Analysis of mutation-specific Epidermal Growth Factor Receptor (E746-A750del) and human papillomavirus in oral squamous cell carcinoma

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Contents

1. Introduction

1.1. Oral Squamous Cell Carcinoma

1.1.1. Epidemiology

Squamous cell carcinoma of the head and neck (SCCHN) is a cancer of the upper aerodigestive tract (including the nasal cavity, oral cavity, pharynx and larynx), the salivary glands and the paranasal sinuses (H. Mehanna, Paleri, West, & Nutting, 2010).

One of the most frequent types of head and neck cancers is oral squamous cell carcinoma (OSCC). It takes the sixth place among the most common cancers worldwide, and leads mostly to death (Jemal et al., 2008). It covers 90% of all neoplastic epithelial neoplasms of the oral cavity and considered accordingly as the most common malignancy in it (Silverman, 1998) and represents 3% of total cancers in the world (Scully & Felix, 2006). It is noticable that the incidence of oral cancer is frequently between the age of 40 and 60 years; men are affected twice more often than women (Messadi, 2013).

Anually 10,000 new cases of oral and pharyngeal cancer are diagnosed in Germany; 4,000 of these lead to death every year (Hertrampf, Wiltfang, Katalinic, Timm, & Wenz, 2012). The global incidence of cancer of the lip and oral cavity is estimated to be 263,900 new cases and 128,000 deaths caused from oral cavity cancer in 2008 (Jemal et al., 2011). The highest incidence rates for oral cancer are characterized in the regions of South and Southeast Asia (e.g. Bangladesh, India, Pakistan, and Sri Lanka), Australia, central and Eastern Europe, Northern America, as well as parts of Southern Africa.

1.1.2. Malignant transformation of oral mucosa

When the squamous epithelium is affected by several genetic alterations, this will lead to a highly complex multi-step process known as oral carcinogenesis. Figure 1 shows the different stages of oral carcinogenesis.

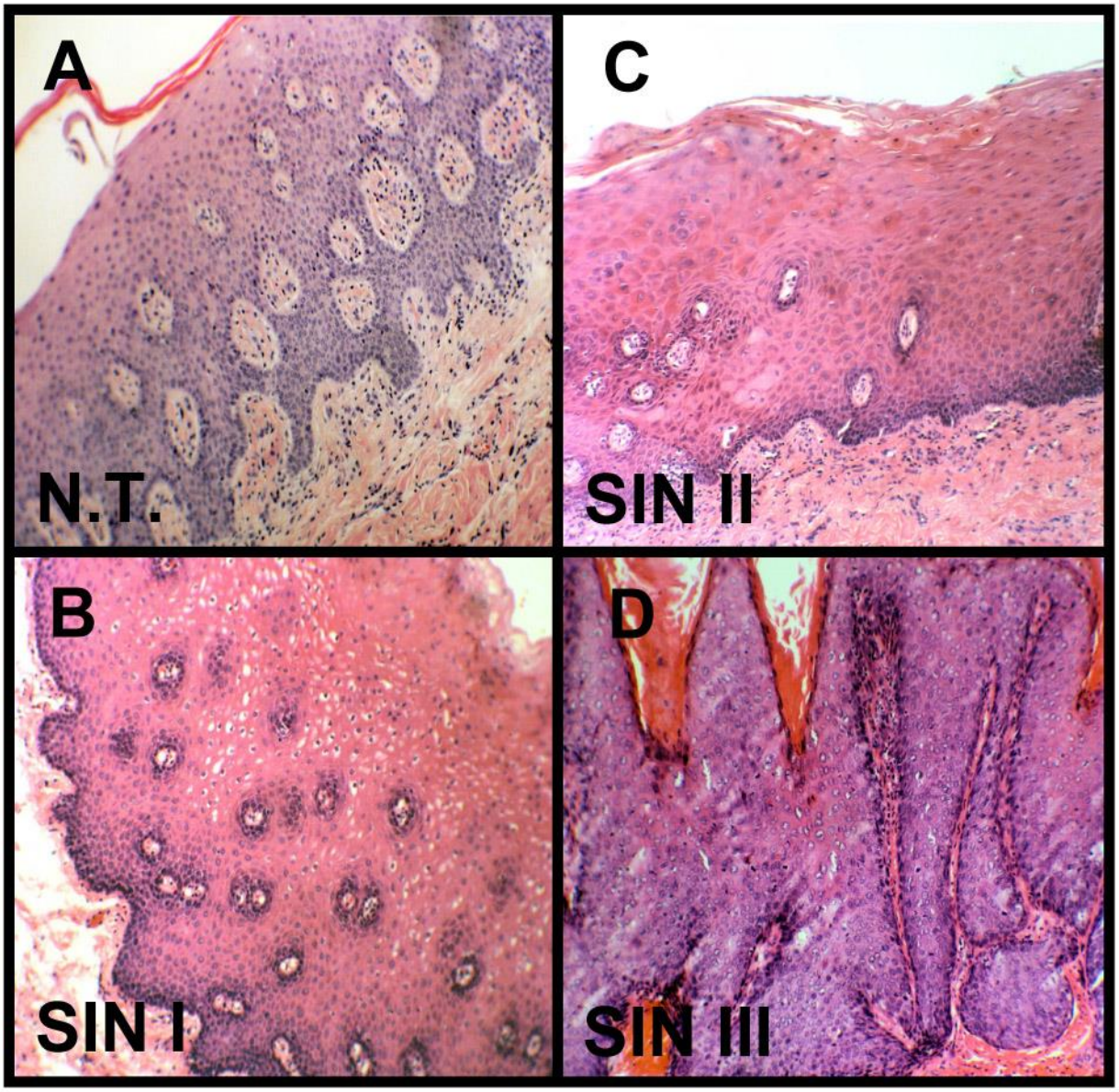

Figure 1: The different stages of oral carcinogenesis

(A) non-malignant tissue, (B) mild dysplasia; slightly atypical cells in the basal layer of epithelium, (C) moderate dysplasia; the atypical cells proliferate into the middle third of the epithelium, (D) severe dysplasia; the atypia arrives at the upper third of the epithelium Abbreviations: N.T. = non-malignant tissue; SIN = squamous intraepithelial neoplasia

The clinical observation of oral precancerous lesions is significant, because these lesions could transform into OSCC (Guillaud, Zhang, Poh, Rosin, & MacAulay, 2008; Ho et al., 2009). Many studies have indicated a rate between 6.6% and 36.4% for the risk of malignant transformation, although a recent study has limited it to 12.1% (Arduino et al., 2009; H. M. Mehanna, Rattay, Smith, & McConkey, 2009). Therefore, an early diagnosis for the precancerous lesions in the oral cavity is recommended using the molecular biology techniques, by which the detection of alterations can be earlier and the identification of high-risk oral cancer patients can be faster (Joseph, 2002; Tanaka, Tanaka, & Tanaka, 2011). The most common precancerous lesions that may develop into OSCC are leukoplakia, erythroplakia, oral submucous fibrosis, palatal lesion of reverse cigar smoking, discoid lupus erythematosus, dyskeratosis congenita and epidermolysis bullosa (Warnakulasuriya, Johnson, & van der Waal, 2007). Leukoplakia and erythroplakia are the most two lesions among the pre-mentioned ones that tend more to malignancy in the oral cavity (Liu et al., 2010; van der Waal, 2009). In addition, oral lichen planus and oral submucous fibrosis are also considered as precancerous with a lower malignant transformation into OSCC as in leukoplakia (van der Waal, 2009). The potential mechanism of oral carcinogenesis is explained by the field cancerization concept, which hypothesizes that the exposure to carcinogenic factors can stimulate genetic defects in the epithelium of the upper aerodigestive tract, leading to development of multiple lesions in this epithelium (Sreedhar, Narayanappa Sumalatha, & Shukla, 2014). The theory of field cancerization proposes that sometimes mutations can be developed in the exons of tumor suppressor genes (Tanaka et al., 2011). An important tumor suppressor is the p53 tumor-suppressor gene. P53 is a well-known gene and is observed in several areas of premalignant leukoplakia and carcinoma in one oral cavity (Boyle et al., 1993).

One of the most important signs of predicting the malignant transformation is the presence of epithelial dysplasia, which can be only histologically diagnosed (Hanken et al., 2013). Nevertheless, epithelial dysplasia can only predict that there is a risk of malignant transformation (Messadi, 2013).

2. The Hallmarks of Cancer

In January 2000, Hanahan and Weinberg published an important paper, in which a list of 6 characteristics for the transformation of normal cells into cancerous had been proposed. (1) Self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) ability to evade programmed cell death (apoptosis), (4) limitless replicative potential, (5) sustained angiogenesis, (6) and tissue invasion and metastasis represent the hallmarks of cancer, which are shared among most if not all cancer cells (Hanahan & Weinberg, 2000). Hanahan and Weinberg have suggested that each of these capabilities must be acquired in the normal human cell in order to enable the malignant transformation. Otherwise, the multiple body anti-cancer mechanisms will inhibit that. Therefore, the risk of developing cancer during the human lifetime is relatively low (Hanahan & Weinberg, 2000). In 2011, Hanahan and Weinberg proposed 4 new hallmarks and they were the ability to evade the immune system, the presence of inflammation, the tendency towards genomic instability, and dysregulated metabolism (Hanahan & Weinberg, 2011). Epidermal growth factor receptor (EGFR) is well-known to be associated with head and neck cancers and anti-EGFR agents are being investigated as a potential adjuvant therapy for oral cancer (Simabuco et al., 2014). An abnormal activation of EGFR gene is associated with self-sufficiency in growth signals, evading apoptosis and other hallmarks of cancer (Tseng & He, 2013).

2.1. Self-sufficiency in growth signals

Normal human cells depend for their division on external growth signals, which are transmitted through the receptors on the cell membrane. The division of normal cells is not possible in the absence of these signals. On the contrary, cancer cells not only show a very low dependence on external growth signals, but also can even produce their signals (Hanahan & Weinberg, 2000). Furthermore, it has become apparent that tumors tend to have cell surface receptors overexpression, in which any binding between the receptor and its

ligand may direct a cell growth (Fedi, 1997). In OSCC, high levels of mRNA for EGFR have been identified. (Grandis & Tweardy, 1993).

2.1.1. Epidermal growth factor receptor (EGFR)

The EGFR family has been reported to be involved in the development of many cancers as well as to be contributed to most hallmarks of cancer (Holbro, Civenni, & Hynes, 2003). Over the past decades, it has been shown that EGFR represents a promising target for cancer therapy medications in advanced head and neck cancers (Aquino et al., 2012). Therefore, a good understanding of this receptor should be obtained to know its precise etiology.

EGFR belongs to a family of tyrosine kinase receptors (RTKs), which includes four members: EGFR (also known as ErbB1 or HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) (Hynes & Lane, 2005). Each of the ErbB receptors is a 170 kDa glycoprotein and has a structure consisting of an extracellular region including a ligand-binding domain, a transmembrane region that attaches the receptor to the cytoplasmic membrane and a cytoplasmic protein tyrosine kinase domain (Reuter, Morgan, & Eckardt, 2007; Tzahar et al., 1996).

When a ligand binds to its corresponding receptor of the ErbB family, a formation of either homodimers (between two identical receptors e.g. EGFR/EGFR) or heterodimers (between two different receptors e.g. ErbB2/ErbB4) is induced. This leads to activation of the intracellular kinase domain through autophosphorylation, followed by initiating lots of intracellular signaling events, such as mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K), AKT, Janus kinase (Jak), mammalian target of rapamycin (mTOR), signal transducer and activator of transcription (STAT) and protein kinase C (PKC) pathways (Kalyankrishna & Grandis, 2006; Rogers, Harrington, Rhys-Evans, P, & Eccles, 2005; Yarden & Sliwkowski, 2001). Afterwards, the downstream signaling regulates many cell functions, such as cell proliferation, survival, metastasis, and angiogenesis (Reuter et al., 2007).

Activation of EGFR (Figure 2) depends on the bind to specific ligands. Currently, the eight known ligands that bind to EGFR are epidermal growth factor (EGF), transforming growth factor α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin (AREG), epiregulin (EPR), betacellulin, epigen and crypto (Fischer, Hart, Gschwind, & Ullrich, 2003). It seems that ErbB2 does not bind to any ligands, while ErbB3 has no tyrosine kinase activity. Therefore, both of them act as heterodimers with the others of the ErbB family, and can be hereby activated (Citri, Skaria, & Yarden, 2003).

EGFR can also be activated indirectly through cellular stresses, Ultraviolet light and γ-irradiation, which lead to phosphorylation (Fischer et al., 2003) .

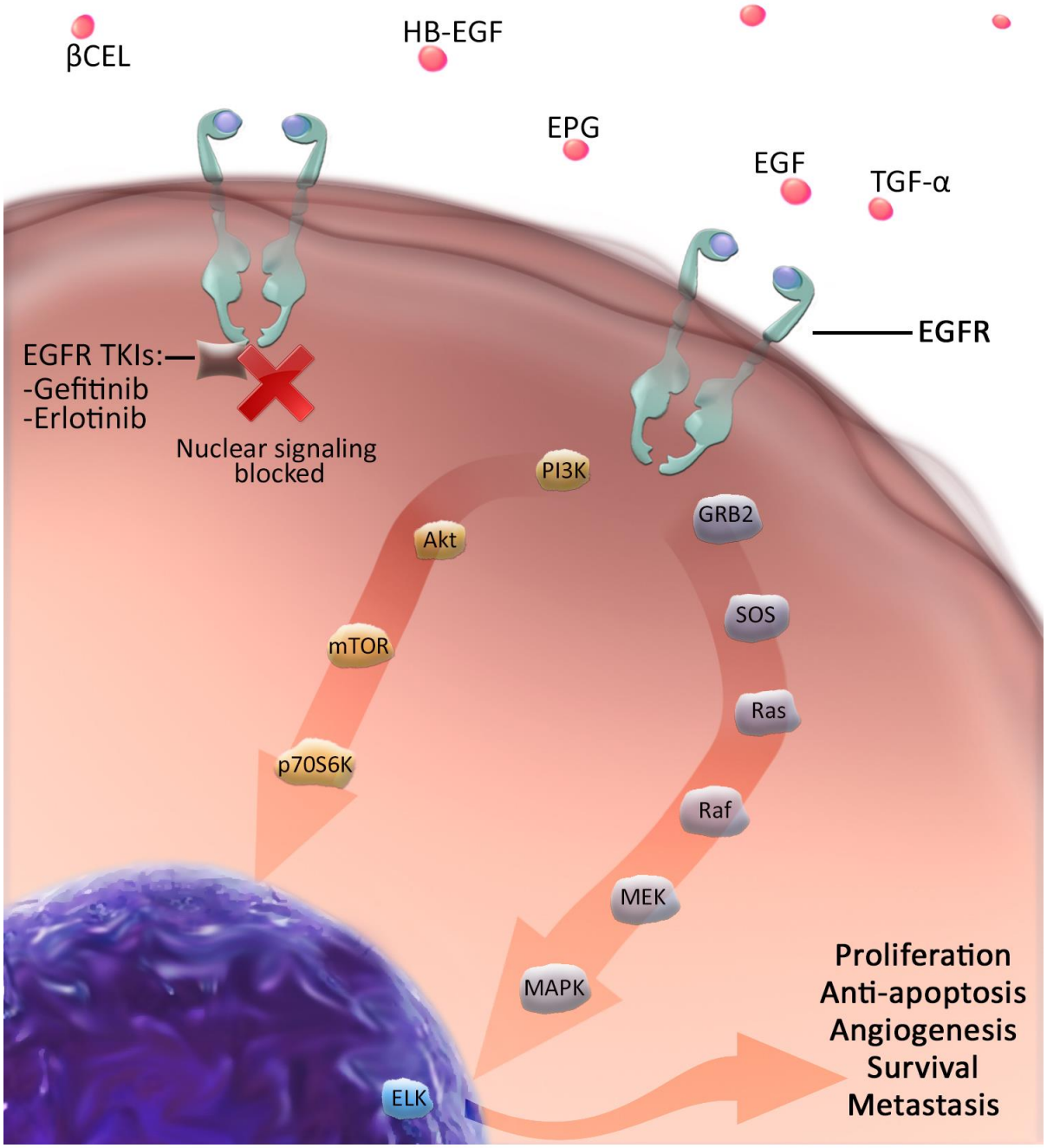

Figure 2: ErbB/HER signaling and the impact of TKIs on it.

The ErbB members signal through MAPK, PI3K, Akt and many other intracellular signaling events regulate cell proliferation, survival, metastasis, and angiogenesis. EGFR is frequently over-expressed, mutated or amplified in many types of cancer, making it an important target for therapies with monoclonal antibodies or special TKI's in those cancers. The mechanism of EGFR-TKI's like gefitinib and erlotinib depends on blocking the tyrosine kinase activity of the cancer cell.
Abbreviations: βCEL = betacellulin; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; EPG = epigen; HB-EGF = heparin-binding EGF; TGF-α = transforming growth factor α

2.1.2. Mutant EGFR (E746-A750del)

Increased activation of EGFR can occur as a result of several mechanisms, such as EGFR overexpression due to gene amplification or transcriptional up-regulation, overproduction of EGFR ligands, and EGFR mutations (Kalyankrishna & Grandis, 2006; Rogers et al., 2005). The overexpression of EGFR has been observed in 80-100% of SCCHN (Kalyankrishna & Grandis, 2006; Reuter et al., 2007). To date, targeting EGFR with cetuximab, which is a chimeric anti-EGFR monoclonal antibody (mAb), has been most successful in gaining favorable clinical outcomes in SCCHN (Cohen, 2013). But unfortunately, the presence of EGFR in OSCC has been correlated with poor treatment outcomes, which has made it controversial whether EGFR is a promising therapeutic target in OSCC (Choi & Myers, 2008; Grobe et al., 2013; Monteiro, Diniz-Freitas, Garcia-Caballero, Forteza, & Fraga, 2010; Oliveira & Ribeiro-Silva, 2011). A study has demonstrated a strong association between EGFR, which has been evaluated by immunohistochemical staining (IHC) with advanced lymph node involvement (Grobe et al., 2013). Nevertheless, EGFR may affect also the survival rate of patients with OSCC according to Laimer et al., since he has considered EGFR overexpression in patients with OSCC and oropharynx as an independent prognostic marker leading to reduced survival. Consequently, EGFR represents an important target for therapies with monoclonal antibodies or special tyrosine-kinase inhibitors (TKI) in these patients (Laimer et al., 2007).

Smoking and alcohol consumption are primary risk factors of OSCC (Wang et al., 2012). However, some research has confirmed a correlation between EGFR and never-smoking in a subset of patients with non-small-cell lung cancer (NSCLC)

(Marchetti et al., 2005; Shigematsu et al., 2005). It seems that there are other genetic factors including DNA mismatch repair, and environmental factors including radiation or second-hand smoke involved in EGFR mutations (Riely, Politi, Miller, & Pao, 2006). Never-smokers with EGFR mutations have shown a positive response to EGFR-TKI's like gefitinib and erlotinib, which inhibit

tyrosine kinase activity by reducing tumor cell proliferation and inducing apoptosis (Lynch et al., 2004; Paez et al., 2004; Pao, Miller, Zakowski, et al., 2004; Riely et al., 2006). EGFR mutations have been reported to occur in exons 18 to 21, which encode the tyrosine kinase domain. Nearly 90 % of all EGFR mutations are deletions of four amino acids (LREA) at positions 747–750 localized in exon 19, and a point mutation (L858R) arising as a result of replacing the amino acid leucine with arginine at position 858 within exon 21. These mutations make the activation of EGFR independent of binding with its ligands, and in case of ligand stimulation, a prolonged receptor kinase activity will be initiated (Amann et al., 2005; Lynch et al., 2004). Most patients with these mutations, especially delE746-A750 and the L858R point mutation, have shown increased sensitivity to EGFR-TKI's such as gefitinib and erlotinib and have survived for a longer time than those patients without EGFR mutations (Figure 3) (Kawahara et al., 2010; Mitsudomi et al., 2005; Ono & Kuwano, 2006; Takano et al., 2005; Yu et al., 2009). In addition, the combination between erlotinib or gefitinib with cytotoxic chemotherapy has improved the overall survival among patients, who had a medical history of NSCLC, EGFR mutations and never-smoking. (Riely et al., 2006).

Recently, direct polymerase chain reaction (PCR) based sequencing of EGFR tyrosine kinase domains have described EGFR mutations in OSCC (Huang et al., 2009; Tushar & Ramanathan, 2013).

IHC staining (is widely used for the detection of biomarkers in tumor cells, and it has a significant role in identification of carcinogenesis and deciding the cancer patient treatment (Cummings, Raynaud, Jones, Sugar, & Dive, 2010). A study has used IHC for the detection of E746-A750del mutation depending on a specific antibody. This method has shown its efficiency in EGFR mutations diagnose when used in combination with DNA sequence (Kawahara et al., 2010).

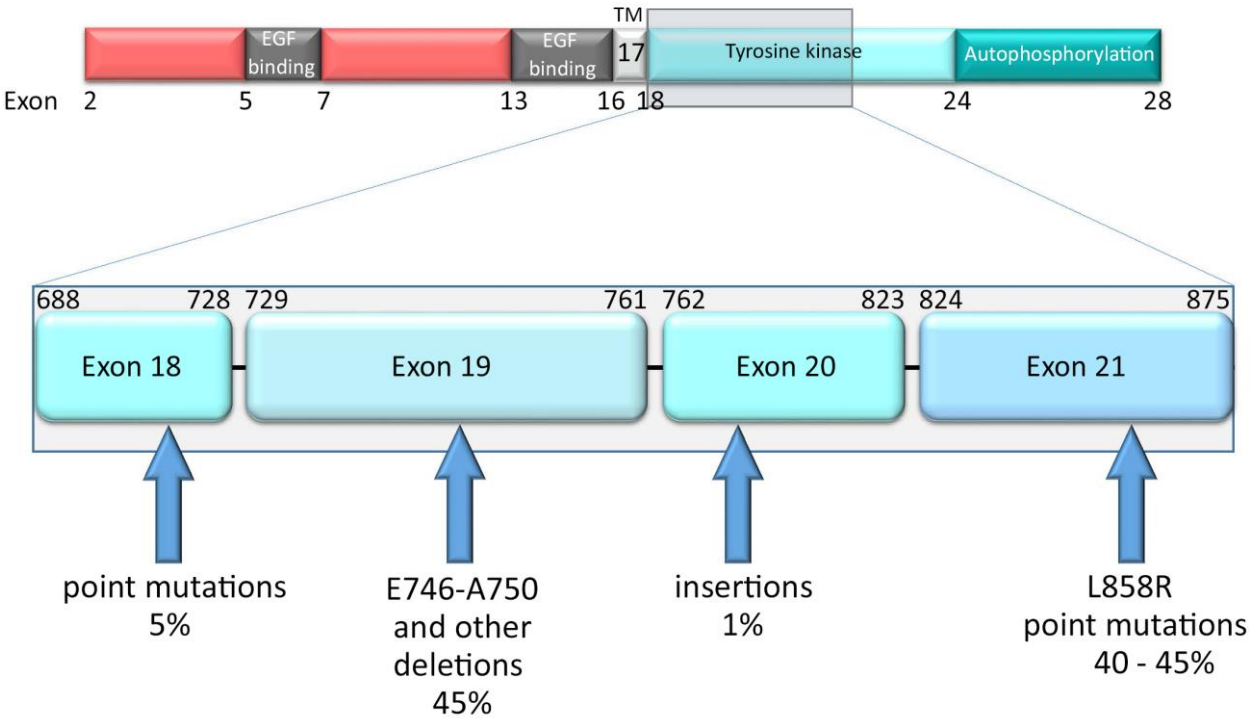

Figure 3: Frequencies of mutations in exons 18-21 in the EGFR gene in the EGFR inhibitor-responsive tumors. Reproduced from (Sharma, Bell, Settleman, & Haber, 2007)

Exons 18–21 in the tyrosine kinase domain of the EGFR gene are enlarged. Approximately 90 % of those mutations are deletion of the codons 746 - 750 localized in exon 19, and (point mutation L858R, substitution Leucin to Arginin in codon 858) localized in exon 21. Abbreviations: TM = Transmembrane region

2.1. Evading programmed cell death (Apoptosis)

When cells become senescent, they will undergo a programmed death (Williams, 1991), which is resisted by most if not all cancer types (Hanahan & Weinberg, 2000). The two responsible components for apoptosis are classified into: sensors, which decide through normal and abnormal situations between survival or death of the cell; and effectors, which lead to apoptosis. Moreover, p53 represents an apoptotic factor (Symonds et al., 1994). However, the cancer cells have the capacity to overcome apoptosis through mutations in p53 (Harris, 1996) and other ways (Hanahan & Weinberg, 2000). Several studies have demonstrated that p53 is mutated in 25-69% of oral cancers (Choi & Myers, 2008). Human papillomavirus (HPV) such as HPV16 and HPV18 subtypes

cause the degradation of p53 (Nagpal, Patnaik, & Das, 2002). Therefore, cancer cells infected with HPV may evade apoptosis.

2.1.1. Human papillomavirus (HPV)

HPVs are members of the papillomaviridae family, which consists of small and non-enveloped DNA viruses. The genome is composed of three regions: the early region, the late region and the non-coding region. The early region covers about 50% of the genome and encodes the early regulatory proteins (E1, E2, E4, E5, E6 and E7 and also E8 in HPV-31) (McLaughlin-Drubin, Meyers, & Munger, 2012; Zheng & Baker, 2006).

It has become evident that HPV16 is the most common HPV type detected in oral cancers, followed by HPV18 (Isayeva, Li, Maswahu, & Brandwein-Gensler, 2012). HPV16/18 has been reported to be involved in lung carcinogenesis in non-smoking patients with NSCLC (Cheng et al., 2001). Another study has also reported an association between HPV infection and non-smokers with OSCC (Laco et al., 2011). Although HPV has shown an etiological relation to a subset of patients with SCCHN, it is still controversial to date, whether HPV infection plays a role in the carcinogenesis of OSCC (Isayeva et al., 2012; Rampias, Sasaki, Weinberger, & Psyrri, 2009). 60 publications studied between the years 2000-2011 were reviewed by Isayeva et al. revealed a weighted prevalence of 20.2% HPV DNA in 4,195 oral cavity cancer patients (range: 0%-94.7%) (Table 1) (Isayeva et al., 2012).

Table 1: HPV DNA detection frequencies in oral cavity carcinomas (Isayeva et al., 2012)

Author	Year	Country	Method, primers, amplicon detection	Number of HPV positive cancers	Total cancers studied	HPV positive cancers (%)
Badaracco	2007	Italy	PCR, MY09/MY11, GP5/GP6	8	60	13.3
Baez	2004	Puerto Rico	PCR, HPV16 E6/E7 ORF	13	36	36.1
Bagan	2007	Spain	PCR, MY09/MY11	0	6	0.0
Balderas-Laenza	2007	Mexico	PCR, MY09/MY11, GP5/GP6	26	62	41.9
Barwad	2011	India	PCR, MY09/MY11, not nested, agarose gel	16	34	47.1
Boscolo-Rizzo	2009	Italy	PCR, HPV16 specific primers	2	10	20.0
Bouda	2000	Greece	PCR	18	19	94.7
Boy	2006	South Africa	PCR, HPV16/18 specific primers	7	59	11.9
Braakhuis	2004	Netherlands	PCR, GP5/GP6, typing	6	106	5.7
Correnti	2004	Venezuela	PCR, MYO9/MY11, not nested, agarose gel, Digene Sharp Signal Assay typing	8	16	50.0
Dahlgren	2004	Scandinavia	PCR, GP5/GP6, CPI/CPIIG, agarose gel	2	85	2.4
Deng	2011	Japan	PCR, MYO9/MY11, GP5/GP6, E1 consensus primers	9	25	36.0
Dong	2003	USA	PCR, HPV16/18 specific primers	3	16	18.8
Elango	2011	India	PCR, MY09/MY11, GP5/GP6, HPV16 specific primers	30	60	50.0
El-Mofty	2003	USA	PCR, SPF10, INNO-LiPA line probe	0	15	0.0
Feher	2009	Hungary	PCR, MY09/MY11, GP5/GP6	31	65	47.7
Fischer	2003	Germany	PCR, L1 consensus primers	0	2	0.0
Fujita	2008	Japan	PCR, SPF10, sequencing	11	23	47.8
Furniss	2007	USA	PCR, SPF1A, SPF2B, HPV16 E6 specific primers	38	150	25.3
Gillison	2000	USA	PCR, MY09/MY11, HPV16E7 HPV18E7 Dot blot	10	84	11.9
Gonzalez	2007	Argentina	PCR, MY09/MY11, GP5/GP6,	15	25	60.0
Gudleviciene	2009	Lithuania	PCR, HPV16/18 specific primers, agarose gel	1	13	7.7
Ha	2002	USA	PCR, HPV16 E6/E7 primers, real time quantitative PCR	1	34	2.9
Halimi	2011	Iran	PCR, MY09/MY11 then typed, agarose gel	6	30	20.0
Hansson	2005	Scandinavia	PCR, MY09/MY11, GP5/GP6, agarose gel, sequenced	15	85	17.6
Harris	2011	USA	PCR, MY09/MY11, GP5/GP6, type specific primers	2	25	8.0
Herrero	2003	Multiple countries	PCR, GP5/GP6, enzyme immune assay typing	30	766	3.9
Ibieta	2005	Mexico	PCR, MY09/M11, GP5/GP6, typed	21	50	42.0
Jalouli	2010	India	PCR, MY09/M11, not nested, agarose gel, typed with HPV16/18 specific primers, and sequenced	15	62	24.2
Kaminagakura	2011	Brazil	PCR, GP5/GP6, agarose gel	22	114	19.3
Kansky	2006	Slovenia	PCR, MY09/M11, GP5/GP6, WD72, WD76, agarose gel, typing by restriction fragment length polymorphism	4	44	9.1
Klozar	2008	Czech	PCR, GP5/GP6, not nested, chemoluminescence detection of hybridized amplicon, sequencing	2	10	20.0
Klussmann	2001	Germany	PCR, consensus primers, HPV16 specific primers, real time PCR	4	22	18.2

Koppikar	2005	India	PCR, L1 primers and GP5/GP6	28	83	33.7
Koskinen	2003	Scandinavia	PCR, MY09/MY11,GP5/GP6, SPF10, INNO-LiPA typing, FAP 59/64, CP65/70, CP66/69, type specific real time PCR	7	13	53.8
Kristoffersen	2012	Scandinavia	PCR, MY09/MY1, GP5/GP6	8	50	16.0
Laco	2011	Czech Republic	PCR, GP5/GP6	3	24	12.5
Lopes	2011	England	PCR, GP5/6 Q-PCR HPV16/18	2	142	1.4
Luo	2007	Tapei	PCR, MY09/M11, GP5/GP6, typed by HPV gene chip	13	51	25.5
Montaldo	2010	Italy	PCR, MY09/M11, agarose gel, sequenced	21	68	30.9
Mork	2001	Scandinavia	PCR, GP5/GP6 CpI, CpII E1, E6 specific primers for HPV6/11/16/18/33	4	91	4.4
Neme	2006	Hungary	PCR, MY09/MY11, type specific, E2 for integration	33	79	41.8
Pannone	2012	Italy	PCR, MY09/M11, GP5/GP6, 8% polyacrylamide gel	3	6	50.0
Popovic	2010	Serbia	PCR, consensus primers typing	6	60	10.0
Ribeiro	2011	Multiple countries	PCR, MY09/MY11, no nesting, HPV16E7 specific primers, agarose gel, typing by restriction fragment length polymorphism	0	483	0.0
Ringstrom	2002	USA	PCR MY09/MY11, agarose gel, typing by restriction fragment length polymorphism	2	41	4.9
Ritchie	2003	USA	PCR, MY09/MY11 agarose gel, dot blot, then heminested PCR MY09. GP5	10	94	10.6
Saghravanian	2011	Iran	PCR, GP5/GP6	3	21	14.3
Sand	2000	Scandinavia	PCR, MY09/MY11, agarose gel	3	24	12.5
Schlecht	2011	USA	PCR, MY09/11, dot blot	5	38	13.2
Seraj	2011	Iran	PCR, HPV 16/18 specific primers, agarose gel	25	94	26.6
Sethi	2011	USA	PCR, SPF10, INNO-LiPA typing	33	120	27.5
Slebos	2006	USA	PCR, MY09/MY11, sequenced	0	15	0.0
Smeets	2007	Netherlands	PCR, GP5/GP6 real time quantitative PCR	9	30	30.0
Smith	2008	USA	PCR,MY09/MY11, GP5/GP6, then typed	27	166	16.3
Soderberg	2008	USA	PCR, MY09/MY11, GP5/GP6, then sequenced	1	18	5.6
Sugiyama	2007	Japan	PCR, HPV16 E7 specific primers, agarose gel	24	66	36.4
Tachezy	2005	Czech Republic	PCR, GP5/GP6, then sequenced	3	12	25.0
van Monsjou	2012	Netherlands	PCR, INNO-LiPA typing	2	20	10.0
Zhang	2004	China	PCR, HPV 16/18 E6 specific primers, agarose gel	54	73	74.0
Total				705	4,195	

Expression of viral E6 and E7 oncoproteins is a well-known genetic alteration identified in the malignant behavior, because E6 degrades the p53 protein, and E7 inhibits the retinoblastoma tumor suppressor protein (pRb) of the host (Rampias et al., 2009). HPV16 E7 also inhibits p53 transcriptional activities and leads to the inactivation of the p53 dependent G1 cell cycle inhibitor (p21CIP1) (McLaughlin-Drubin et al., 2012). Another mechanism that is followed by HPV infected cell to drive proliferation is the fact that E7 inhibits the bind between retinoblastoma protein and E2F. As a result, E2F are released and can activate the S-phase genes (Doorbar, 2006).

E6 and E7 oncogene repression has been reported by Rampias et al. to restore p53 and retinoblastoma tumor suppressor pathways and induce apoptosis in HPV16+ oropharyngeal cancer cell lines (Rampias et al., 2009). Therefore, patients with HPV+ tumors tend to have a better prognosis and substantially better survival rates after the radiation therapy or chemotherapy (Ang et al., 2010; Posner et al., 2011; Rischin et al., 2010). Among patients with HPV+ tumors, the 5-year survival rates should be of approximately 75 to 80%, versus 45 to 50% among patients with HPV- tumors (Ang et al., 2010). A study has confirmed that HPV+ SCCHN cancer cells are sensitive to radiation as compared to HPV-, which has an activating mutation in EGFR resulting in phosphorylation of Akt, which can be down-regulated by the HIV protease inhibitor Nelfinavir (NFV), resulting in sensitization to radiation (Gupta et al., 2009). Moreover, HPV+ cancers have fewer mutations than tobacco-induced cancers, which make the treatment of HPV+ less aggressive (Psyrri & Cohen, 2011).

Multiple studies mentioned an inverse relationship between HPV and EGFR expression, since the low EGFR expression in HPV-related SCCHN might be associated with the favorable outcome of patients (Kumar et al., 2008). However, it remains unclear, whether HPV and EGFR mutations have a clinical impact in OSCC.

3. Aims

The overall aim of this study was the investigation of the clinical impact of EGFR (E746-A750del) mutation and HPV on the survival of patients with OSCC.

Specific aims were:

1- To investigate the involvement of the EGFR (E746-A750del) mutation and HPV in OSCC.

2- To determine, whether HPV infection is related to EGFR mutation or non-smokers in OSCC.

3- To evaluate, whether the mutation-specific EGFR (E746-A750del) expression and HPV is relevant for the survival of patients with OSCC.

4. Materials and Methods

4.1. Patients and Tumor Specimen

The records of 211 OSCC patients were reviewed retrospectively (Freudlsperger, Alexander, Reinert, & Hoffmann, 2010) after primary radical R0 tumor resection in the Department of Oral and Maxillofacial Surgery, University Hospital Tuebingen over a period of ten years. We retrospectively reviewed the records of ten healthy individuals (normal oral mucosa tissues, n = 10), as well. The local ethics committee of the University Hospital Tuebingen approved this study (approval number: 001/2012BO2). An informed consent was obtained from each patient prior to surgical resection. The clinicopathological and follow-up data were available for 191 out of 211 patients (n = 191/211). From 161 out of 191 patients, FFPE blocks for a representative immunohistochemical staining were available. Patients who presented insufficient follow-up data, nonresectable disease, and patients who were treated with preoperative antineoplastic therapies (chemoradiation / chemotherapy) were excluded from this study. The diagnosis of squamous cell carcinoma was confirmed by the Department of Pathology, University Hospital Tuebingen, from which the specimens were retrieved retrospectively.

The used material was archival formalin-fixed and paraffin-embedded tissue (FFPE; formalin-fixed, paraffin-embedded) from routine histopathological work-up. Experienced pathologists have selected the tumor blocks of paraffin-embedded tissue using the routine Haematoxylin-Eosin (H.E.) stained sections. Regardless of the prior histopathology report, sections from all available tumors underwent intensive histopathological evaluation. Serial tissue sections were cut at 2 µm from formalin-fixed paraffin-embedded (FFPE) blocks using a microtome and fixed on microscope slides. Surgical margin status was determined on final histopathological assessment. Negative margins were considered to be greater than or equal to 10 mm from resection margin after tissue fixation, whereas close margins were deemed to be positive in all analysis.

Tumor grading and staging was done according to WHO criteria, and the 7th edition of the TNM staging system by the UICC/ AJCC of 2010, respectively (Hamilton, 2000; Sobin LH & Ch., 2010). Tumor characteristics (UICC stage, pT-categories, pN-categories, cM-categories, infiltrated lymph nodes, residual tumor status, tumor size, site distribution) and patient characteristics (gender, age, personal history, habitual history) were collected in a database using (Excel, Microsoft). Tumor and patient characteristics are summarized in Table 2.

Follow-up data was obtained from the local tumor registry and the last follow-up was recorded from the last outpatient visit or the date of locoregional recurrence or tumor-specific death, respectively.

Table 2: Clinicopathological characteristics and prognostic factors of 161 patients with OSCC measured by negative and positive EGFR E746-A750del specific mutation expression.

Characteristics	Number of Patients			p-value
	Total n=161	EGFR E746-A750del negative (<10%) n=121 (75%)	EGFR E746-A750del positive (>10%) n=40 (25%)	
Age (y)				0.0916
<60±11.8	80 (49.7%)	55 (69%)	25 (31%)	
≥60±11.8	81 (50.3%)	66 (81%)	15 (19%)	
Gender				0.5273
Male	125 (77.6%)	92 (74%)	33 (26%)	
Female	36 (22.4%)	29 (80%)	7 (20%)	
Site distribution of OSCC				0.2026
Lips	10 (6.2%)	10 (100%)	0 (0%)	
Tongue	36 (22.4%)	28 (78%)	8 (22%)	
Floor of the mouth	66 (41.0%)	48 (73%)	18 (27%)	
Palate	15 (9.3%)	10 (67%)	5 (33%)	
Buccal mucosa	9 (5.6%)	8 (89%)	1 (11%)	
Alveolar ridge	25 (15.5%)	17 (68%)	8 (32%)	
Histological Grading				0.9888*
G1	39 (24.2%)	30 (77%)	9 (23%)	
G2	108 (67.1%)	80 (74%)	28 (26%)	
G3	13 (8.1%)	10 (77%)	3 (23%)	
G4	1 (0.6%)	1 (100%)	0 (0%)	
Tumor size				0.5263**
pT1	64 (39.8%)	48 (75%)	16 (25%)	
pT2	40 (24.8%)	28 (70%)	12 (30%)	
pT3	17 (10.6%)	14 (82%)	3 (18%)	
pT4	40 (24.8%)	31 (78%)	9 (22%)	
Cervical lymph node metastasis				0.7363

pN0	118 (73.3%)	90 (76%)	28 (24%)	
pN1-3	43 (26.7%)	31 (72%)	12 (28%)	
UICC stage				0.8927***
UICC I	48 (29.8%)	39 (81%)	9 (19%)	
UICC II	36 (22.4%)	24 (67%)	12 (33%)	
UICC III	31 (19.3%)	25 (81%)	6 (19%)	
UICC IV	46 (28.6%)	33 (72%)	13 (28%)	
Smoking history				0.8598
Never-smoker	44 (27.3%)	34 (77%)	10 (23%)	
Smoker	117 (72.7%)	87 (74%)	30 (26%)	
Alcohol consumption				0.2755
Never	55 (34.2%)	38 (69%)	17 (31%)	
Ever	106 (65.8%)	83 (78%)	23 (22%)	
Locoregional recurrence				0.9047
No	117 (72.7%)	87 (74%)	30 (26%)	
Yes	44 (27.3%)	33 (75%)	11 (25%)	

Abbreviations: y = years; G = grading; UICC = International Union against Cancer; *G1/2 vs. G3/4; **pT1/2 vs. pT3/4; ***UICC I/II vs. UICC III/IV

4.2. EGFR staining procedure and quantification of immunohistochemistry

Immunohistochemistry was performed in 161 patients with OSCC. Unconjugated mutation-specific (Yu et al., 2009) EGFR (E746-A750del) (6B6) (Cell Signaling, Frankfurt am Main, Germany, rabbit anti-human mAb, dilution: 1:250), and isotype control antibodies (BD Pharmingen, Heidelberg, Germany) were used for immunohistochemical analysis. The staining with unconjugated EGFR antibody (Dako, Glostrup, Denmark, mouse anti-human mAb, dilution: 1:50) confirmed the presence of EGFR in tumor specimen. The tumor tissue FFPE blocks were used to cut 2 µm thick sections, which were mounted from warm water onto adhesive glass slides and dried for 24 h at 37°C. Then, the sections were put in xylene (2x10 minutes) to remove paraffin (deparaffinization), and then rehydrated through a graded ethanol series (3 minutes 100% ethanol, 3 minutes 95% ethanol, 3 minutes 90% ethanol, 3 minutes 80% ethanol, 3 minutes 70% ethanol) to distilled water. Subsequently, the sections were immersed into the pre-heated target retrieval solution using EDTA buffer pH 9.0 or citrate buffer pH 6.1 (Dako, Glostrup, Denmark) and boiled in a steamer. Endogenous peroxidase activity was blocked in a solution containing 3% H2O2, and endogenous biotin was blocked by Streptavidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA) according to the

manufactures instructions. After incubation of the sections overnight with primary antibody to EGFR (E746-A750del specific) (6B6) or control antibody in a humidified chamber, sections were washed with TBS, and incubated for 30 min with the biotinylated secondary antibody (LSAB2 system multi-link swine anti-goat/mouse/rabbit immunoglobulin; Dako, Glostrup, Denmark) and horseradish peroxidase (HRP)-conjugated streptavidin (label). After washing and incubating for 5 minutes, the sections were visualized using 3,3′-diaminobenzidine (DAB), which is oxidized by HRP in the presence of 0.3% H2O2. At the end, sections were counterstained in hematoxylin and covered with a glycergel (Dako, Glostrup, Denmark).

For each case, five representative chosen high power fields (1 HPF = 0.237 mm2) were studied and averaged to confirm the histological diagnosis of EGFR expression in the tumor tissue, The extent of the staining, defined as the relative area of positive staining within the tumor cells relative to the whole tissue area, was semiquantitatively scored on a scale of 0 to 3 as follows: 0, <10%; 1, 10–30%; 2, 30–60%; 3, >60%. The intensity of staining was scored using the following scale: no staining, 0; weak staining, 1+; moderate staining, 2+; and strong staining, 3+. The values of these two categories were multiplied and the combined score (0–9) for each specimen was approved. Cases were classified as: EGFR negative, 0 points; EGFR positive, 1–9 points. The immunostained sections were evaluated by two observers, who independently performed scoring while blinded to the diagnosis.

In addition, ImageJ software (http:/rsbweb.nih.gov/ij/) coupled with immunomembrane plug-in (http://imtmicroscope.uta.fi/immunomembrane) was used for computer-assisted semi-quantitative analysis of EGFR expression, in order to assess the quantification of EGFR immunoreactivity in microscopically acquired JPEG images of OSCC samples. Staining completeness (0–10 points) and intensity (0–10 points) were added for a combined score (0–20 points) (Tuominen, Tolonen, & Isola, 2012). Cases were classified as the following: EGFR negative, 0 points; EGFR positive, 1–20 points. 5 images were collected per sample from EGFR positive slides to show representative tumor areas using 10x and 20x objectives to assess precision (reproducibility/ repeatability) of

computer-assisted (semi-) quantitative analysis. Pictures were analyzed using a Canon camera (Krefeld, Germany). The photographed images were imported into the Microsoft Office Picture Manager.

4.3. HPV genotyping

In this study 211 patients with OSCC were screened based on SPF-10-PCR and Reverse Line Probe Assay LiPA Extra (SPF-10-PCR) for the presence of HPV genotypes. DNA isolation from FFPE samples was performed using a Qiasymphony device and the FFPE protocol (Qiagen, Hilden, Germany) and then analyzed with the INNO-LiPA Extra HPV prototype assay (Innogenetics, Inc, Gent, Belgium) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed in a separate room in the laboratory by following good laboratory practice.

Each PCR reaction contained 10 µl input DNA in a total volume of 50 µl using reagents provided by Innogenetics. The reaction mixture was first heated at 37°C for 10 minutes, and then at 94°C for 9 minutes, followed by 40 cycles each at 94°C for 30 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 45 seconds run on a MJ Thermocycler PCT 200. The PCR product was then denatured, and a 10-µl aliquot was hybridized at 49°C for 60 minutes onto one strip, followed by multiple washing steps.

The reading of the hybridized strips was performed using a flatbed scanner with the use of LiRAS prototype software (Innogenetics, Inc), which displays the patterns and relative intensity of positive bands as arbitrary grey-tone values between 0.1 and 1.0.

The INNO-LiPA Extra test allowed identification of established high-risk-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) (Gillison et al., 2012; Schiffman, Clifford, & Buonaguro, 2009), and five known or putative high-risk types (26, 53, 66, 73 and 82) (Cogliano et al., 2005; Munoz et al., 2003).

4.4. Statistical analysis

Statistical analysis was performed using MedCalc Software (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013). The disease-free survival (DFS) time was defined as the time from tumor resection until appearance of obvious locoregional recurrence or tumor conditional death, respectively. The Kaplan-Meier method was used to estimate the disease free survival times (Kaplan & Meier, 1958). For determination of the most significant variables contributing to survival status in univariate analysis, Hazard ratios (HR) were provided with 95% Confidence Interval (CI). The relation between two categorical variables was investigated by using Chi-Square test ($\chi 2$) and Fisher's exact test. The estimation of the accuracy (the degree of closeness of measurements of a quantity to that quantity's true value) between the two quantification methods of immunohistochemical analysis was performed by measuring non-parametric Kendall´s tau (τ) correlation coefficient. All P values presented were 2-sided. P values less than 0.05 were considered statistically significant.

5. Results

5.1. Comparison of observer semi-quantitative scoring with computer-assisted semi-quantitative analysis of EGFR (E746-A750del) expression

A preliminary study was carried out to assess the accuracy between the two quantification methods of immunohistochemical analysis. There were significant correlations between the first (observer related semi-quantitative scoring) and the second (computer-assisted (semi)-quantitative analysis) assessment. EGFR (E746-A750del) expression: $\tau = 0.983$, $p < 0.0001$, 95% CI 0.974 to 0.990.

5.2. Mutant EGFR (E746-A750del) expression is not associated with clinicopathological characteristics of OSCC

EGFR (E746-A750del) expression was not detected in human normal oral squamous epithelium (n = 0/10 normal oral mucosa samples). On the contrary to stromal cells, EGFR (E746-A750del) expression was only located in the cytoplasm and the membrane of cancer cells (Figure 4). According to the analysis of EGFR (E746-A750del) expression, 25% of the patients (n = 40/161) had positive EGFR (E746-A750del) expression. Table 2 shows the clinicopathological characteristics and prognostic factors of 161 patients with OSCC. There was no association between positive EGFR (E746-A750del) expression (Figure 4) and any clinicopathological characteristics, prognostic factors, or social habits like smoking or alcohol consumption (Table 2).

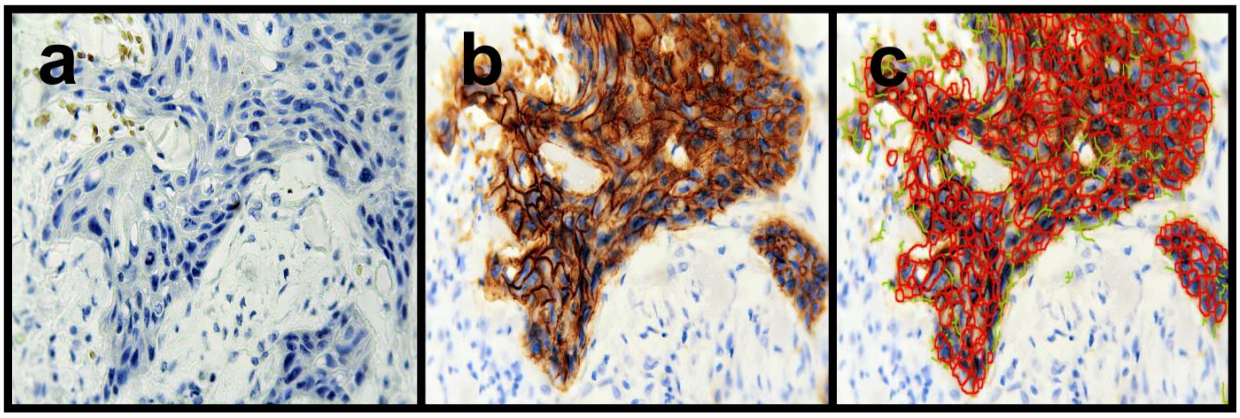

Figure 4: Immunohistochemical single staining of mutant EGFR (E746–A750del) in OSCC.

Representative images of IgG-control (a, no staining) and mutant positive EGFR expression (b and c) in OSCC (membranous and cytoplasmic staining pattern, brown). (b) The brown chromogen colour (3,3′- diaminobenzidine) indicates positive mutant EGFR staining, the blue colour shows the nuclear counterstaining by haematoxylin. (c) The pseudocoloured image shows the staining components of computer-assisted semi-quantitative analysis in mutant EGFR-positive OSCC cells. The computer-assisted red label indicates strong or complete staining, the green label indicates weak or incomplete staining. Original magnification: 200x.

5.3. Prognostic value of mutant EGFR (E746-A750del) in OSCC

Patients were divided into two subgroups as follows: positive mutant (E746-A750del) expressors and negative mutant EGFR (E746-A750del) expressors (dichotomous variables), in order to analyze the survival rates after successful curative surgical (R0) resection of OSCC.

In this study population, cervical lymph node metastasis (pN1-3, HR = 2.1145, 95% CI = 1.0272 to 4.3528, p = 0.0139) was shown to be an unfavorable predictor for the survival by univariate analysis of all (n = 161) OSCCs. The univariate analysis showed that tumor size (pT3/4, HR = 1.3865, 95% CI = 0.6887 to 2.7914, p = 0.3080), grading (G3/4, HR = 0.9199, 95% CI = 0.2969 to 2.8506, p = 0.8885), and advanced tumor stages (UICC III/IV, HR = 1.6734, 95% CI = 0.9145 to 3.0623, p = 0.08) were not found to be unfavorable prognostic factors (Grimm et al., 2013). Social habits like alcohol consumption (alcohol consumption, HR = 2.1337, 95% CI = 1.1022 to 4.1304, p = 0.0593) or smoking (smoking history, HR = 1.6794, 95% CI = 0.8364 to 3.3721, p = 0.2015) showed no significant impact on tumor specific survival.

Another two subgroups of patients were divided into positive and negative mutant EGFR (E746-A750del) expressors, to analyze the impact of EGFR (E746-A750del) in OSCC patients on the tumor related survival. The subgroup with positive EGFR (E746-A750del) mutation was not associated significantly with a better survival by contrast with the subgroup of patients without EGFR (E746-A750del) mutation (E746-A750del+: n = 40/161, p = 0.3397, HR = 0.7008, 95% CI = 0.3574 to 1.3744, Figure 5a).

As the cervical lymph node metastasis in this study population was found to be the only significant unfavorable factor in univariate analysis, multivariate analysis using the Cox Proportional Hazards Model was not performed.

5.4. Prevalence and prognostic value of HPV status

3 out of 211 OSCC samples were only HPV16+ (HPV16+: n = 3/211, 1.42%), using INNO-LiPA. The floor of the mouth was involved in each of the 3 cases as the affected anatomical site. All of the patients with HPV16 DNA positive had a positive history of smoking and alcohol consumption, but none of them were positively detected for EGFR (E746-A750del) mutation. The positive HPV status had no impact on tumor specific survival (HPV+: n = 3/191, p = 0.9188, HR = 1.1078, 95% CI = 0.1385 to 8.8626, Figure 5b).

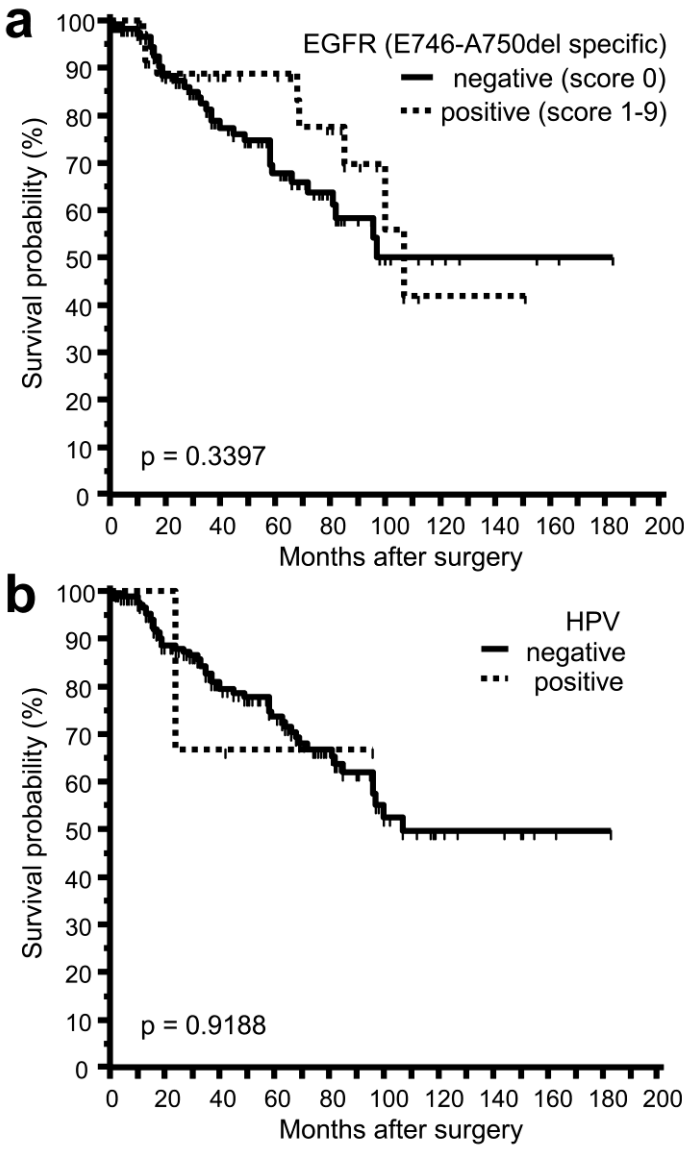

Figure 5: Survival curves for OSCC patients measured by mutant EGFR (E746–A750del) expression (n = 161/211) and HPV status (n = 191/211)

Kaplan–Meier survival curves for disease-free survival (DFS) are stratified by positive mutant EGFR expression (EGFR+, dashed line, a), negative mutant EGFR expression (EGFR-, solid line, a), positive HPV status (HPV+, dashed line, b), and negative HPV status (HPV-, solid line, b). In the univariate Kaplan–Meier analysis, mutant EGFR expression (a) and HPV status (b) were not significantly associated with survival. The times at which the data were censored are indicated with short vertical lines.

6. Discussion

The aim of this study was to analyze the role of EGFR (E746-A750del) mutation in the carcinogenesis of OSCC, since EGFR mutations have been reported to be involved in the development of many cancers such as lung cancers. Moreover, several studies have confirmed EGFR overexpression as the independent prognostic marker, which may probably decrease radiation sensitivity, increase the risk of recurrence and increase the tumor size (Ang et al., 2002; Chen et al., 2003; Gupta et al., 2002; Rubin Grandis et al., 1998; Shiraki et al., 2005).

The incidence of OSCC has been decreased in the recent years because of the early detection and diagnosis of this cancer (Aquino et al., 2012). However, the correlation between EGFR mutations in OSCC with poor prognosis is still one of the biggest challenges in oral cancer research (Choi & Myers, 2008; Grobe et al., 2013; Monteiro et al., 2010; Oliveira & Ribeiro-Silva, 2011). The conventional grading, staging and site of tumor have been used for many years as major parameters of OSCC (Aquino et al., 2012). A better understanding of the molecular mechanisms and identification of potential oncogenes in oral cancer may provide more useful prognostic markers and probably a more effective treatment.

One of the most useful diagnostic methods for detecting EGFR mutations is the use of mutation-specific antibodies when used in combination with DNA sequencing. Yu and Colleagues have generated specific antibodies for identifying EGFR mutations in exon 19 (E746-A750del) and in exon 21 (L858R point mutation). This method was performed on 340 paraffin-embedded NSCLC tissues, showing a sensitivity of 92% as compared with a sensitivity of 99% for DNA sequencing (Yu et al., 2009). In this study we were able to identify EGFR mutations by the EGFR mutation-specific antibodies.

Our studies have found no association between EGFR (E746-A750del) mutation expression with any clinicopathological characteristics, prognostic factors, social habits (smoking, alcohol consumption), or tumor-specific survival.

Although EGFR has been reported in never-smoking patients with NSCLC (Marchetti et al., 2005; Shigematsu et al., 2005), our data shows no relation between EGFR mutations and never-smoking.

Lee and colleagues have confirmed the prevalence of EGFR mutation in 7.3% of Asian patients with HNSCC by using RT-PCR (J. W. Lee et al., 2005). The single EGFR mutation has been detected in exon 19 (E746-A750del), which has been exclusively detected in our study. On the other hand, they have suggested that EGFR mutations may be rather functional alterations than non-functional passenger changes (J. W. Lee et al., 2005; Pao, Miller, Venkatraman, & Kris, 2004). This differs from our study, since our survival analysis does not show any differences between positive and negative expressors, although EGFR (E746-A750del) expression has been detected in 25% of the patients of our study.

Our results support the findings by Na et al showing a prevalence of EGFR (E746-A750del) mutation in OSCC (Na et al., 2007). Although these observations could not confirm its prevalence in the dysplastic lesions by the histopathological examination. These findings raise the question of whether EGFR (E746-A750del) mutation represents a predictor for TKI therapy in OSCC (J. W. Lee et al., 2005), because to date there is no evidence that EGFR (E746-A750del) mutation is involved in the multi-step carcinogenesis of OSCC.

EGFR mutations have been described to have a high response to small molecules tyrosine kinases. However, the clinical outcomes of EGFR mutations in NSCLC may be probably different from those in OSCC or tongue and tonsil carcinoma. Previous studies have indicated that TKI therapy has enhanced the survival in nearly 80% of patients with mutations, especially E746-A750del and the L858R point mutation in NSCLC (Kawahara et al., 2010; Lynch et al., 2004). Taking our data together with a previously published study from Na et al. on patients with tongue and tonsil carcinoma, EGFR mutations seem not to be associated with the survival outcome (Na et al., 2007). This result is in agreement with our study indicating that there is no significance between positive and negative expressors ($p = 0.3397$), in spite of the tendency of our

survival curve towards a better overall survival for patients with positive EGFR (E746-A750del) expression within the 5-year survival rate.

Theoretically, all EGFR mutations may react to TKI in OSCC patients. Contrary to monoclonal antibodies, that block the extracellular ligand binding domain of EGFR, small-molecule EGFR inhibitors such as gefitinib and erlotinib inhibit its tyrosine kinase activity by binding directly to EGFR tyrosine kinase domain. Accordingly, cancers with EGFR tyrosine kinase domains mutations show relatively increased sensitivity to EGFR-TKI's (Gazdar, 2009). The results of a previous study, wherein none of OSCC samples were found to carry mutations in exon 19 and 21, have suggested that OSCC lesions may not response to EGFR-TKI's unlike NSCLCs (Mehta, Annamalai, & Ramanathan, 2014). Another study has found disparate survival outcomes according to the subtype of EGFR mutation, as the better survival outcomes were identified in Patients with NSCLC and EGFR exon 19 deletions in comparison with those with the L858R mutation (Jackman et al., 2006). It is unclear whether the survival outcomes could be enhanced in patients with EGFR mutations after the treatment of TKI in OSCC. Studies are ongoing to investigate the prognostic significance of EGFR mutations in OSCC and the effect of small-molecule targeted therapy on it. Increased knowledge in this field will probably spot the etiology of this mutation and its clinical impact.

The prevalence of HPV DNA in oral carcinoma varies considerably between studies. Isayeva et al reviewed 60 publications on 4,195 patients with oral cavity SCC studied between the years 2000-2011, and determined the weighted prevalence of HPV DNA to be 20.2% (range: 0%-94.7%) (Isayeva et al., 2012). Our findings of HPV DNA in 1.42% of 211 OSCC patients show a low prevalence of HPV in OSCC, and are close to the results of recent several reports, which have determined the HPV prevalence to be from <2% (Francis, Dileep Kumar, Nalinakumari, Jayasree, & Kannan, 2013) to <6% (Lingen et al., 2013). Although the results of Kantola et al could not detect any prevalence of HPV among 105 patients with tongue tumors (Kantola et al., 2000).

It was suggested that the occasional detection of HPV DNA in OSCC samples might be due to an incidental HPV colonization of the oral mucosa and not because of a viral infection (Kansky et al., 2003). While the high presence of HPV in some studies may refer to false-positive results, which overestimate the infection rates (Kansky et al., 2003; Miller & Johnstone, 2001; Rivero & Nunes, 2006). The prevalence of HPV DNA in OSCC and the possibility of its involvement as a causative agent in the oral carcinogenesis are still controversial. Only few studies have examined the continuous prevalence of HPV in the Lymph Node Metastasis, tumoral recurrence or necropsies of OSCC samples (Hoffmann et al., 2005; Howell & Gallant, 1992). A study among patients with advanced OSCC has found that most of the HPV+ samples have been detected only in the matched samples of recidives and not in the primary tumor, which can demonstrate HPV to have a temporary prevalence in the progression of oral cancer (Oliveira, Ribeiro-Silva, Ramalho, Simoes, & Zucoloto, 2008).

The disparately detected HPV prevalence between the studies may result from the different way in preserving, preparing and storing the HPV samples, distinct study populations, the different detection techniques and mixed anatomical areas. It is important to detect the location from which the samples were collected. Some previous studies have not mentioned the origin of the collected samples whether from the mobile tongue, which is a part of the oral cavity, or from the base of the tongue, which is a part of the oropharynx (Oliveira et al., 2008). The distinction between oral cavity and oropharyngeal SCC is the subject of intense research interest and is believed to have a wide variety of effects on HPV prognosis, since HPV is more likely to be positive in oropharyngeal and Waldeyer's ring sites consisting of submucosal and subepithelial lymphatic tissues (Boy et al., 2006; Kreimer, Clifford, Boyle, & Franceschi, 2005). In both normal tissue or oropharyngeal SCC, the tonsillar crypt epithelium is capable of capturing and processing antigens, which let the virus enter the host basal cells. Furthermore, the crypt epithelium and lymphoid tissue may increase the chances of virus persistence in them. The oral rinse collected samples have shown to have much a higher rate of HPV than the

samples collected by oral swabs. This data raised the possibility of presenting the tonsillar tissue in the upper aerodigestive tract a reservoir of HPV. Moreover, It might be important for the immune response to HPV its persistence in tonsillar tissue (Syrjanen, 2004). Dahlgren et al have shown that HPV was positively detected in 2 (2.4%) of 85 of the tumor samples collected from the mobile tongue versus 10 (40%) of 25 of tumor samples collected from the base of tongue (Dahlgren et al., 2004). Similar to what has been observed by Dahlgren et al, another study has found an increased prevalence of HPV16 in oropharyngeal cancer patients compared with patients with oral cancer (Dahlstrom et al., 2003). HPV16 is believed to be found primarily in cancers originating from inflammation sites such as the tonsil, the cervix and the base of tongue (zur Hausen, 1996, 1999).

Gillison et al have supposed that the survival of patients with HNSCC and HPV+ was better than the survival of those with HPV- (Gillison et al., 2000). The genotoxic chemotherapy may play a role in enhancing the survival in patients with HPV-positive cancer cell lines, as it induces apoptosis in these cells and reduces the expression of E6/E7 (Butz, Geisen, Ullmann, Spitkovsky, & Hoppe-Seyler, 1996). Hence, cancers with HPV+ may be more sensitive to radiation and chemotherapy (DeWeese et al., 1997). As reported by other investigators, HPV-positive OSCC appears not to be significantly associated with better survival outcomes (Kaminagakura et al., 2012; Sugiyama et al., 2007). On the contrary, previous published data suggested that HPV is correlated with poor survival and an increased risk of recurrence in OSCC patients who receive radical surgery (L. A. Lee et al., 2012). Therefore, there is still a lack of information on clinical impact of HPV in the context of OSCC due to small samples of enrolled patients, relatively low rates of detected HPV (less than 10%), inclusion patients with cancers in both the oral cavity and oropharynx, different treatment modalities and different cultural behaviors and regions (Dahlgren et al., 2004; Herrero et al., 2003; Joo et al., 2012; S. Y. Lee et al., 2010; Liang, Lewis, Foote, Smith, & Kademani, 2008; Machado et al., 2010; Na et al., 2007; Pathare et al., 2011; Shima et al., 2000).

HPV16 is the most frequent subtype detected in oral cancers (Isayeva et al., 2012), as found in this study, since the single observed genotype was HPV16. According to the smoking and alcohol drinking history, all HPV16+ patients in our study were smokers and alcoholics. In accordance with our findings, a recent study reported that among heavy smokers or heavy drinkers, the risk of OSCC is greater in HPV+ patients compared with HPV- patients (Smith, Rubenstein, Haugen, Pawlita, & Turek, 2012). Accordingly, the most of the patients with HPV-positive OSCC are smokers and/or alcoholics (L. A. Lee et al., 2012). Based on the study of Lee et al, long-lasting betel quid chewing may result in damaging the HPV-infected oral epithelium and can accumulate chemicals, which can play a role in the HPV carcinogenesis (L. A. Lee et al., 2012).

In our study, EGFR (E746-A750del) mutation was negative in all HPV16+ patients. Due to the small number of HPV-positive samples in our study, which may lead to random results, it was not possible to draw any conclusion from this context and the detected affected anatomical site (floor of the mouth).

There are some limitations to this work. It was a retrospective study, not a prospective cohort research. Accordingly, the collecting of precise information during the patient follow-up period was not always possible. In addition, FFPE tissue blocks for HPV PCR were collected from 211 patients, whereas the follow-up data were available from only 191 patients. Of these 191 patients, FFPE blocks for a representative immunohistochemical staining were available for 161 patients. Validation of these findings requires future prospective studies providing fresh tumor samples and a multi-method approach for analyzing the HPV status.

The detection sensitivity of HPV may be decreased for the FFPE samples that contained degraded DNA and RNA (Lingen et al., 2013). Therefore, our observed rate of 1.42% may have been underestimated. Perhaps the most used HPV DNA detection is usually PCR, and the detection of HPV-associated carcinogenesis depends on p16 overexpression, demonstration of HPV E6/E7 RNA, and wild type p53, which have not been analyzed in this survey (Isayeva

et al., 2012). A lot of researches do not focus attention on P16 expression as an surrogate biomarker for HPV-associated OSCC, because these studies do not identify the sensitivity and specificity of p16 overexpression. Therefore, future studies must be conducted in order to fully understand the P16 expression in OSCC (Isayeva et al., 2012).

7. Summary

Oral squamous cell carcinoma (OSCC) takes the sixth place among the most common cancers worldwide. In spite of the vast amount of research and the advances in diagnosis and treatment modalities, the survival rates of patients with oral cancer have not significantly improved in the last decades. Prognostic and predictive biomarkers of treatment outcome have been identified as causative factors for other tumor entities but they are still lacking for OSCC. The primary purpose of this study is the analysis of two important biomarkers - Epidermal Growth Factor Receptor (EGFR) and human papillomavirus (HPV), which may have a promising impact on the diagnosis and therapy of OSCC.

It has become apparent that an abnormal activation of EGFR gene is correlated with self-sufficiency in growth signals, evading apoptosis and other hallmarks of cancer. Moreover, EGFR (E746-A750del) mutations can increase the sensitivity to EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib in patients with advanced non-small-cell lung cancer. Therefore, testing for mutations in EGFR is an important step in the treatment-decision-pathway. However, the prognostic impact of EGFR (E746-A750del) mutation and HPV on OSCC remains unclear.

This study was designed to analyze the clinical impact of EGFR (E746-A750del) mutation and human papillomavirus (HPV) in OSCC. 211 patients with OSCC treated by primary radical tumor resection were retrospectively enrolled in this study. Using INNO-LiPA Extra, high-risk-HPV types were analyzed in all 211 OSCC samples. The EGFR (E746-A750del) expression was analyzed in 161 OSCC samples by immunohistochemistry. The expression results were associated with clinicopathological characteristics and survival outcome. The disease-free survival times were estimated using the Kaplan-Meier method. Multivariate analysis using the Cox Proportional Hazards Model was not performed for these parameters.

Our findings showed low prevalence of EGFR (E746-A750del) expression and HPV in all cancer specimens. Positive EGFR (E746-A750del) expression was

detected in 25% of the patients (n = 40/161), while 1.42% of them were HPV16 positive (HPV16+: n = 3/211). EGFR (E746-A750del) mutation was not significantly associated with survival of the patients. Lymph node metastasis was shown to be the only significant unfavorable factor in multivariate analysis. Social habits like alcohol consumption or smoking had no significant effect on the tumor specific survival. Positive HPV status had no impact on tumor specific survival in OSCC.

On the basis of the results of this research it can be concluded that in OSCC EGFR (E746-A750del) expression is not associated with clinicopathological characteristics, prognostic factors, or social habits; HPV does not seem to be correlated with the survival of patients. Our results may contribute to a better understanding of the prognostic impact of EGFR mutations and HPV in OSCC leading to guidance on better diagnosis and therapeutic decisions in the future. Further studies are needed to investigate the prognostic impact of EGFR mutations in OSCC particularly in relation to small molecules.

8. References

Amann, J., Kalyankrishna, S., Massion, P. P., Ohm, J. E., Girard, L., Shigematsu, H., Peyton, M., Juroske, D., Huang, Y., Stuart Salmon, J., Kim, Y. H., Pollack, J. R., Yanagisawa, K., Gazdar, A., Minna, J. D., Kurie, J. M., & Carbone, D. P. (2005). Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. *Cancer Res, 65*(1), 226-235.

Ang, K. K., Berkey, B. A., Tu, X., Zhang, H. Z., Katz, R., Hammond, E. H., Fu, K. K., & Milas, L. (2002). Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res, 62*(24), 7350-7356.

Ang, K. K., Harris, J., Wheeler, R., Weber, R., Rosenthal, D. I., Nguyen-Tan, P. F., Westra, W. H., Chung, C. H., Jordan, R. C., Lu, C., Kim, H., Axelrod, R., Silverman, C. C., Redmond, K. P., & Gillison, M. L. (2010). Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med, 363*(1), 24-35. doi: 10.1056/NEJMoa0912217

Aquino, G., Pannone, G., Santoro, A., Liguori, G., Franco, R., Serpico, R., Florio, G., De Rosa, A., Mattoni, M., Cozza, V., Botti, G., Losito, S., Longo, F., Staibano, S., Cuda, G., Lo Muzio, L., Sbordone, C., Bufo, P., Grimaldi, A., Caraglia, M., & Di Domenico, M. (2012). pEGFR-Tyr 845 expression as prognostic factors in oral squamous cell carcinoma: a tissue-microarray study with clinic-pathological correlations. *Cancer Biol Ther, 13*(11), 967-977. doi: 10.4161/cbt.20991

Arduino, P. G., Surace, A., Carbone, M., Elia, A., Massolini, G., Gandolfo, S., & Broccoletti, R. (2009). Outcome of oral dysplasia: a retrospective hospital-based study of 207 patients with a long follow-up. *J Oral Pathol Med, 38*(6), 540-544. doi: 10.1111/j.1600-0714.2009.00782.x

Boy, S., Van Rensburg, E. J., Engelbrecht, S., Dreyer, L., van Heerden, M., & van Heerden, W. (2006). HPV detection in primary intra-oral squamous cell carcinomas--commensal, aetiological agent or contamination? *J Oral Pathol Med, 35*(2), 86-90. doi: 10.1111/j.1600-0714.2006.00385.x

Boyle, J. O., Hakim, J., Koch, W., van der Riet, P., Hruban, R. H., Roa, R. A., Correo, R., Eby, Y. J., Ruppert, J. M., & Sidransky, D. (1993). The incidence of p53 mutations increases with progression of head and neck cancer. *Cancer Res, 53*(19), 4477-4480.

Butz, K., Geisen, C., Ullmann, A., Spitkovsky, D., & Hoppe-Seyler, F. (1996). Cellular responses of HPV-positive cancer cells to genotoxic anti-cancer agents: repression of E6/E7-oncogene expression and induction of apoptosis. *Int J Cancer, 68*(4), 506-513. doi: 10.1002/(sici)1097-0215(19961115)68:4<506::aid-ijc17>3.0.co;2-2

Chen, I. H., Chang, J. T., Liao, C. T., Wang, H. M., Hsieh, L. L., & Cheng, A. J. (2003). Prognostic significance of EGFR and Her-2 in oral cavity cancer in betel quid prevalent area cancer prognosis. *Br J Cancer, 89*(4), 681-686. doi: 10.1038/sj.bjc.6601171

Cheng, Y. W., Chiou, H. L., Sheu, G. T., Hsieh, L. L., Chen, J. T., Chen, C. Y., Su, J. M., & Lee, H. (2001). The association of human papillomavirus

16/18 infection with lung cancer among nonsmoking Taiwanese women. *Cancer Res, 61*(7), 2799-2803.

Choi, S., & Myers, J. N. (2008). Molecular pathogenesis of oral squamous cell carcinoma: implications for therapy. *J Dent Res, 87*(1), 14-32. doi: 87/1/14 [pii]

Citri, A., Skaria, K. B., & Yarden, Y. (2003). The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res, 284*(1), 54-65.

Cogliano, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., & Cancer, W. H. O. I. A. f. R. o. (2005). Carcinogenicity of human papillomaviruses. *Lancet Oncol, 6*(4), 204.

Cohen, R. B. (2013). Current challenges and clinical investigations of epidermal growth factor receptor (EGFR)- and ErbB family-targeted agents in the treatment of head and neck squamous cell carcinoma (HNSCC). *Cancer Treat Rev*. doi: 10.1016/j.ctrv.2013.10.002

Cummings, J., Raynaud, F., Jones, L., Sugar, R., & Dive, C. (2010). Fit-for-purpose biomarker method validation for application in clinical trials of anticancer drugs. *Br J Cancer, 103*(9), 1313-1317. doi: 10.1038/sj.bjc.6605910

Dahlgren, L., Dahlstrand, H. M., Lindquist, D., Hogmo, A., Bjornestal, L., Lindholm, J., Lundberg, B., Dalianis, T., & Munck-Wikland, E. (2004). Human papillomavirus is more common in base of tongue than in mobile tongue cancer and is a favorable prognostic factor in base of tongue cancer patients. *Int J Cancer, 112*(6), 1015-1019. doi: 10.1002/ijc.20490

Dahlstrom, K. R., Adler-Storthz, K., Etzel, C. J., Liu, Z., Dillon, L., El-Naggar, A. K., Spitz, M. R., Schiller, J. T., Wei, Q., & Sturgis, E. M. (2003). Human papillomavirus type 16 infection and squamous cell carcinoma of the head and neck in never-smokers: a matched pair analysis. *Clin Cancer Res, 9*(7), 2620-2626.

DeWeese, T. L., Walsh, J. C., Dillehay, L. E., Kessis, T. D., Hedrick, L., Cho, K. R., & Nelson, W. G. (1997). Human papillomavirus E6 and E7 oncoproteins alter cell cycle progression but not radiosensitivity of carcinoma cells treated with low-dose-rate radiation. *Int J Radiat Oncol Biol Phys, 37*(1), 145-154.

Doorbar, J. (2006). Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond), 110*(5), 525-541. doi: 10.1042/cs20050369

Fedi, P., Tronick, S.R., and Aaronson, S.A. (1997). Growth factors. *Cancer Med*, pp. 41–64.

Fischer, O. M., Hart, S., Gschwind, A., & Ullrich, A. (2003). EGFR signal transactivation in cancer cells. *Biochem Soc Trans, 31*(Pt 6), 1203-1208. doi: 10.1042/

Francis, G., Dileep Kumar, U., Nalinakumari, K. R., Jayasree, K., & Kannan, S. (2013). Accumulation of inactive p53 protein in oral squamous cell carcinoma: stabilization by protein interaction. *Eur J Oral Sci, 121*(1), 21-28. doi: 10.1111/eos.12007

Freudlsperger, C., Alexander, D., Reinert, S., & Hoffmann, J. (2010). Prognostic value of c-Met expression in oral squamous cell carcinoma. *Exp Ther Med, 1*(1), 69-72. doi: 10.3892/etm_00000012

Gazdar, A. F. (2009). Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. *Oncogene, 28 Suppl 1*, S24-31. doi: 10.1038/onc.2009.198

Gillison, M. L., Alemany, L., Snijders, P. J., Chaturvedi, A., Steinberg, B. M., Schwartz, S., & Castellsague, X. (2012). Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. *Vaccine, 30 Suppl 5*, F34-54. doi: 10.1016/j.vaccine.2012.05.070

Gillison, M. L., Koch, W. M., Capone, R. B., Spafford, M., Westra, W. H., Wu, L., Zahurak, M. L., Daniel, R. W., Viglione, M., Symer, D. E., Shah, K. V., & Sidransky, D. (2000). Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst, 92*(9), 709-720.

Grandis, J. R., & Tweardy, D. J. (1993). Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. *Cancer Res, 53*(15), 3579-3584.

Grimm, M., Schmitt, S., Teriete, P., Biegner, T., Stenzl, A., Hennenlotter, J., Muhs, H. J., Munz, A., Nadtotschi, T., Konig, K., Sanger, J., Feyen, O., Hofmann, H., Reinert, S., & Coy, J. F. (2013). A biomarker based detection and characterization of carcinomas exploiting two fundamental biophysical mechanisms in mammalian cells. *BMC Cancer, 13*, 569. doi: 10.1186/1471-2407-13-569

Grobe, A., Eichhorn, W., Fraederich, M., Kluwe, L., Vashist, Y., Wikner, J., Smeets, R., Simon, R., Sauter, G., Heiland, M., & Blessmann, M. (2013). Immunohistochemical and FISH analysis of EGFR and its prognostic value in patients with oral squamous cell carcinoma. *J Oral Pathol Med*. doi: 10.1111/jop.12111

Guillaud, M., Zhang, L., Poh, C., Rosin, M. P., & MacAulay, C. (2008). Potential use of quantitative tissue phenotype to predict malignant risk for oral premalignant lesions. *Cancer Res, 68*(9), 3099-3107. doi: 10.1158/0008-5472.can-07-2113

Gupta, A. K., Lee, J. H., Wilke, W. W., Quon, H., Smith, G., Maity, A., Buatti, J. M., & Spitz, D. R. (2009). Radiation response in two HPV-infected head-and-neck cancer cell lines in comparison to a non-HPV-infected cell line and relationship to signaling through AKT. *Int J Radiat Oncol Biol Phys, 74*(3), 928-933. doi: 10.1016/j.ijrobp.2009.03.004

Gupta, A. K., McKenna, W. G., Weber, C. N., Feldman, M. D., Goldsmith, J. D., Mick, R., Machtay, M., Rosenthal, D. I., Bakanauskas, V. J., Cerniglia, G. J., Bernhard, E. J., Weber, R. S., & Muschel, R. J. (2002). Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction. *Clin Cancer Res, 8*(3), 885-892.

Hamilton, S. R., Aaltonen, L.A. (2000). Pathology and Genetics. Tumours of the Digestive System. *Lyon, IARC Press, Third Edition*.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

Hanken, H., Kraatz, J., Smeets, R., Heiland, M., Blessmann, M., Eichhorn, W., Clauditz, T. S., Grobe, A., Kolk, A., & Rana, M. (2013). The detection of oral pre- malignant lesions with an autofluorescence based imaging system (VELscope) - a single blinded clinical evaluation. *Head Face Med, 9*, 23. doi: 10.1186/1746-160x-9-23

Harris, C. C. (1996). p53 tumor suppressor gene: from the basic research laboratory to the clinic--an abridged historical perspective. *Carcinogenesis, 17*(6), 1187-1198.

Herrero, R., Castellsague, X., Pawlita, M., Lissowska, J., Kee, F., Balaram, P., Rajkumar, T., Sridhar, H., Rose, B., Pintos, J., Fernandez, L., Idris, A., Sanchez, M. J., Nieto, A., Talamini, R., Tavani, A., Bosch, F. X., Reidel, U., Snijders, P. J., Meijer, C. J., Viscidi, R., Munoz, N., & Franceschi, S. (2003). Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *J Natl Cancer Inst, 95*(23), 1772-1783.

Hertrampf, K., Wiltfang, J., Katalinic, A., Timm, O., & Wenz, H. J. (2012). Recent trends in incidence and mortality of oral and pharyngeal cancer in Schleswig-Holstein in Northern Germany. *Community Dent Health, 29*(4), 268-273.

Ho, P. S., Chen, P. L., Warnakulasuriya, S., Shieh, T. Y., Chen, Y. K., & Huang, I. Y. (2009). Malignant transformation of oral potentially malignant disorders in males: a retrospective cohort study. *BMC Cancer, 9*, 260. doi: 10.1186/1471-2407-9-260

Hoffmann, M., Gottschlich, S., Gorogh, T., Lohrey, C., Schwarz, E., Ambrosch, P., & Kahn, T. (2005). Human papillomaviruses in lymph node neck metastases of head and neck cancers. *Acta Otolaryngol, 125*(4), 415-421. doi: 10.1080/00016480510028528

Holbro, T., Civenni, G., & Hynes, N. E. (2003). The ErbB receptors and their role in cancer progression. *Exp Cell Res, 284*(1), 99-110.

Howell, R. E., & Gallant, L. (1992). Human papillomavirus type 16 in an oral squamous carcinoma and its metastasis. *Oral Surg Oral Med Oral Pathol, 74*(5), 620-626.

Huang, S. F., Chuang, W. Y., Chen, I. H., Liao, C. T., Wang, H. M., & Hsieh, L. L. (2009). EGFR protein overexpression and mutation in areca quid-associated oral cavity squamous cell carcinoma in Taiwan. *Head Neck, 31*(8), 1068-1077. doi: 10.1002/hed.21067

Hynes, N. E., & Lane, H. A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer, 5*(5), 341-354. doi: 10.1038/nrc1609

Isayeva, T., Li, Y., Maswahu, D., & Brandwein-Gensler, M. (2012). Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. *Head Neck Pathol, 6 Suppl 1*, S104-120. doi: 10.1007/s12105-012-0368-1

Jackman, D. M., Yeap, B. Y., Sequist, L. V., Lindeman, N., Holmes, A. J., Joshi, V. A., Bell, D. W., Huberman, M. S., Halmos, B., Rabin, M. S., Haber, D. A., Lynch, T. J., Meyerson, M., Johnson, B. E., & Janne, P. A. (2006). Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients

treated with gefitinib or erlotinib. *Clin Cancer Res, 12*(13), 3908-3914. doi: 10.1158/1078-0432.ccr-06-0462

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA Cancer J Clin, 61*(2), 69-90. doi: 10.3322/caac.20107

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., & Thun, M. J. (2008). Cancer statistics, 2008. *CA Cancer J Clin, 58*(2), 71-96. doi: 10.3322/ca.2007.0010

Joo, Y. H., Jung, C. K., Sun, D. I., Park, J. O., Cho, K. J., & Kim, M. S. (2012). High-risk human papillomavirus and cervical lymph node metastasis in patients with oropharyngeal cancer. *Head Neck, 34*(1), 10-14. doi: 10.1002/hed.21697

Joseph, B. K. (2002). Oral cancer: prevention and detection. *Med Princ Pract, 11 Suppl 1*, 32-35.

Kalyankrishna, S., & Grandis, J. R. (2006). Epidermal growth factor receptor biology in head and neck cancer. *J Clin Oncol, 24*(17), 2666-2672. doi: 10.1200/jco.2005.04.8306

Kaminagakura, E., Villa, L. L., Andreoli, M. A., Sobrinho, J. S., Vartanian, J. G., Soares, F. A., Nishimoto, I. N., Rocha, R., & Kowalski, L. P. (2012). High-risk human papillomavirus in oral squamous cell carcinoma of young patients. *Int J Cancer, 130*(8), 1726-1732. doi: 10.1002/ijc.26185

Kansky, A. A., Poljak, M., Seme, K., Kocjan, B. J., Gale, N., Luzar, B., & Golouh, R. (2003). Human papillomavirus DNA in oral squamous cell carcinomas and normal oral mucosa. *Acta Virol, 47*(1), 11-16.

Kantola, S., Parikka, M., Jokinen, K., Hyrynkangs, K., Soini, Y., Alho, O. P., & Salo, T. (2000). Prognostic factors in tongue cancer - relative importance of demographic, clinical and histopathological factors. *Br J Cancer, 83*(5), 614-619. doi: 10.1054/bjoc.2000.1323

Kaplan, E. L., & Meier, P. (1958). Nonparametric estimation from incomplete observations. *J Am Stat Assoc, 75*, 457-487.

Kawahara, A., Yamamoto, C., Nakashima, K., Azuma, K., Hattori, S., Kashihara, M., Aizawa, H., Basaki, Y., Kuwano, M., Kage, M., Mitsudomi, T., & Ono, M. (2010). Molecular diagnosis of activating EGFR mutations in non-small cell lung cancer using mutation-specific antibodies for immunohistochemical analysis. *Clin Cancer Res, 16*(12), 3163-3170. doi: 10.1158/1078-0432.CCR-09-3239

Kreimer, A. R., Clifford, G. M., Boyle, P., & Franceschi, S. (2005). Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiol Biomarkers Prev, 14*(2), 467-475. doi: 10.1158/1055-9965.epi-04-0551

Kumar, B., Cordell, K. G., Lee, J. S., Worden, F. P., Prince, M. E., Tran, H. H., Wolf, G. T., Urba, S. G., Chepeha, D. B., Teknos, T. N., Eisbruch, A., Tsien, C. I., Taylor, J. M., D'Silva, N. J., Yang, K., Kurnit, D. M., Bauer, J. A., Bradford, C. R., & Carey, T. E. (2008). EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer. *J Clin Oncol, 26*(19), 3128-3137. doi: 10.1200/jco.2007.12.7662

Laco, J., Vosmikova, H., Novakova, V., Celakovsky, P., Dolezalova, H., Tucek, L., Nekvindova, J., Vosmik, M., Cermakova, E., & Ryska, A. (2011). The role of high-risk human papillomavirus infection in oral and oropharyngeal squamous cell carcinoma in non-smoking and non-drinking patients: a clinicopathological and molecular study of 46 cases. *Virchows Arch, 458*(2), 179-187. doi: 10.1007/s00428-010-1037-y

Laimer, K., Spizzo, G., Gastl, G., Obrist, P., Brunhuber, T., Fong, D., Barbieri, V., Jank, S., Doppler, W., Rasse, M., & Norer, B. (2007). High EGFR expression predicts poor prognosis in patients with squamous cell carcinoma of the oral cavity and oropharynx: a TMA-based immunohistochemical analysis. *Oral Oncol, 43*(2), 193-198. doi: 10.1016/j.oraloncology.2006.02.009

Lee, J. W., Soung, Y. H., Kim, S. Y., Nam, H. K., Park, W. S., Nam, S. W., Kim, M. S., Sun, D. I., Lee, Y. S., Jang, J. J., Lee, J. Y., Yoo, N. J., & Lee, S. H. (2005). Somatic mutations of EGFR gene in squamous cell carcinoma of the head and neck. *Clin Cancer Res, 11*(8), 2879-2882. doi: 10.1158/1078-0432.CCR-04-2029

Lee, L. A., Huang, C. G., Liao, C. T., Lee, L. Y., Hsueh, C., Chen, T. C., Lin, C. Y., Fan, K. H., Wang, H. M., Huang, S. F., Chen, I. H., Kang, C. J., Ng, S. H., Yang, S. L., Tsao, K. C., Chang, Y. L., & Yen, T. C. (2012). Human papillomavirus-16 infection in advanced oral cavity cancer patients is related to an increased risk of distant metastases and poor survival. *PLoS ONE, 7*(7), e40767. doi: 10.1371/journal.pone.0040767

Lee, S. Y., Cho, N. H., Choi, E. C., Baek, S. J., Kim, W. S., Shin, D. H., & Kim, S. H. (2010). Relevance of human papilloma virus (HPV) infection to carcinogenesis of oral tongue cancer. *Int J Oral Maxillofac Surg, 39*(7), 678-683. doi: 10.1016/j.ijom.2010.03.014

Liang, X. H., Lewis, J., Foote, R., Smith, D., & Kademani, D. (2008). Prevalence and significance of human papillomavirus in oral tongue cancer: the Mayo Clinic experience. *J Oral Maxillofac Surg, 66*(9), 1875-1880. doi: 10.1016/j.joms.2008.04.009

Lingen, M. W., Xiao, W., Schmitt, A., Jiang, B., Pickard, R., Kreinbrink, P., Perez-Ordonez, B., Jordan, R. C., & Gillison, M. L. (2013). Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral Oncol, 49*(1), 1-8. doi: 10.1016/j.oraloncology.2012.07.002

Liu, W., Wang, Y. F., Zhou, H. W., Shi, P., Zhou, Z. T., & Tang, G. Y. (2010). Malignant transformation of oral leukoplakia: a retrospective cohort study of 218 Chinese patients. *BMC Cancer, 10*, 685. doi: 10.1186/1471-2407-10-685

Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haserlat, S. M., Supko, J. G., Haluska, F. G., Louis, D. N., Christiani, D. C., Settleman, J., & Haber, D. A. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med, 350*(21), 2129-2139. doi: 10.1056/NEJMoa040938

Machado, J., Reis, P. P., Zhang, T., Simpson, C., Xu, W., Perez-Ordonez, B., Goldstein, D. P., Brown, D. H., Gilbert, R. W., Gullane, P. J., Irish, J. C.,

& Kamel-Reid, S. (2010). Low prevalence of human papillomavirus in oral cavity carcinomas. *Head Neck Oncol, 2*, 6. doi: 10.1186/1758-3284-2-6

Marchetti, A., Martella, C., Felicioni, L., Barassi, F., Salvatore, S., Chella, A., Camplese, P. P., Iarussi, T., Mucilli, F., Mezzetti, A., Cuccurullo, F., Sacco, R., & Buttitta, F. (2005). EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol, 23*(4), 857-865. doi: 10.1200/JCO.2005.08.043

McLaughlin-Drubin, M. E., Meyers, J., & Munger, K. (2012). Cancer associated human papillomaviruses. *Curr Opin Virol, 2*(4), 459-466. doi: 10.1016/j.coviro.2012.05.004

Mehanna, H., Paleri, V., West, C. M., & Nutting, C. (2010). Head and neck cancer--Part 1: Epidemiology, presentation, and prevention. *Bmj, 341*, c4684. doi: 10.1136/bmj.c4684

Mehanna, H. M., Rattay, T., Smith, J., & McConkey, C. C. (2009). Treatment and follow-up of oral dysplasia - a systematic review and meta-analysis. *Head Neck, 31*(12), 1600-1609. doi: 10.1002/hed.21131

Mehta, D. T., Annamalai, T., & Ramanathan, A. (2014). Lack of mutations in protein tyrosine kinase domain coding exons 19 and 21 of the EGFR gene in oral squamous cell carcinomas. *Asian Pac J Cancer Prev, 15*(11), 4623-4627.

Messadi, D. V. (2013). Diagnostic aids for detection of oral precancerous conditions. *Int J Oral Sci, 5*(2), 59-65. doi: 10.1038/ijos.2013.24

Miller, C. S., & Johnstone, B. M. (2001). Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982-1997. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 91*(6), 622-635. doi: 10.1067/moe.2001.115392

Mitsudomi, T., Kosaka, T., Endoh, H., Horio, Y., Hida, T., Mori, S., Hatooka, S., Shinoda, M., Takahashi, T., & Yatabe, Y. (2005). Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol, 23*(11), 2513-2520. doi: 10.1200/jco.2005.00.992

Monteiro, L. S., Diniz-Freitas, M., Garcia-Caballero, T., Forteza, J., & Fraga, M. (2010). EGFR and Ki-67 expression in oral squamous cell carcinoma using tissue microarray technology. *J Oral Pathol Med, 39*(7), 571-578. doi: 10.1111/j.1600-0714.2009.00876.x

Munoz, N., Bosch, F. X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K. V., Snijders, P. J., Meijer, C. J., & International Agency for Research on Cancer Multicenter Cervical Cancer Study, G. (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med, 348*(6), 518-527. doi: 10.1056/NEJMoa021641

Na, II, Kang, H. J., Cho, S. Y., Koh, J. S., Lee, J. K., Lee, B. C., Lee, G. H., Lee, Y. S., Yoo, H. J., Ryoo, B. Y., Yang, S. H., & Shim, Y. S. (2007). EGFR mutations and human papillomavirus in squamous cell carcinoma of

tongue and tonsil. *Eur J Cancer, 43*(3), 520-526. doi: 10.1016/j.ejca.2006.09.025

Nagpal, J. K., Patnaik, S., & Das, B. R. (2002). Prevalence of high-risk human papilloma virus types and its association with P53 codon 72 polymorphism in tobacco addicted oral squamous cell carcinoma (OSCC) patients of Eastern India. *Int J Cancer, 97*(5), 649-653.

Oliveira, L. R., & Ribeiro-Silva, A. (2011). Prognostic significance of immunohistochemical biomarkers in oral squamous cell carcinoma. *Int J Oral Maxillofac Surg, 40*(3), 298-307. doi: 10.1016/j.ijom.2010.12.003

Oliveira, L. R., Ribeiro-Silva, A., Ramalho, L. N., Simoes, A. L., & Zucoloto, S. (2008). HPV infection in Brazilian oral squamous cell carcinomapatients and its correlation with clinicopathological outcomes. *Mol Med Rep, 1*(1), 123-129.

Ono, M., & Kuwano, M. (2006). Molecular mechanisms of epidermal growth factor receptor (EGFR) activation and response to gefitinib and other EGFR-targeting drugs. *Clin Cancer Res, 12*(24), 7242-7251. doi: 10.1158/1078-0432.ccr-06-0646

Paez, J. G., Janne, P. A., Lee, J. C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F. J., Lindeman, N., Boggon, T. J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M. J., Sellers, W. R., Johnson, B. E., & Meyerson, M. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science, 304*(5676), 1497-1500. doi: 10.1126/science.1099314

Pao, W., Miller, V., Zakowski, M., Doherty, J., Politi, K., Sarkaria, I., Singh, B., Heelan, R., Rusch, V., Fulton, L., Mardis, E., Kupfer, D., Wilson, R., Kris, M., & Varmus, H. (2004). EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proceedings of the National Academy of Sciences of the United States of America, 101*(36), 13306-13311. doi: 10.1073/pnas.0405220101

Pao, W., Miller, V. A., Venkatraman, E., & Kris, M. G. (2004). Predicting sensitivity of non-small-cell lung cancer to gefitinib: is there a role for P-Akt? *J Natl Cancer Inst, 96*(15), 1117-1119. doi: 10.1093/jnci/djh244

Pathare, S. M., Gerstung, M., Beerenwinkel, N., Schaffer, A. A., Kannan, S., Pai, P., Pathak, K. A., Borges, A. M., & Mahimkar, M. B. (2011). Clinicopathological and prognostic implications of genetic alterations in oral cancers. *Oncol Lett, 2*(3), 445-451. doi: 10.3892/ol.2011.271

Posner, M. R., Lorch, J. H., Goloubeva, O., Tan, M., Schumaker, L. M., Sarlis, N. J., Haddad, R. I., & Cullen, K. J. (2011). Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. *Ann Oncol, 22*(5), 1071-1077. doi: 10.1093/annonc/mdr006

Psyrri, A., & Cohen, E. (2011). Oropharyngeal cancer: clinical implications of the HPV connection. *Ann Oncol, 22*(5), 997-999. doi: 10.1093/annonc/mdr124

Rampias, T., Sasaki, C., Weinberger, P., & Psyrri, A. (2009). E6 and e7 gene silencing and transformed phenotype of human papillomavirus 16-

positive oropharyngeal cancer cells. *J Natl Cancer Inst, 101*(6), 412-423. doi: 10.1093/jnci/djp017

Reuter, C. W., Morgan, M. A., & Eckardt, A. (2007). Targeting EGF-receptor-signalling in squamous cell carcinomas of the head and neck. *Br J Cancer, 96*(3), 408-416. doi: 10.1038/sj.bjc.6603566

Riely, G. J., Politi, K. A., Miller, V. A., & Pao, W. (2006). Update on epidermal growth factor receptor mutations in non-small cell lung cancer. *Clin Cancer Res, 12*(24), 7232-7241. doi: 10.1158/1078-0432.ccr-06-0658

Rischin, D., Young, R. J., Fisher, R., Fox, S. B., Le, Q. T., Peters, L. J., Solomon, B., Choi, J., O'Sullivan, B., Kenny, L. M., & McArthur, G. A. (2010). Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. *J Clin Oncol, 28*(27), 4142-4148. doi: 10.1200/jco.2010.29.2904

Rivero, E. R., & Nunes, F. D. (2006). HPV in oral squamous cell carcinomas of a Brazilian population: amplification by PCR. *Braz Oral Res, 20*(1), 21-24. doi: /S1806-83242006000100005

Rogers, S. J., Harrington, K. J., Rhys-Evans, P., P, O. C., & Eccles, S. A. (2005). Biological significance of c-erbB family oncogenes in head and neck cancer. *Cancer Metastasis Rev, 24*(1), 47-69. doi: 10.1007/s10555-005-5047-1

Rubin Grandis, J., Melhem, M. F., Gooding, W. E., Day, R., Holst, V. A., Wagener, M. M., Drenning, S. D., & Tweardy, D. J. (1998). Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst, 90*(11), 824-832.

Schiffman, M., Clifford, G., & Buonaguro, F. M. (2009). Classification of weakly carcinogenic human papillomavirus types: addressing the limits of epidemiology at the borderline. *Infect Agent Cancer, 4*, 8. doi: 10.1186/1750-9378-4-8

Scully, C., & Felix, D. H. (2006). Oral medicine--update for the dental practitioner oral cancer. *Br Dent J, 200*(1), 13-17. doi: 10.1038/sj.bdj.4813117

Sharma, S. V., Bell, D. W., Settleman, J., & Haber, D. A. (2007). Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer, 7*(3), 169-181. doi: 10.1038/nrc2088

Shigematsu, H., Lin, L., Takahashi, T., Nomura, M., Suzuki, M., Wistuba, II, Fong, K. M., Lee, H., Toyooka, S., Shimizu, N., Fujisawa, T., Feng, Z., Roth, J. A., Herz, J., Minna, J. D., & Gazdar, A. F. (2005). Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst, 97*(5), 339-346. doi: 10.1093/jnci/dji055

Shima, K., Kobayashi, I., Saito, I., Kiyoshima, T., Matsuo, K., Ozeki, S., Ohishi, M., & Sakai, H. (2000). Incidence of human papillomavirus 16 and 18 infection and p53 mutation in patients with oral squamous cell carcinoma in Japan. *Br J Oral Maxillofac Surg, 38*(5), 445-450. doi: 10.1054/bjom.2000.0162

Shiraki, M., Odajima, T., Ikeda, T., Sasaki, A., Satoh, M., Yamaguchi, A., Noguchi, M., Nagai, I., & Hiratsuka, H. (2005). Combined expression of p53, cyclin D1 and epidermal growth factor receptor improves estimation

of prognosis in curatively resected oral cancer. *Mod Pathol, 18*(11), 1482-1489. doi: 10.1038/modpathol.3800455

Silverman, S. J. (1998). Epidemiology. In S. J. Silverman (Ed.), *Oral Cancer* (4th ed., pp. 1-6). Hamilton, Ontario, Canada: BC Decker Inc.

Simabuco, F. M., Kawahara, R., Yokoo, S., Granato, D. C., Miguel, L., Agostini, M., Aragao, A. Z., Domingues, R. R., Flores, I. L., Macedo, C. C., Della Coletta, R., Graner, E., & Paes Leme, A. F. (2014). ADAM17 mediates OSCC development in an orthotopic murine model. *Mol Cancer, 13*, 24. doi: 10.1186/1476-4598-13-24

Smith, E. M., Rubenstein, L. M., Haugen, T. H., Pawlita, M., & Turek, L. P. (2012). Complex etiology underlies risk and survival in head and neck cancer human papillomavirus, tobacco, and alcohol: a case for multifactor disease. *J Oncol, 2012*, 571862. doi: 10.1155/2012/571862

Sobin LH, & Ch., W. (2010). UICC. TNM Classification of Malignant Tumors *Berlin, Springer Verlag 7th edition*.

Sreedhar, G., Narayanappa Sumalatha, M., & Shukla, D. (2014). An overview of the risk factors associated with multiple oral premalignant lesions with a case report of extensive field cancerization in a female patient. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. doi: 10.5507/bp.2013.092

Sugiyama, M., Bhawal, U. K., Kawamura, M., Ishioka, Y., Shigeishi, H., Higashikawa, K., & Kamata, N. (2007). Human papillomavirus-16 in oral squamous cell carcinoma: clinical correlates and 5-year survival. *Br J Oral Maxillofac Surg, 45*(2), 116-122. doi: 10.1016/j.bjoms.2006.04.012

Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., & Van Dyke, T. (1994). p53-dependent apoptosis suppresses tumor growth and progression in vivo. *Cell, 78*(4), 703-711.

Syrjanen, S. (2004). HPV infections and tonsillar carcinoma. *J Clin Pathol, 57*(5), 449-455.

Takano, T., Ohe, Y., Sakamoto, H., Tsuta, K., Matsuno, Y., Tateishi, U., Yamamoto, S., Nokihara, H., Yamamoto, N., Sekine, I., Kunitoh, H., Shibata, T., Sakiyama, T., Yoshida, T., & Tamura, T. (2005). Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. *J Clin Oncol, 23*(28), 6829-6837. doi: 10.1200/jco.2005.01.0793

Tanaka, T., Tanaka, M., & Tanaka, T. (2011). Oral carcinogenesis and oral cancer chemoprevention: a review. *Patholog Res Int, 2011*, 431246. doi: 10.4061/2011/431246

Tseng, H. H., & He, B. (2013). Molecular markers as therapeutic targets in lung cancer. *Chin J Cancer, 32*(2), 59-62. doi: 10.5732/cjc.013.10011

Tuominen, V. J., Tolonen, T. T., & Isola, J. (2012). ImmunoMembrane: a publicly available web application for digital image analysis of HER2 immunohistochemistry. *Histopathology, 60*(5), 758-767. doi: 10.1111/j.1365-2559.2011.04142.x

Tushar, M. D., & Ramanathan, A. (2013). Tyrosine 1045 codon mutations in exon 27 of EGFR are infrequent in oral squamous cell carcinomas. *Asian Pac J Cancer Prev, 14*(7), 4279-4282.

Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., & Yarden, Y. (1996). A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol, 16*(10), 5276-5287.

van der Waal, I. (2009). Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. *Oral Oncol, 45*(4-5), 317-323. doi: 10.1016/j.oraloncology.2008.05.016

Wang, W., Han, S., Yao, Z., Li, X., Huang, P., Zhang, M., Chen, Y., & He, J. (2012). A study of epidemiologic and recurrence factors of oral cancer. *J Oral Maxillofac Surg, 70*(9), 2205-2210. doi: 10.1016/j.joms.2011.09.040

Warnakulasuriya, S., Johnson, N. W., & van der Waal, I. (2007). Nomenclature and classification of potentially malignant disorders of the oral mucosa. *J Oral Pathol Med, 36*(10), 575-580. doi: 10.1111/j.1600-0714.2007.00582.x

Williams, G. T. (1991). Programmed cell death: apoptosis and oncogenesis. *Cell, 65*(7), 1097-1098.

Yarden, Y., & Sliwkowski, M. X. (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol, 2*(2), 127-137. doi: 10.1038/35052073

Yu, J., Kane, S., Wu, J., Benedettini, E., Li, D., Reeves, C., Innocenti, G., Wetzel, R., Crosby, K., Becker, A., Ferrante, M., Cheung, W. C., Hong, X., Chirieac, L. R., Sholl, L. M., Haack, H., Smith, B. L., Polakiewicz, R. D., Tan, Y., Gu, T. L., Loda, M., Zhou, X., & Comb, M. J. (2009). Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. *Clin Cancer Res, 15*(9), 3023-3028. doi: 10.1158/1078-0432.ccr-08-2739

Zheng, Z. M., & Baker, C. C. (2006). Papillomavirus genome structure, expression, and post-transcriptional regulation. *Front Biosci, 11*, 2286-2302.

zur Hausen, H. (1996). Papillomavirus infections--a major cause of human cancers. *Biochim Biophys Acta, 1288*(2), F55-78.

zur Hausen, H. (1999). Papillomaviruses in human cancers. *Proc Assoc Am Physicians, 111*(6), 581-587.

9. Zusammenfassung

Mundhöhlenkarzinome gehören zu der sechsthäufigsten Tumorentität weltweit. Trotz Fortschritten in Diagnose und therapeutischen Maßnahmen konnte die Überlebensrate von Patienten mit Mundhöhlenkarzinom in den letzten Jahrzehnten nicht signifikant gesteigert werden.

Während für andere Tumorentitäten bereits standardisierte prognostische Biomarker identifiziert wurden, fehlen diese bis heute für das Mundhöhlenkarzinom.

Das Ziel dieser Studie war anhand der „Hallmarks of Cancer“ (Krebsmerkmale) gewebebasierte Biomarker zu analysieren, wodurch möglicherweise langfristig eine schonendere und bessere Therapie für Patienten mit Mundhöhlenkarzinom angeboten werden kann.

Der Nachweis eines abnormal aktivierten sowie mutierten epidermalen Wachstumsfaktor Rezeptors (EGFR) in Tumorzellen kann als Hinweis auf Apoptoseresistenz und Selbstversorgung mit Wachstumssignalen (Hallmarks of Cancer) verstanden werden. In bisherigen Arbeiten konnte gezeigt werden, dass Mutationen im EGFR-Gen (E746-A750del) die Sensitivität von Patienten mit fortgeschrittenem nicht-kleinzelligem Lungenkarzinom gegenüber EGFR-basierten Tyrosinkinaseninhibitoren wie Gefitinib und Erlotinib erhöhen. Aus diesem Grund ist die Untersuchung von EGFR-Mutationen ein wichtiger Schritt auf dem Weg zu neuen Behandlungsmethoden.

In der Literatur wird das humane Papillomaviren (HPV) mit Karzinogenese von Kopf-Hals Tumoren (Plattenepithelkarzinomen des Kopf-/Halsbereiches) assoziiert und mit Apoptoseresistenz von Tumorzellen in Verbindung gebracht, insbesondere mit orpharyngealen Plattenephithelkarzinomen.

Die Analyse der Prävalenz der EGFR-Mutation (E746-A750del) beim Mundhöhlenkarzinom ist bislang unbekannt. Diese Studie befasste sich daher insbesondere mit der Analyse der EGFR-Mutation (E746-A750del) sowie des HPV Status auf die Prognose des Mundhöhlenkarzinoms.

In dieser Studie wurde retrospektiv 211 Tumorgewebeproben mit histologisch gesichertem oralen Plattenepithelkarzinom mittels INNO-LiPa Extra auf Hochrisikotypen von HPV hin untersucht. Die Expression der EGFR-Mutante E746-A750del wurde in 161 Tumorgewebeproben immunhistochemisch analysiert. Die Ergebnisse dieser Expressionsanalysen wurden anschließend mit klinisch-pathologischen Verlaufsparametern und Überlebensraten assoziiert. Die Überlebensrate der Tumorpatienten wurde in Bezug auf die EGFR (E746-A750del) und HPV Expression univariat mit der Kaplan-Meier-Methode ermittelt.

Die Untersuchungen zeigten eine geringe Expression von EGFR (E746-A750del) und HPV in den analysierten Proben. In 25% der Patientenproben (n=40/161) konnte eine Expression der EGFR-Mutante nachgewiesen werden, während nur 1.42% der Proben positive Resultate für HPV16 aufwiesen (n=3/211). In Assoziation mit den klinisch-pathologischen Verlaufsparametern zeigte sich, dass die Subpopulation der EGFR (E746-A750del)-positiv getesteten Patienten keine signifikant bessere oder schlechtere Überlebensrate, verglichen mit den EGFR (E746-A750del)-negativen Patienten aufwies. In der multivariaten Analyse war Lymphknotenmetastasierung der einzig prognostisch unabhängig Faktor für signifikant schlechtes tumorbedingtes Überleben. Personenbezogene Risikofaktoren wie Alkoholkonsum oder Rauchen hatten keinen signifikanten Einfluss auf die tumorspezifische Überlebensrate. Des Weiteren konnte auch kein Einfluss des HPV-Status auf die tumorspezifische Überlebensrate für das Mundhöhlenkarzinom gezeigt werden.

Auf Basis der erzielten Ergebnisse aus dieser Studie kann schlussgefolgert werden, dass die Expression der EGFR-Mutante (E746-A750del) keinen Einfluss auf klinisch-pathologische Verlaufsparameter, prognostische Faktoren oder persönliche Risikofaktoren wie Nikotin- und Alkoholabusus hat. Auch das Vorhandensein einer HPV-Infektion scheint nicht mit dem Überleben der entsprechenden Patienten assoziiert zu sein.

Die in dieser Arbeit erzielten Ergebnisse können jedoch zu einem besseren Verständnis der prognostischen Relevanz von EGFR-Mutationen und HPV beim Mundhöhlenkarzinom beitragen. Diese basiswissenschaftlichen Ergebnisse tragen dazu bei, in Zukunft notwendige Therapieentscheidungen besser zu untermauern. Weiterführende Arbeiten zur prognostischen Relevanz von EGFR-Mutationen sind jedoch unerlässlich, besonders im Zusammenhang mit klinischen Studien und Therapieansätzen durch spezifische Tyrosinkinaseinhibitoren (sog. `small molecules´).

10. Author's declaration

I hereby declare that this thesis is my own work, which was performed under the supervision of PD Dr. Dr. Martin Grimm. Hazem Altaki carried out immunohistochemistry studies, statistics, data analysis and interpretation.

11. Abbreviations

AJCC	American Joint Cancer Committee
AKT	Protein kinase B
AREG	Amphiregulin
CI	Confidence Interval
DAB	3,3'-Diaminobenzidine
DFS	The disease-free survival
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Epiregulin
FFPE	Formalin-fixed, paraffin-embedded
H.E.	Hematoxylin and eosin
H2O2	Hydrogen Peroxide
HB-EGF	Heparin-binding EGF
HPF	High power fields
HPV	Human papillomavirus
HR	Hazard ratios
HRP	Horseradish peroxidase
IHC	Immunohistochemical staining
Jak	Janus kinase
kDa	KiloDaltons
LiPA	Line Probe Assay
LSAB	Labelled streptavidin biotin method
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NFV	Nelfinavir

NSCLC	Non-small-cell lung cancer
NT	Non-malignant tissue
OSCC	Oral squamous cell carcinoma
P	*P*-value
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
RTKs	Receptor tyrosine kinases
SCCHN	Squamous cell carcinoma of the head and neck
SIN	squamous intraepithelial neoplasia
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TGF-a	Transforming growth factor alpha
TKI	Tyrosine-kinase inhibitors
TM	Transmembrane region
TNFα	Tumor necrosis factor alpha
TNM	Tumor, Nodes, Metastases
UICC	Union Internationale Contre le Cancer
WHO	World Health Organization

12. List of figures

13. List of Tables

14. Acknowledgments

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