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Publikationshinweis

Publiziert am: 12. Februar 2013

Journal: Im European Journal of Cancer, Bd. 49, S. 1915-1922
High sex determining region Y-box 2 expression is a negative predictor of occult lymph node metastasis in early squamous cell carcinomas of the oral cavity

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Available online 12 February 2013

KEYWORDS
Head and neck squamous cell carcinoma (HNSCC)
Oral cavity
SOX2 (sex determining region Y-box 2)
Immunohistochemistry
Sentinel node biopsy

Abstract  Background: The transcription factor sex determining region Y (SRY)-box 2 (SOX2) (3q26.3–q27) has been recently identified as a recurrently activated major oncogene in squamous cell carcinoma of various sites. Its prognostic value in head and neck squamous cell carcinoma (HNSCC) is currently unclear.

Aim: To correlate SOX2 protein expression with the occurrence of occult lymph node metastasis and relapse free survival in early oral SCC.

Methods: SOX2 expression in 120 T1/T2 oral SCC patients was evaluated using a tissue microarray technique. Intensity of SOX2 expression was quantified by assessing the Intensity/Reactivity Scores (IRSs). These scores were correlated with the lymph node status of biopsied sentinel lymph nodes and recurrence. Log rank univariate and Cox regression multivariate analysis was used to determine statistical significance.

Results: Twenty-six of 120 primary tumours (21.7%) showed high SOX2 expression. High expression levels of SOX2 significantly correlated with negative lymph node status in univariate (p = 0.001) and multivariate analysis (p = 0.003). Sensitivity was found to be 95.6% with a negative predictive value of 92.3%. Specificity was 32% with a positive predictive value of 45.7%.
1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the fifth-most common solid tumour diagnosed in the world. The oral cavity is the most commonly affected subsite (274,000 cases) and ranks as the 12th most common cancer.1 Advances in surgical and radio-oncological management over the years have unfortunately not significantly improved oncological outcomes for advanced cases, though ever more selective approaches to the neck and though innovative flap/reconstruction techniques have lowered morbidity and improved function. Involvement of regional lymph nodes at diagnosis roughly halves 5-year survival.2

Several oncogenes have been identified in oral SCC. Sex determining region Y (SRY)-box 2 (SOX2), a transcription factor-coding gene located on 3q26.3–27,3 has recently been shown to be frequently amplified in SCCs of oesophagus and lung SCC.4,5 In oral squamous cell carcinoma, SOX2 has also been reported to be amplified and overexpressed.6 It encodes a transcription factor involved in the determination of cell fate and regulation of embryonic development. After creating a protein complex with other proteins it acts as a transcriptional activator that is essential to maintain self-renewal of undifferentiated embryonic stem cells.7–10 As forced Oct4 expression induces pluripotency in SOX2-null cells, a group of researchers have concluded that the primary role of SOX2 in induced pluripotent stem cells is controlling Oct4 expression, and that they perpetuate their own expression when expressed concurrently.11 Several recent publications implicate a substantial role of SOX2 as prognostic factor for squamous cell carcinoma of various sites. SOX2 amplification was identified by FISH in 20–23% of lung cancer; 11–15% of oesophageal cancer, 5–28% of cervix SCC and 28% of skin cancer.3,5,12 In lung cancer, SOX2 overexpression is associated with favourable prognosis, whereas the relevance of SOX2 expression for prognosis of HNSCC is controversial.13–15

Sentinel node biopsy (SNB) of the N0 neck in early oral SCC has been shown in several studies to reliably detect occult neck metastasis with negative predictive values >95%. To clarify a potential prognostic relevance for occult metastasis of SOX2 protein expression, we consequently analysed a very well defined patient cohort with early oral SCC (T1/T2-stage) undergoing sentinel lymph node biopsy.16,17

2. Patients and methods

Permission for performing the study was obtained from the local ethics committee.

Detailed information of patient recruitment and surgical procedure (including surgical resection of the primary tumour and SNB) can be found in former articles by our group.18,19 In brief, 133 patients of the ear nose and throat departments of the University Hospital of Zurich and the Kantonsspital St. Gallen, Switzerland, with early stage (T1/T2) SCC of the oral cavity and the tonsillar pillar without preoperative clinical and imaging evidence of regional lymph node metastasis were selected for this study. SCCs of the tonsillar pillar were included, because they behave similarly to oral cavity carcinoma rather than Human Papilloma Virus (HPV)-positive tonsillar carcinoma.20 Due to the established fact, that a subgroup of oral and oropharyngeal SCC is caused by HPV infection,21 p16 immunohistochemistry was used as a surrogate marker of HPV status.22

For SNB, the radioactive tracer was injected preoperatively into the region of the primary tumour. Sentinel lymph nodes (SLN) were intraoperatively located by use of a gammaprobe, excised and subjected to immediate frozen section analysis. If occult metastases were identified, a completion modified radical neck dissection was performed in the same sitting.17 Patients whose definitive histology showed a positive SNB underwent interval neck dissection.

Formalin-fixed, paraffin-embedded primary tumour tissues of the 133 patients were histopathologically re-evaluated. 13 patients were excluded from the analysis because of loss of follow-up or inability to appropriately evaluate the paraffin block. The mean clinical follow-up of the remaining 120 patients (111 from Zurich, nine from St. Gallen) was 81 months (range 11–122). Fig. 1 summarises the patients enrolled in this study (consort diagram). Mean patient age was 59 years (range 28–88). The tumour locations were in the oral cavity proper (110/120 (91.7%); mostly tongue) and the palatine arch (10/120 (8.3%)). Male:female ratio was 82:38 (2.16:1). 64 patients were staged pT1 and 56 pT2. Both sides of the oral cavity were equally involved. 11% of tumours were found in the midline. Table 2 gives a synopsis over the clinical parameters.

For the construction of a tissue microarray (TMA) a morphologically representative region of the “donor” - paraffin blocks with primary tumour tissue was selected. From this representative section two core biopsies...
(diameter, 0.6 mm; height, 3–4 mm) from the invading front were taken and precisely arrayed into a new “recipient” paraffin block using a custom-built instrument. After the block production was finished, 4.0-μm sections of the resulting tumour TMA block were cut for further analysis as recently described.

SOX2 immunohistochemistry was performed using the rabbit monoclonal SOX2 antibody (1:100, Clone EPR3131, Epitomics, Inc.) and a monoclonal mouse anti-p16 antibody (clone JC8, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) on a Ventana Benchmark automated staining system (Ventana Medical Systems Inc., Tucson, AZ, USA) as recently described. For SOX2, extranuclear staining was regarded to be negative or unspecific. Immunohistochemistry was evaluated in two cores per tumour. The average percentage was taken for statistical analysis. SOX2 staining intensity was measured by the Intensity/Reactivity Score (IRS), which is frequently applied for a detailed expression analysis of transcription factors. Staining intensity (SI) was assessed to be negative (=0), weak (=1), moderate (=2) or strong staining (=3). Reactivity (R) was determined by the percentage of positive tumour cells (PP) and scored as follows: negative (=0), 1–10% positive cells (=1), 11–30% (=2), 31–50% (=3), 51–80% (=4) and >80% positive cells (=5). IRS was calculated by multiplying PP with SI (minimum 0/maximum 15). High expression was defined as ≥8 according to the median IRS of positive stained cells.

According to recent literature, a strong and diffuse, nuclear and cytoplasmic staining reaction of p16 in a majority of tumour cells was considered as indicative for an HPV-associated carcinoma.

2.1. Statistics

The relationship between presence of lymph node metastasis and SOX2 expression was evaluated using Fisher’s exact test. The predictive power of SOX2 expression for lymph node metastasis was evaluated with a logistic regression controlling for grade of differentiation and tumour stage. Tumour differentiation was not included as a factor in the logistic regression model for the reason of “quasi” complete separation in the data. In order to evaluate the potential of SOX2 as a predictive marker for occult metastasis and eventually for relapse time, a logistic regression and a Kaplan–Meier survival analysis was performed. The possible value of SOX2 expression as a predictive marker for occult metastasis was additionally specified with calculation of sensitivity, specificity, positive and negative predictive values. How sensitivity and specificity were calculated is illustrated in Table 1. Positive and negative predictive values were calculated accordingly.

A Kaplan–Meier curve with calculation of log rank statistics was performed to compare survival and relapse rates, respectively, between patients with high and low expression. PASW Statistics 18.0.0 for windows (SPSS) was used for statistical analyses.

3. Results

SOX2 protein expression was virtually exclusively identified in the nucleus of the tumour cells (Fig. 2). Twenty-six of 120 (21.7%) of the primary tumours showed a high SOX2 expression and 23/120 (19.2%) were negative. For statistical analysis, tumours were categorised according to the median value of stained

Fig. 1. Consort diagram of the patients included in the study. ITC = Isolated tumour cells/IRS = Intensity/Reactivity Score.
positive cells into 3 categories: negative, low expressors (IRS = 1–7) and high expressors (IRS = 8–15). These groups were then compared to clinical course. There was no association between SOX2 protein expression and tumour differentiation grade or with tumour stage (Table 2). High SOX2 expression correlated with negative lymph node status ($p = 0.001$) (Table 2, Fig. 3), regardless of p16 state. After controlling for tumour stage, SOX2 expression remained a significant predictor for lymph node status (odds ratio (OR) = 0.102, 95% confidence interval (CI): 0.023–0.457, $p = 0.003$; Table 3).

SOX2 expression showed a sensitivity of 95.6% (95% CI: 84.9–99.5%) and a specificity of 32.0% (95% CI: 21.7–43.8%) with regard to presence or absence of lymph node metastasis. The positive predictive value was 45.7% and the negative predictive value was 92.3%.

SOX2 expression was also evaluated for a possible effect on relapse rates. However, the log rank test did not indicate any influence of SOX2 expression on relapse rates. Gender, tumour location, side and T-stage were not found to relate to occult metastasis.

3.1. p16 immunohistochemistry

Strong and diffuse, nuclear and cytoplasmic immunohistochemical p16 expression was detected in eleven of 120 tumours (9.2%). Ten of 110 tumours of the oral cavity (9.1%) and one of ten oropharyngeal SCC (10%) were p16-positive, suggesting an HPV-associated carcinogenesis. p16 could therefore be excluded as a relevant confounder.

4. Discussion

In our study, we demonstrate that approximately 80% of early stage SCC of the oral cavity expresses SOX2. High expression of SOX2 in these tumours is associated with a significantly lower incidence of occult metastasis in neck lymph nodes.
The high SOX2 expression in early SCC of the oral cavity is consistent with the hypothesis that SOX2 is the target of chromosomal amplifications observed in the 3q26-qter region by previous comparative genomic hybridisation (CGH) studies. Copy number increases of the 3q26-qter region have been identified in different SCCs, including lung, oesophagus and cervix. The presence of frequent copy number gains/amplifications suggested the presence of a relevant proto-oncogene in this region.

SOX2 was first reported to be amplified in SCCs of oesophagus and lung, but also in SCC of the cervix uteri, the skin and the penis. Freier et al. identified SOX2 as potential target of the 3q gains/amplifications in HNSCC by array-CGH and demonstrated high SOX2 protein expression in about 18.1% (49/271) of oral SCC, using a polyclonal rabbit anti-SOX2 antibody (Chemicon, Millipore, MA). We observed a similar frequency of high SOX2 expression (21%) with a different monoclonal antibody (Epitomics).

Our result is consistent with a very high expression level of nuclear SOX2 protein in the majority of primary SCC, but high SOX2 expression has also been found in breast cancer, testicular germ cell tumours, as well as gastric and pancreatic adenocarcinoma. The mechanisms for SOX2 protein overexpression are unclear. SOX2 protein expression has also been reported in tumours without gene amplifications. Interestingly, SOX2 amplifications are obviously specific for SCC.

Fig. 2. SOX2 immunohistochemistry of primary tumours: (a) absent expression, (b) low nuclear expression, (c) high nuclear expression.

Fig. 3. Sentinel status according SOX2 expression levels: While in none- and low expressors (Intensity/Reactivity Score (IRS) 0–7) sentinel node negative patients outweigh sentinel node positive patients, in high expressors (8–15) most patients were found to be negative for occult metastatic disease.
Such controversial results: high SOX2 score. There are different explanations for chemical results, but a different antibody (Novus Biologicals). In their cohort of oral tongue SCC, 17.1% had a similar interpretation method of the immunohistochemical results (95% CIs) and $p$-value of SOX2 expression and tumour stage as predictors for lymph node status. Because of quasi-complete separation in the data, differentiation grade was not included as a factor in the logistic regression model.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>OR</th>
<th>95% CI</th>
<th>$p$-Value</th>
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<tbody>
<tr>
<td>SOX2 expression</td>
<td></td>
<td></td>
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<tr>
<td>High versus low</td>
<td>0.102</td>
<td>0.023–0.457</td>
<td>0.003</td>
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<tr>
<td>Tumour stage</td>
<td></td>
<td></td>
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<tr>
<td>pT2 versus pT1</td>
<td>1.665</td>
<td>0.758–3.659</td>
<td>0.204</td>
</tr>
</tbody>
</table>

Pathogenesis, because adenocarcinomas of the lung or cervix have no SOX2 amplifications, but high SOX2 protein expression. In our well characterised cohort of early SCC of the oral cavity, we were able to study the process of early lymphatic metastasis and demonstrate a significant association between high cancer cell-expressed SOX2 protein and significant lower risk of lymph node metastasis. This result is consistent with findings in lung SCC, reporting high SOX2 protein expression levels and SOX2 amplification to be correlated with better overall survival. The relationship between SOX2 expression and tumour behaviour is highly controversial. It is intriguing that there are some studies reporting increased aggressive behaviour of tumour cells with SOX2 expression. Du et al. showed a significant association of high SOX2 expression in nodal negative oral tongue carcinoma with poor overall and disease-free survival using the same interpretation method of the immunohistochemical results, but a different antibody (Novus Biologicals). In their cohort of oral tongue SCC, 17.1% had a high SOX2 score. There are different explanations for such controversial results:

(i) Different results may be explained by the design of retrospective studies. The problem of many studies investigating predictive markers is the heterogeneity of primary tumours (location, T-stage and N-stage), which are then analysed together. Since patients qualifying for a sentinel node biopsy yield a highly selected cohort (regarding tumour location and N-stage) without any clinical evidence of lymph node disease, we had the possibility to detect cancers with occult metastatic disease at the earliest time point.

(ii) Discrepancies could also be explained by the selection of tumours with different pT stages. Our study included only T1/T2 carcinoma, whereas Du et al. also included T3 SCC (30% of the cases). The size of their primary tumours correlated significantly with SOX2 expression. The multivariate analysis by Du et al. showed that SOX2 as well as T-stage were independent prognostic factors for unfavourable overall, cancer-specific and disease-free survival. Therefore, it remains unclear, which factor (T-stage, lymph node metastasis or SOX2 expression) was most important for patients’ outcome.

(iii) A biological reason for a different prognostic role of SOX2 in different tumour types could be the involvement of additional oncogenes. In a meta-phase comparative genomic hybridisation (CGH) analysis, overrepresentation of the chromosomal region 3q21-q29 was associated with decreased overall and disease-free survival suggesting that 3q gains are involved in HNSCC progression. Several candidate proto-oncogenes are located within this chromosomal arm, including PIK3CA and CCNL1. Amplification and protein expression of PIK3CA and CCNL1 were reported in primary HNSCC and implicated in the progression of HNSCC. CCNL1 amplification was associated with the presence of lymph node metastasis, but the prevalence of this amplification was relatively low.

(iv) In a recent review by Hussenet et al., a pleiotropic oncogenic role of SOX2 in lung SCC was discussed: SOX2 overexpression seems to increase the precursor basal cell and may be a triggering event in the lung SCC pathogenesis. Furthermore it seems, that SOX2 overexpression in cancer stem cells (CSC) of lung SCC did not mediate self-renewal like in CSC of glioblastoma, but leads to more differentiated cancer cells. Finally the binding of SOX2 to different co-factors and the activation of different downstream targets can result in a very different aggressiveness of the tumour. It is tempting to speculate that SOX2 has a similar influence on tumour progression of HNSCC.

According to our data, SOX2 is a potential predictive marker for a negative neck SLN in early SCC of the oral cavity. Sentinel node biopsy has been successfully validated for early HNSCC. However, use of SNB alone or SNB assisted elective neck dissection is still controversially discussed for staging and treatment of the cN0 neck in patients with early HNSCC. In addition, SNB is not abundantly available and to date mostly reserved for tertiary referral centres. The hitherto published predictive factors for metastatic disease in SNB include histomorphological parameters of the primary tumour including mode of invasion (morphological appearance of the infiltrating tumour front), grade of differentiation, lymphatic invasion and intratumoural lymphatic density. In our recent study, all these parameters failed to predict metastasis in SNB of oral SCC. Therefore, additional biomarkers may be of help for better patient stratification to select for SNB or elective neck dissection. In a previous study, we demonstrated that expression of E-Cadherin (ECAD) significantly correlated with occult metastatic disease. In the future, a combination of molecular
markers like SOX2, ECAD, podoplanin, p16, bmi-1, lysyl oxidase (LOX) and histomorphologic parameters may be used to stratify patients for the appropriate surgical lymph node procedure (SNB alone versus SNB assisted elective neck dissection) according to their risk for occult nodal disease. This would allow individual risk stratification with implications then for treatment strategy.

In summary, we provide evidence that up-regulation of cancer cell-expressed SOX2 correlates with lower incidence of lymph node metastasis in early SCC of the oral cavity. While SOX2 might not be applicable as a predictive marker as a single test, SOX2 immunohistochemistry in combination with other molecular markers might contribute to a more patient tailored treatment.

Conflict of interest statement

None declared.

Acknowledgements

We like to thank Mrs. Martina Storz for the TMA production and technical support, Dr. Thomas Périz for the manuscript revision and our Grant sponsor: Olga Mayenfisch Stiftung, Toblerstrasse 83, 8044 Zürich.

References


Verdankungen

Gebührender Dank für die herausragende Betreuung an meinen Doktorvater PD Dr. med. Gerhard F. Huber, der diese Dissertation mit viel Fachwissen und Engagement geleitet und mir einen spannenden, interessanten und lehrreichen Einblick in die Welt der Forschung und Kongresse ermöglicht hat.

Martina Storz und ihrem Team danke ich herzlichst für die kompetente Unterstützung bei der Arbeit im TMA-Labor.

Ebenfalls meinen Dank aussprechen möchte ich meiner Familie und meinen Freunden für das stets vorhandene Interesse an meiner Dissertation sowie für die persönliche Unterstützung im Laufe der Arbeit.
Erläuterung zur Arbeit


Jeder einzelne Core wurde von der Dissertantin und dem Dissertationsbetreuer mit dem virtuellen Mikroskop des Universitätsspitals Zürich analysiert und nach dem Immunreactivity Score (IRS) bewertet. Der IRS ist eine Skala zur Quantifizierung der immunhistochemischen Anfärbung, die durch das Multiplizieren der Anteile der angefarbten Zelle mit der Färbeintensität entsteht. Da pro Patient zwei Cores gefärbt wurden, wurde durch die Dissertantin jeweils der Durchschnitt der IRSs berechnet und für die weiteren statistischen Analysen verwendet.
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