated carcinogenesis in the group of young adults. Infection with high-risk HPV is recognized as an independent risk factor, particularly in oropharyngeal tumor sites. HPV-positive patients with oropharyngeal carcinomas demonstrate a better survival, irrespective of



**Figure 2: Immunohistochemistry (20x) of p53 (a, b), p16 (c, d), EGFR (e, f), and CXCR4 (g, h).** Tissue samples of the cohort of patients  $\leq 45$  years are visualized on the left, the cohort of patients  $>45$  years on the.



**Figure 3: Quantitative PCR of oncogenes and tumor suppressors.** The relative expression is visualized for patients ≤45 years, normalized by patients >45 years. Significant differences were marked black.

the treatment regimen. HPV associated carcinogenesis in oral carcinomas has been discussed controversially so far [47, 48]. Beside a HPV induced p16 over-expression, abrogation of the *CDKN2A-CCND1-CDK4-RB1* pathway is a frequent event in head and neck squamous cell carcinoma. In our cohort, the *CDKN2A* over-expression in the absence of a p16 protein refers most likely to *CDKN2A* deficiency. Mutation, hypermethylation, and allelic alteration (loss of heterozygosity (LOH), microsatellite size alteration) of *CDKN2A* were associated with the development of dysplastic lesions [22, 49–52]. Particularly, LOH and absence of p16 protein might be associated with the progression of normal mucosa to hyperplastic lesion or carcinoma in situ [49, 53, 54]. Recently, in malignant melanoma a *CDKN2A* deficient mouse cell line demonstrated *MET* gene amplification [55]. The *HGF-MET* pathway plays a pivotal role in the progression of head and neck squamous cell carcinoma [56]. Particularly HPV negative tonsillar carcinomas with *HGF* or *MET* over-expression were associated with a reduced survival [46]. In our cohort, we identified a *MET-HGF* over-expression in patients ≤45 years.

Contradictory results in survival data might refer to different study populations and treatment regimens. In agreement with the present literature, we observed a high incidence of T1/2 tumors and solitary neck metastases [8, 12, 45]. Radical and subsequent reconstructive surgery resulted in a R0-resection the majority of patients. 111 patients (45%) underwent an adjuvant radio-(chemo-) therapy. We achieved a significant better loco-regional

control with a 5-year RFI of 84% in patients ≤45 years and 75% in patients >45 years respectively. Other studies with a comparable study population and treatment regimen also demonstrated a significant higher OS and DSS in patients ≤45 years when compared with their older counterparts [6, 8, 12]. An enhanced radiosensitivity can constitute the better survival in our cohort. Tumor suppressors *ATM*, *BRCA1*, and *E2F1* were significantly higher expressed in patients ≤45 years. *ATM* plays a pivotal role in the homologous recombination after DNA double strand breaks. *ATM* or *ATR* kinases phosphorylate and therefore stabilize *E2F1* transcription factor. *E2F1* represents a member of the *E2F* family that exclusively interacts with the *Rb* tumor suppressor. Furthermore, *E2F1* regulates *p53* dependent apoptosis [57]. Pusapati et al. demonstrated an accelerated *Myc* induced tumorigenesis in the oral cavity in *E2F1* deficient mice [58]. Wang et al. established the association of *BRCA1* with *ATM* and DNA repair proteins in the “BRCA1-associated genome surveillance complex (BASC)” [59]. The derogation of the *ATM-ATR-CHEK1* pathway was associated with radioresistance in HNSCC [60, 61]. *FHIT* represents a further tumor suppressor gene that was associated with patients’ prognosis in oral squamous cell carcinoma. *FHIT* negativity correlated with cervical lymph node metastasis and poor disease-specific survival [62, 63]. *FHIT* interacts with the *CHEK1* pathway in an opposing manner. *Fhit* negative cells demonstrate an over-activated *ATM-ATR-CHEK1* pathway that is associated with an increased mutation frequency and therefore disturbed functional integrity [64]. QPCR

revealed an 8-fold higher expression of *FHIT* in young adults. The higher expression of *ATM*, *BRCA1*, *E2F1*, and *FHIT* in patients  $\leq 45$  years can result in an enhanced radiosensitivity and therefore better survival in our cohort.

## CONCLUSION

The derogation of the *WNT-CTNNB1-STK11* and *CDKN2A-HGF-MET* pathway can constitute the carcinogenesis in young patients with SCCT where a longstanding nicotine or alcohol abuse is missing. The higher expression of *ATM*, *BRCA1*, *E2F1* and *FHIT* and subsequent enhanced radiosensitivity can explain the better OS/DSS in patients  $\leq 45$  years.

## MATERIALS AND METHODS

### Patient selection

The study included 66 patients with SCCT  $\leq 45$  years and 210 patients  $>45$  years. SCCT tumor samples were histologically reviewed by at least two experienced pathologists. Dysplasia, carcinoma in situ and other histologic subtypes were excluded. Clinical parameters and survival data were retrospectively collected including age, sex, alcohol and nicotine abuse, TNM-staging, grading, treatment modalities, recurrence and death/loss to follow-up. Patients with lacking data, incomplete staging, and refused/not finished treatment were excluded from survival analysis. The mean follow-up time was 54 months [range: 0–301 months]. Paraffin-embedded tumor (FFPE) samples from 15 SCCT patients  $\leq 45$  years and 15 patients  $>45$  years were randomly selected and analyzed in quantitative PCR (qPCR) and immunohistochemistry (IHC).

### Statistical analysis

Differences between both groups were analyzed using the Chi square test and Fisher exact test for categorical, and the unpaired student's *t*-test for continuous variables. As main endpoints the overall survival (OS), disease-specific survival (DSS) and recurrence-free interval (RFI) were assessed measuring the time from treatment to death of any cause, tumor-related death and loco-regional recurrence, and/or distant metastasis. Survival rates and curves were calculated and illustrated by the Kaplan-Meier method and further analyzed by the log-rank test for univariate analysis. *P*-values  $< 0.05$  were considered statistically significant (Graph Pad Prism, La Jolla, USA).

### Immunohistochemistry

FFPE tumor sections (2.5  $\mu\text{m}$ ) were p16 (Ventana, Tuscon, USA, ready to use), p53 (Do-7, Dako, Hamburg, Germany, 1:200), EGFR (US Biological, Hamburg,

Germany, 1:200), and CXCR4 (R&D, Wiesbaden, Germany, 1:200) stained and visualized with the Bond Polymer Refine Detection Kit (Leica, Nussloch, Germany). Expression levels were classified using a scoring system analyzing the staining intensity (0 = no staining, 1 = low, 2 = moderate, 3 = strong staining intensity) and the relative proportion of stained cells (0, 1 =  $<10\%$ , 2 = 10–39%, 3 = 40–69%, 4 =  $>70$  of the tumor cells). A cumulative score (range 0–7 points) was assessed by adding both scores. A positive staining was defined by a cumulative score equal or greater than 3.

### RNA extraction and quantitative PCR

Tumor areas were identified after hematoxylin staining and corresponding areas micro-dissected from untreated tumor sections (10  $\mu\text{m}$ ). RNA extraction was done according to the manufacturer's protocol, including a DNase digestion (FFPE RNA Micro Kit, Roche, Mannheim, Germany). RNA concentration and purity was determined using a Nanodrop system (Thermo Scientific, Wilmington, USA). 250 ng of total RNA was reverse transcribed and pre-amplified using the RT<sup>2</sup> FFPE PreAMP cDNA Synthesis Kit (Qiagen, Hilden, Germany). Subsequent qPCR was performed using the RT<sup>2</sup> qPCR Master Mix and the RT<sup>2</sup> Profiler<sup>TM</sup> PCR Array for Oncogenes and Tumor Suppressor (*CDK4*, *CDKN2A*, *TP53*, *MDM2*, *AKT1*, *PIK3CA*, *NRAS*, *HRAS*, *KRAS*, *HGF*, *MET*, *EGF*, *ATM*, *BRCA1*, *E2F1*, *FHIT*, *RUNX3*, *STK11*, *BCL2*, and *CTNNB1*) (Qiagen). Gene-specific PCR products were continuously measured during 40 cycles with the BioRad D-CFX96 Cycler (München, Germany). Results were evaluated using the 2- $\Delta\Delta\text{CT}$  method. Data analysis was done using the RT<sup>2</sup> Profiler PCR Array Data Analysis version 3.5 (Qiagen). Target gene expression was normalized between different samples based on the values of *GAPDH* expression. A fold difference  $>2.5$  was considered statistically significant.

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### Conflict of interest

All authors state no conflict of interest.

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