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Prognostic value of HMGA2, P16, and HPV in oral squamous cell carcinomas

Siegfried Loeschke [a, 1], Anne Katharina Ohlmann [b, *], Jan Hinrich Bräsen [c, 2], René Holst [d], Patrick H. Warnke [b, 3]

[a] Aalborg University Hospital, Hobrovej 18-22, 9100 Aalborg, Denmark
[b] Department of Oral and Maxillofacial Surgery, University Hospital of Schleswig-Holstein, Campus Kiel, Arnold-Heller-Str. 3, 24105 Kiel, Germany
[c] Department of Pathology, University Hospital Schleswig Holstein, Campus Kiel, Arnold-Heller-Str. 3, 24105 Kiel, Germany
[d] University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark

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ABSTRACT

Purpose: Molecular markers are only occasionally used in diagnostics of oral squamous cell carcinoma (OSCC), even though they could influence decision making in individually designed cancer therapies. We analyzed the predictive value of the markers HPV, p16, and HMGA2 and the TNM classification in regard to survival and recurrence rates.

Material and methods: A total of 91 OSCC cases were included in this study, with a follow up of up to 131 months. HPV-DNA was present in 7 carcinomas. p16 was detected by immunohistochemical staining in 14 samples. HMGA2 expression was determined by real-time quantitative polymerase chain reaction (qRT-PCR). Overexpression of HMGA2 was found to vary between 32-fold and 32,000-fold compared to nondysplastic tissue.

Results: Cox regression analysis showed that age, sex, smoking status, use of alcohol, human papillomavirus (HPV), and tumor size had no significant effect on overall and progression-free survival. HMGA2 and N-status showed significant effects on overall (HMGA2: $p = 0.049$; N1: $p = 0.019$; N2: $p = 0.02$) and disease-free survival (HMGA2: $p = 0.057$; N1: $p = 0.198$; N2: $p = 0.02$). P16 appeared to be borderline significant but the χ^2 indicated that p16 and N were correlated.

Conclusion: Our results suggest that HMGA2 expression may have the potential to allow a more precise prognosis on survival in patients with OSCC.

1. Introduction

In recent years, different markers for the prognosis of OSCC have been suggested (Miyazawa et al., 2004; D'Silva and Ward, 2007). However, to assess the prognosis for survival and recurrence for OSCC, the TNM classification is still the most important instrument. To investigate different pathways, we concentrated on HPV, p16, and HMGA2.

HPV has oncogenic potential due to its ability to insert certain genes (E6 and E7) into the genome of the host cell, which contribute to complex chromosomal changes (Duensing and Munger, 2002). These changes lead to malfunction of cell cycle regulation and DNA repair (Feller et al., 2010). It is thought that this HPV-induced subset of OSCC has a better response to radio- and chemotherapy (Pannone et al., 2011).

P16 is a protein that acts as a tumor suppressor (Rocco and Sidransky, 2001). It controls the cell cycle progression from the G1-to the S-phase (Schafer, 1998). Loss or mutation of the p16INK4a-gen, which is located on chromosome 9p21, has been detected in a large number of tumor cell lines (Rocco and Sidransky, 2001).

* Corresponding author.
E-mail addresses: siggil@bbsyd.dk (S. Loeschke), anne.k.ohlmann@gmail.com (A.K. Ohlmann), braesen.jan@mh-hannover.de (J.H. Bräsen), rholst@health.sdu.dk (R. Holst), prof.warnke@mkg-flensburg.de (P.H. Warnke).
[1] University of Southern Jutland, Sydvang 1, 6400 Sønderburg, Denmark.
[2] Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany.
[3] Oral and Maxillofacial Surgery Flensburg, Ballastkai 5, 24937 Flensburg, Germany.

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HMGA2 is a non-histone chromosomal protein (Goodwin et al., 1973). It changes chromatin structure by binding to AT-rich DNA, thus enhancing or suppressing a large number of transcriptional factors (Wolffe, 1994) that are necessary for proliferation and differentiation during embryogenesis (Zhou et al., 1995, 1996; Fusco and Fedele, 2007). HMGA2 is hardly detectable in adult tissue (Rogalla et al., 1996; Zhou et al., 1996) but is overexpressed in tumors such as thyroid carcinomas (Belge et al., 2008), uterine leiomyomas (Klemke et al., 2009), non-small cell lung cancer (Meyer et al., 2007), breast cancer (Rogalla et al., 1997), pancreatic cancer (Abe et al., 2003), colorectal cancer (Wang et al., 2011), ovarian cancer (Kim et al., 2015), tongue cancer (Zhao et al., 2016), and squamous carcinomas of the oral cavity (Miyazawa et al., 2004; Sterenczak et al., 2014). It was shown that HMGA2 works as a stem cell regulator in hematopoiesis (Ikeda et al., 2011).

Oral carcinomas are staged according to the Union International Contre le Cancer (UICC) criteria by their extension at the primary tumor site (T), involvement of regional lymph nodes (N), and distant metastatic spread (M). Survival and recurrence rates are worse if adjacent structures are infiltrated or lymph nodes involved (Kademani et al., 2005; Kovács et al., 2007; Pulte and Brenner, 2010; Vázquez-Mahía et al., 2012; Institut für Krebsepidemiologie e.V., 2014). Oral cancer in an early stage (UICC stage I and II) carries a 5-year survival rate of 68–84%. Affected lymph nodes (UICC stage III) decrease the survival rate to 61%. If distant metastatic spread is found (UICC stage IV), survival rates are especially low (31–40%) (Institut für Krebsepidemiologie e.V., 2014).

In recent years, survival and quality of life in survivors of extended carcinomas has been improved by advanced pre-surgical 3D imaging and free-flap reconstructive surgery. However, prognosis depends on access to adequate therapy and planning as well as modern treatment centers, as there are still countries even in the western world with sometimes poorer results, such as Australia (Crombie et al., 2012), where overall 5-year survival rates as low as 30% have been reported recently when surgery was not possible.

The aim of this study is to reassess the results of the Miyazawa et al. study (Miyazawa et al., 2004) in a larger group, and to compare the predictive value of HMGA2, p16, HPV, and TNM on survival and disease recurrence. To our knowledge, this is the first study of this kind in the literature.

2. Material and methods

A total of 91 patients (59 male and 32 female) with OSCC who underwent R0-surgery at the UKSH in Kiel, Germany during the years 2000–2004 were included in this study. All tissue samples were formalin fixed and paraffin embedded (FFPE). Histological staging and grading data were obtained from clinical and pathological diagnosis undertaken during patient treatment. Ethics approval was obtained from the Medical Faculty ethics board of Christian-Albrechts-University of Kiel (D 418/08). Patients had been regularly followed up until November 2011. The age of patients ranged from 36 to 82 years.

For HPV detection, DNA isolation was performed with the Roche MagNA Pure LC DNA Isolation Kit II according to the manufacturer's protocol. DNA concentration was quantified by spectrophotometry. Integrity of DNA was confirmed by β-actin polymerase chain reaction (PCR). A total of 85 patients were eligible for human papillomavirus (HPV) testing. HPV DNA was detected by PCR with short PCR fragment primers, which amplify a 65-bp fragment (Kleter et al., 1998). Positive samples were identified on a 2% agarose gel with ethidium bromide staining.

Immunohistochemical staining of p16 was performed on 4-µm-thick sections with the CINtec® Histology Kit (Ventana) and the *ultra*View Universal DAB Detection Kit (Ventana) on a Benchmark XT workstation (Ventana) according to the manufacturer's instructions. The sections were counterstained with hematoxylin. Brown nuclear and/or cytoplasmatic staining of the carcinoma was defined as positive reaction. Positive and negative controls were carried along.

For HMGA2 analysis, hematoxylin and eosin (H&E)-stained sections were assessed. For RNA isolation, three 5-µm sections of the FFPE sample blocks were cut. When containing normal and cancerous tissue, cancer cells were isolated from H&E-stained slides by laser microdissection on a Olympus LMD workstation. Isolation was performed with the Roche MagNA Pure LC RNA Isolation Kit III according to the protocol. RNA concentration was determined by spectrophotometry. The integrity of RNA was checked with the Agilant 2100 Bioanalyzer and showed a severe RNA degradation of our tumor samples in contrast to FFPE control material (data not shown). Reverse transcription of total RNA was performed with Roche Transcriptor First Strand cDNA Synthesis Kit using Random Hexamer Primer. The transcription was done according to the kit protocol. Real-time quantitative PCR (RT-PCR) was carried out on the Applied Biosystems 7900HT FAST Real-Time PCR System. A relative quantification approach with 18S RNA as endogenous control was chosen. Reactions were performed in triplicate using 25 ng total RNA for HMGA2 and 2.5 ng total RNA for 18S detection. HMGA2 and 18S specific TaqMan primers and probe were ordered from Applied Biosystems (HMGA2: No. Hs00171569_m1; 18S: No. Hs03003631_g1). Conditions for RT-PCR were set to the following parameters: 50 °C for 2 min, 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative amount of HMGA2 was calculated using the ΔCt method.

Univariate analysis was performed using the Kaplan–Meier method and the log-rank test. For a better statistical model that would account for the missing data, all data were then analyzed by Cox regression for both overall and disease-free survival. The initial model was reduced sequentially by removing insignificant variables one at a time until only significant variables were left. The assumption on proportional hazards were tested and we found no cause for concern. Multiple imputation (Rubin, 1987) was used to account for the missing observations. Analyses were performed using R.

Table 1
Clinicopathological parameters and incidence of HPV and p16 in OSCC.

	No. of cases	%
T stage		
T1	41	45.1
T2	27	29.7
T3	7	7.7
T4	16	17.6
N stage		
N0	50	54.9
N1	18	19.8
N2	23	25.3
HPV[a]		
Positive	7	8.2
Negative	78	91.8
p16		
Positive	14	15.4
Negative	77	84.6

[a] Data for six cases not available due to insufficient DNA integrity.