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CD44, SHH and SOX2 as novel biomarkers in esophageal cancer patients treated with neoadjuvant chemoradiotherapy.

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ABSTRACT

Background and purpose: Neoadjuvant chemoradiotherapy (nCRT) improves survival in esophageal cancer (EC) patients, but the response to treatment is heterogeneous and little is known regarding prognostic and predictive markers in these patients. CD44, SOX2 and SHH have been implicated in resistance to CRT, possibly through an association with a cancer stem cell phenotype.

Material and Methods: 101 EC patients treated with nCRT and surgery were included. Sufficient pre-treatment biopsy material was present in 71 patients, of which 53 patients were non-complete responders on nCRT (nCR). Protein expression was examined using immunohistochemistry (IHC). Prognostic factors were determined using Cox regression analysis for disease free survival (DFS) and cause specific survival (CSS) in the complete cohort, the pre-treatment biopsies group and post-treatment nCR group.

Results: Low CD44 expression in the nCR group was an independent prognostic factor for both DFS and CSS (DFS HR 2.81, $p=0.002$ and CSS HR 3.48, $p=0.002$). Absent SOX2 expression in pretreatment biopsies was related to systemic recurrence ($p=0.029$) while low SHH in pretreatment biopsies was an independent prognostic factor for a poor DFS (HR 2.27, $p=0.036$). No relation between marker expression and response to nCRT was observed.

Conclusions: Low expression of CD44 and SHH are associated with a poor survival outcome in EC patients treated with nCRT.

Introduction

Esophageal cancer (EC) is a common cancer with a poor prognosis. Two major histological EC subtypes are recognized: Esophageal Adenocarcinoma (EAC) and Esophageal Squamous Cell Carcinoma (ESCC). The treatment for both subtypes is similar, and historically consisted of a radical esophagectomy. In the past decade neoadjuvant chemoradiotherapy (nCRT) has emerged as a valuable addition to surgery, increasing median survival from 24.0 to 49.4 months [1]. However, the response to nCRT is heterogeneous. Whereas approximately 25% of EAC patients will achieve a pathological complete response (pCR) after nCRT, others respond partially or not at all [1]. Currently, the differences underlying the response to therapy are poorly understood, and predictive and prognostic biomarkers are lacking in EC patients treated with CRT.

The glycoprotein CD44, the Sex determining region Y-box 2 (SOX2) transcription factor and the hedgehog (HH) signalling pathway ligand Sonic Hedgehog (SHH) have been associated with resistance to chemo- and radiotherapy. CD44 and SOX2 are potential markers of Cancer Stem Cells (CSCs), a subpopulation of tumor cells with stem cell characteristics such as the ability to self-renew and generate multilineage offspring. CD44 and SOX2 expression has associated with CSCs in colon cancer and glioblastoma [2,3]. CSCs have been linked to chemo- and radiotherapy resistance [reviewed in 4], and CD44 expression has been associated with radiation-resistance in colon, head and neck and esophageal cancers [5-7] while SOX2 expression has been associated with both a good response to radiotherapy [8,9] as well as with radiotherapy resistance [10]. Previous work from our and other groups revealed that low CD44 and SOX2 expression are related with poor prognosis in EC patients treated with surgery alone [11,12], but the prognostic and predictive value in EC patients treated with nCRT is unknown.

The hedgehog signalling pathway is a known regulator of the CSC in various solid malignancies (reviewed in [13]). Increased expression of SHH has been implicated in the

development of EC and EC radiotherapy resistance *in vitro* [14-16], but the prognostic and predictive value of SHH expression in tissue of EC patients undergoing nCRT is unknown.

The aim of this study was to determine the prognostic value and the predictive value of response to nCRT for the expression of CD44, SHH and SOX2 in pre-treatment biopsies and post-nCRT resection material of EC patients treated with combined nCRT and surgery.

Patients and methods

Patient material

This retrospective study consisted of 101 patients with EC who were treated with nCRT followed by an esophagectomy with curative intent at the University Medical Center Groningen, Groningen, the Netherlands between 2006 and 2013. Patients who died within 90 days after surgery or those treated with surgery alone were not included. Follow-up was recorded until March 2013, with a minimum follow-up of one year. The median follow-up was 22.2 (inter-quartile range 12.8-35.5) months. All data were prospectively collected from the patients' medical records.

pCR was defined as the absence of microresidual disease (mRD) in the resection specimen. mRD was defined as the presence of vital tumor cells, either at the primary tumor site (ypT) and/or in the lymph nodes (ypN) at postoperative pathological evaluation. Pathological response to therapy was determined on a 1-5 scale according to the Mandard scoring system [17]. A positive circumferential margin (CRM) was defined by the presence of tumor cells within 1mm ($\leq 1\text{mm}$) from the resection margin. Figure 1 gives a schematic representation of the patient material included. Thirty patients were excluded because insufficient biopsy material was present to be used for research purposes as determined by the Dutch Code for proper use of Human Tissue (www.federa.org). Two groups were analyzed for marker expression in relation to survival; the pre-treatment biopsy group (n=71) consisting of biopsy material and the post-treatment non-complete response (nCR) group consisting of resection material at the primary tumor site. The latter group consisted of 53 of the 71 patients

that had a non-complete response to nCRT. The study was conducted according to the guidelines of the Ethical Board of our institute (www.ccmo.nl). Archival tissue of all patients was handled according to the Dutch Code for proper use of Human Tissue (www.federa.org).

Neoadjuvant chemoradiotherapy

Neoadjuvant chemoradiotherapy was given according to the CROSS-scheme [1]. Carboplatin (AUC 2) and paclitaxel (50mg/m²) were given weekly during radiotherapy for five weeks. Radiotherapy was given in 23 fractions of 1.8Gy with a total of 41.4Gy. Three patients whose tumor was located at the gastro-esophageal junction received 45Gy. The median interval between neoadjuvant chemoradiotherapy and surgery was six weeks.

Commented [J.1]: Klopt dit?

Surgical procedure

Patients underwent surgery by an experienced surgical team at the University Medical Center Groningen, Groningen, the Netherlands. Resection was performed via a transthoracic route and a laparotomy with a two-field nodal dissection in the mediastinum and abdomen respectively, as previously described [18].

Immunohistochemistry (IHC)

Paraffin-embedded tumor specimens were retrieved from pathological archives of hospitals in the northeast of the Netherlands. Antigen retrieval was performed using citrate pH 6.0 (CD44, SOX-2, SHH) or EDTA buffer pH 9.0 (cytokeratin), followed by quenching of endogenous peroxidase. The sections were incubated with primary antibody for 1 hour at room temperature. Primary antibodies used were directed against: Cytokeratin AE1/AE3 (1:400, Dako, Heverlee, Belgium), CD44 (clone IM7 recognizing all isoforms of CD44, 1:100, Biolegend, London, UK), SOX-2 (clone L1D6A2, 1:400; Cell Signaling, Leiden, the Netherlands) and SHH (ab53281, 1:750, Abcam, Cambridge, UK). Subsequently, tissue sections were incubated for 30 minutes with HRP-conjugated secondary (rabbit anti rat, rabbit

anti mouse, goat anti rabbit) and tertiary antibodies (goat anti rabbit, rabbit anti goat, all 1:50 dilution, all from Dako). Staining was visualized using 3,3'-diaminobenzidine and haematoxylin counterstaining. Positive and negative controls (immunoglobulin class-matched control sera) were included for each staining.

Analysis of immunohistochemistry

Slides were digitized using the Digital Slide Scanner NanoZoomer and NDP software (Hamamatsu, Shizuoka, Japan). HE and cytokeratin staining was used to identify vital tumor areas. Scoring was performed by two independent observers (JH and KP) blinded for patient outcome and random samples were validated by a blinded expert gastrointestinal pathologist (AK). Protein expression of CD44 and SHH was determined by scoring localization, intensity and percentage of positive cells. CD44 and SHH expression was initially scored according to the Immunoreactivity Score (IRS); the percentage of positive cells was scored into six categories: 0) no staining, 1) ≥ 1 -<5%, 2) ≥ 5 -<25%, 3) ≥ 25 -<50%, 4) ≥ 50 -<75% and 5) ≥ 75 -100% positive cells. Intensity was scored as 0) negative, 1) weak, 2) medium and 3) strong. The IRS was calculated by multiplying the percentage of positive cells with the intensity score, resulting in a score from 0 to 15. For evaluation of the CD44 staining the IRS was divided into two groups: negative or low staining (IRS 0-5), and high staining (IRS 6-15). Since almost all specimens showed SHH expression in 75-100% of the tumor cells, only intensity for SHH was determined as the consensus of 2 out of 3 observers (JH, KP, AK) and dichotomized into low (intensity 0-1) and high (intensity 2-3). SOX2 staining was determined by either nuclear or cytoplasmic staining and groups were divided in either no staining or positive staining as described previously [12].

Statistics

Cause-specific survival (CSS) was defined as the time between end of treatment i.e. surgery and the documented day of death or last follow-up, with death due to recurrence of the primary tumor.

Disease-free survival (DFS) was defined as the time between date of surgery and date of recurrence, death, or last follow up alive without a recurrence. Differences in patient characteristics with regard to marker expression and co-expression of markers were calculated using the Fisher's exact test. Differences in expression of markers between samples of one patient (pre-treatment biopsy, resection specimen, lymph node, recurrence) were calculated using a McNemar test. Survival curves were calculated with the Kaplan-Meier method using the log-rank test. Univariate Cox-regression analyses were performed to identify prognostic factors for CSS and DFS. Factors with multiple categories were made dichotomous by creating two subgroups. Clinical and pathological tumor stage (cT-stage and pT-stage) were subdivided into: low (cT1-2/pT0-T2) and high (cT3-4/pT3). Clinical and pathological nodal stage (cN-stage and pN-stage) were divided into negative (cN0/pN0) and positive nodal involvement (cN \geq 1/pN \geq 1). Factors with a *P*-value of *P*<0.200 for either the CSS or DFS in the univariate analysis were included in a stepwise (backwards conditional method) multivariate Cox-regression. A *P*-value of *P* \leq 0.05 was considered significant in a multivariate analysis. Two groups were analyzed in the univariate and multivariate analysis: 1) patients with pre-treatment biopsy material (n=71), 2) patients with residual tumor after nCRT in the resection material (n=53). Statistical analysis was performed by using the International Business Machines Statistical Package for Social Sciences (IBM SPSS, Armonk, New York, USA) version 22.0.

Results

In Table 1 patient and tumor characteristics are shown for patients with pre-treatment biopsies available for IHC (n=71) and the post-treatment nCR group (n=53). The majority of tumors were EAC and 20 patients had a pCR.

Supplementary figure 1 shows representative pictures of the immunohistochemical stainings. The CD44 staining failed in 1 resection specimen and the SHH staining in 1 biopsy specimen; these slides were excluded from further analysis. Supplementary Table 1 shows patient and tumor

characteristics in relation to the expression of CD44, SOX2 and SHH. Absent SOX2 expression in the pre-treatment biopsies group was related to systemic recurrence ($P=0.029$).

In the group of patients in whom pre-treatment biopsies were available positive pN-stage (HR 2.40 95% Confidence interval (CI) 1.21-4.74 $P=0.012$) and positive CRM (HR 2.17 CI 1.02-4.61 $P=0.044$) were independent prognostic factors for a poor DFS in both univariate and multivariate analysis (Table 2a). CD44 expression was not correlated with DFS or CSS in the pre-treatment biopsy group. In a univariate analysis, absent SOX2 expression in pre-treatment biopsies showed a trend for a worse DFS (HR 1.99 1.04-4.89 $P=0.039$, Table 2a and Figure 2c) and CSS (HR 2.16 1.08- 4.34 $P= 0.030$, Table 2a and Supplementary figure 2c) but did not retain significance in a multivariate analysis.

Analyzing only nuclear (instead of both cytoplasmic and nuclear) SOX2 expression did not alter the findings of our study (data not shown). Of note, low SHH expression was not significantly associated with a poor DFS or CSS in a univariate analysis ($P=0.117$ for DFS and $P=0.054$ for CSS, Table 2a and Figure 2e,f and Supplementary figure 2e,f), but low SHH expression was an independent prognostic factor for a poor DFS in the multivariate analysis (HR 2.27 CI 1.05-4.89 $P=0.036$, Table 2a).

In the post-treatment nCR group positive pN-stage (HR 3.75 CI 1.67-8.42 $P=0.001$ for DFS and HR 2.96 CI 1.27-6.89 $P=0.012$ for CSS) and low CD44 expression (HR 3.25 CI 1.46-7.24 $P=0.004$ for DFS and HR 4.08 CI 1.69-9.86 $P=0.002$ for CSS) were independent prognostic factors for poor survival (Table 2b, Figure 2b and Supplementary figure 2b) and remained independent prognostic factors for DFS and CSS when a subgroup analysis was performed on only EAC patients (data not shown). SOX2 expression in the post-treatment nCR group was not correlated with DFS or CSS in either uni-or multivariate analysis (Table 2b, Figure 2c and Supplementary figure 2c). In contrast to the pre-treatment biopsy group, SHH expression was not correlated with DFS or CSS in the post-treatment nCR group (Table 2b, Figure 2f and Supplementary figure 2f).

No predictive value of the tested markers was found for either pCR or response to nCRT therapy as determined by the Mandard score (Table 3).

A positive, non-significant correlation between low CD44 expression and absence of SOX2 expression was observed in both the pre-treatment biopsies group and the post-treatment nCR group (Supplementary Table 2, $P=0.052$ and $P=0.087$ respectively).

To study the changes in expression of CD44, SOX2 and SHH during treatment, we used a subset of patients within the post-treatment nCR group ($n=53$) for whom samples of lymph nodes ($n=23$) and material of recurrent disease ($n=12$) were available. The proportion of patients with absent SOX2 expression increased in the sequence of biopsy, resection specimen, lymph node and recurrence (25%, 47%, 57%, and 70% respectively). This increase was significant between biopsies and resection specimens (McNemar test, $P=0.029$). No difference in the expression of CD44 or SHH was found between the matched patient samples.

Discussion

To our best knowledge this is the first study to determine the expression of CD44, SOX2 and SHH in both pre- and post-treatment samples of EC patients treated with nCRT. Low expression of CD44, SOX2 and SHH was associated with either recurrence or a poor survival in EC patients. The results of our study suggest that CD44 and SHH may be of use to stratify EC patients treated with nCRT and the use of these markers may help identifying patients eligible for adjuvant therapy.

CD44 and SOX2 have been associated with CSCs and resistance to therapy in other solid tumors [2,3]. Nevertheless, we did not find a relation with response to nCRT in this study. In line with our previous study, in which a cohort of surgically treated EAC patients was examined [12], low CD44 expression was also an independent prognostic factor for poor survival in the post-treatment nCR group, while in the pre-treatment biopsy group low CD44 expression showed a trend for poor survival that did not reach statistical significance. Low CD44 expression has previously been related

to a worse survival in EAC patients and a decrease of CD44 expression was observed in EAC development [11,19]. However, another study found that a high percentage of CD44-positive tumor cells was associated with poor survival in EAC patients treated with nCRT and surgery, but this study used only resection material without pre-treatment biopsies and had a smaller cohort of patients (n=39) [20]. Our finding that loss of CD44 is an adverse prognostic factor in EC is unexpected given the previously reported association between CD44 with CSCs and radiotherapy resistance. A possible explanation could be that the CSC compartment might be heterogeneous with CD44-positive CSCs being primarily important in EAC tumor initiation, but less so in established EAC. CD44 is a downstream target of the Wnt signalling pathway, which is known to regulate CSCs in colon cancer [21]. Activation of the Wnt pathway occurs during the malignant transformation of BE towards dysplasia, with no further increase of Wnt activity in EAC [22]. The subsequent emergence of more aggressive, CD44-negative clones in established EC could possibly explain the negative correlation between CD44 expression and survival observed in the current study. Peitzsch et al. suggested that CSC markers might be flexible targets due to the heterogeneity between different clones derived from different CSC-lineages, which could explain why in some studies targets are positively and in other negatively associated with CSC characteristics [23]. Alternatively, CD44 may be a marker for CSC *in vitro*, but not *in vivo*. This possibility is supported by a recent study in non-small cell lung cancer that found that CD44 expression was associated with stem cell characteristics *in vitro*, while loss of CD44 expression in patient biopsies was associated with a poor survival [24].

We found that absence of SOX2 was correlated with systemic recurrence and that SOX2 expression decreased in patient-matched samples during disease progression. Together with the association between absent SOX2 staining and poor survival outcome (though not significant in multivariate analysis) our findings suggest that SOX2 has a tumor suppressor role in EC. This finding fits well with our previous observation that absence of SOX2 in EAC patients treated with surgery alone is related to poor survival in EAC patients [12]. A tumor-suppressor role of SOX2 is further supported by *in vitro* studies in gastric and colon cancer cells showing that overexpression of SOX2

has a tumor suppressor effect by reducing cell proliferation [25,26] and by a recent study that reported loss of SOX2 expression during progression of BE towards EAC and found this to be an independent predictive factor for malignant progression [27]. Taken together, low SOX2 expression might contribute to the development of EAC and a poor prognosis of EAC patients.

HH signalling increases significantly in both ESCC and EAC development [14,15]. High SHH expression has been described in post-treatment resection specimens of EAC treated with nCRT [16]. However, thus far the prognostic and predictive value of SHH expression in EC has not been examined. Our finding that low SHH expression is a prognostic factor for a decreased DFS is in contrast with previous *in vitro* studies reporting that HH antagonists reduced cell growth and induced apoptosis in EAC and ESCC cell lines [14]. The mechanisms underlying the discrepancy between our *in vivo* and previous *in vitro* findings are currently unknown. Differences between *in vitro* and *in vivo* tissue microenvironment could possibly explain these contradictory findings, but further research is required to establish the role of SHH in EC biology.

Radiation therapy has become a cornerstone in EC treatment, but it is not effective in all patients and associated with potentially severe side effects. The identification of prognostic and predictive biomarkers could contribute to a personalized treatment that reflects the biological heterogeneity of EC cases. Such novel biomarkers could be further combined with novel imaging strategies such as diffusion-weighted MRI, which in a recent pilot was useful in predicting response to neoadjuvant chemoradiation [28]. However, at the moment too little is known about the role of these markers in the pathogenesis of esophageal cancer to extrapolate these findings to the clinic. As previously discussed by Butof et al., proteins associated with a CSC phenotype are attractive targets for use as biomarkers [29]. To our best knowledge, this study is the first to examine the relation between potential CSC-markers and therapy response in EC patients treated with radiotherapy. While the potential of CD44, SOX2 and SHH to identify CSC in EC awaits further *in vitro* validation, our results suggest that the expression of these biomarkers does affect prognosis in EC

patients. While EAC and SCC have a different biology, both tumor types are currently treated using the same neoadjuvant chemoradiotherapy regimen, and therefore we included both histological subtypes in this study.

In conclusion, this is the first study to report that low CD44 and SHH expression are independent prognostic factors for poor survival in EC patients treated with nCRT, and that loss of SOX2 is related to disease recurrence. While the elucidation of the function of CD44, SOX2 and SHH in EC requires further study, our results suggest that these markers have a potential to identify EC patients with a poor prognosis.

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Figure legends

Figure 1: Schematic representation of patient material included.

Patient material used in this study consisted of: 1) diagnostic endoscopic biopsies obtained before therapy; 71 of the 101 samples had sufficient material to be used for research purposes, 2) resection material of the patients with microscopic residual disease ($N=53$) and 3) positive lymph nodes of patients ($N=23$) with microscopic residual disease and 4) material of recurrent disease ($N=12$). The pre-treatment biopsy group ($N=71$) and post-treatment non-complete responder (nCR) group ($N=53$) are highlighted in grey.

One patient with a Mandard 2 score had no vital tumor cells in the resection specimens.

Figure 2: Disease Free Survival (DFS) curves based on expression of CD44, SOX2 and SHH in the pre-treatment biopsy group and post-treatment non-complete responder (nCR) group. *The CD44 failed in 1 resection specimen and the SHH staining in 1 biopsy specimen; these were excluded from analysis

Supplementary figure 1: Representative images of immunohistochemical stainings

Supplementary figure 2: Cause Specific Survival (CSS) curves based on expression of CD44, SOX2 and SHH in the pre-treatment biopsy group and post-treatment non-complete responder (nCR) group.

*The CD44 failed in 1 resection specimen and the SHH staining in 1 biopsy specimen; these were excluded from analysis

Supplementary Table 1: Patient and tumor characteristics in relation to marker expression

Supplementary Table 2: Marker co-expression