Enrichment of Cells with Cancer Stem Cell-Like Markers in Relapses of Chemoresistant Patients with Locally Advanced Head and Neck Squamous Cell Carcinoma

Juan J. Grau¹ Ricard Mesía³ Maria de la Iglesia-Vicente⁴
Estrelania S. Williams⁴ Miren Taberna³ Miguel Caballero² Ana-Belen Larque¹
Jorge de la Oliva¹ Carlos Córdon-Cardo⁴ Josep Domingo-Domenech⁴

Departments of ¹Medical Oncology and ²Otorhinolaringology, University of Barcelona, IDIBAPS, Hospital Clinic Barcelona, and ³Institut Català d’Oncologia, IDIBELL, l’Hospitalet de Llobregat, Barcelona, Spain; ⁴Department of Pathology, Mount Sinai Medical Center, New York, N.Y., USA

Key Words
Chemoresistance · Head and neck cancer · Stem-like cells

Abstract
Background: Patients with head and neck squamous cell carcinoma (HNSCC) present different responses to chemotherapy and radiotherapy. One explanation may be the differences in the individual rates of stem cell-like cells. Methods: We included patients with HNSCC and tumor progression or relapse. Tumor samples were obtained before and after primary chemotherapy, and immunohistochemical analyses were performed for CD44, HLA class I (HLA-I), pancytokeratin, and phosphorylated epidermal growth factor receptor (p-EGFR). Differences in expression between the first and second specimens were assessed. Results: Expression between the first and second specimens varied as follows: CD44 increased by 14.67% (95% confidence interval, CI: 6.94 to 22.40; p < 0.01); HLA-I decreased by 16.72% (95% CI: –23.87 to –9.47; p < 0.01); pancytokeratin decreased by 24.91% (95% CI: –32.8 to –17.7; p < 0.01), and p-EGFR expression decreased by 12.30% (95% CI: –20.61 to –3.98; p < 0.005). Conclusions: Among patients with HNSCC, there is an enrichment of cells with stem-like markers in relapsed tumors when compared with the primary tumor. This finding should be considered when developing treatment strategies.

Introduction

When induction chemotherapy is administered with or without radiotherapy in patients with locally advanced head and neck squamous cell carcinoma (HNSCC), only some patients will achieve complete response [1]. This indicates that tumors are heterogeneous; that is, some cells are resistant to treatment, and some of these resistant cells are also capable of proliferation and tumor progression. In other words, it appears that chemotherapy-resistant tumor tissue is rich in cancer stem cells (CSCs), in both local and distant relapses.
Further salvage surgery offers an opportunity to obtain samples of tumor tissue from chemotherapy-resistant patients in order to compare their characteristics with those of the primary tumor. We hypothesize that residual or recurrent tumor tissue has a high content of chemotherapy-resistant cancer cells which can cause tumor regeneration. Several lines of research support this hypothesis [2–4].

The existence of a subpopulation of cells expressing the cell surface glycoprotein CD44 with stem cell potential was first demonstrated by Prince et al. in 2007 [2]. Animal studies have demonstrated that HNSCC CD44-positive cells had a greater ability to develop new tumors in mouse xenograft models. This suggests that CD44 may be a cellular marker of CSCs in head and neck cancer, though less specific than in other tumors such as prostate, colon, or breast cancer.

It has also been shown that prostate cancer cells with stemness capacity lacked differentiation markers and the HLA class I marker (HLA-I). Tumor cells with a low expression of HLA-I were more resistant to chemotherapy than tumor cells that had a high expression of the HLA-I tumor antigen [3]. A similar loss of HLA-I expression could be expected in tumors with high numbers of CSCs. Also, a loss of pancytokeratin (pan-CK) expression by immunohistochemical analysis has been used as an indicator of the poor cell differentiation typically observed in tumors with CSC enrichment [4].

Another important finding is that epidermal growth factor receptor (EGFR) is overexpressed in almost all HNSCCs [5]. Although the mechanism is not well understood, EGFR has been implicated in the resistance to chemotherapy and an increase in resistant cell populations in HNSCC [6]. Furthermore, in HNSCC, it has been shown that CD44 interacts with the phosphorylation of EGFR in vitro [7]. Another study in HNSCC cell lines with stem cell phenotype showed that those cells with a high expression of CD44 and a low expression of EGFR have a negative impact on radiotherapy response [8]. These in vitro results should be confirmed in HNSCC patients treated with radiochemotherapy.

We assume that persistent or recurrent tumor tissue following chemotherapy and radiotherapy will have a higher content of stem cells than the primary tumor tissue. The analysis of cell markers associated with CSCs could help prove the hypothesis that relapsed tumor tissues contain higher levels of CSCs and that this could influence the outcome of the disease and the survival of patients.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Age, years</th>
<th>mean range</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>18–74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gender</th>
<th>male</th>
<th>55 (92)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>female</td>
<td>5 (8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primary</th>
<th>Oropharynx</th>
<th>28 (47)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glottis</td>
<td>8 (13)</td>
</tr>
<tr>
<td></td>
<td>Supraglottis</td>
<td>8 (13)</td>
</tr>
<tr>
<td></td>
<td>Hypopharynx</td>
<td>16 (27)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage</th>
<th>III</th>
<th>5 (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IVA</td>
<td>22 (37)</td>
</tr>
<tr>
<td></td>
<td>IVB</td>
<td>23 (38)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ChT</th>
<th>8 (13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RDT/ChT</td>
<td>11 (18)</td>
</tr>
<tr>
<td></td>
<td>RDT/Cet</td>
<td>20 (34)</td>
</tr>
<tr>
<td></td>
<td>ChT plus RDT/ChT</td>
<td>13 (22)</td>
</tr>
<tr>
<td></td>
<td>ChT plus RDT/Cet</td>
<td>8 (13)</td>
</tr>
</tbody>
</table>

All data, except for age, are expressed as number (%). ChT = Chemotherapy; RDT = radiotherapy; Cet = cetuximab.

Material and Methods

Study Design and Patient Selection

We included 30 patients from the Hospital Clinic Barcelona and 15 other patients from the Institut Català d’Oncologia of Barcelona diagnosed between 1995 and 2011 with locally advanced HNSCC who had received at least one cycle of induction chemotherapy with or without radiotherapy and who further presented with disease progression or recurrence within 5 years after the treatment (radiochemoresistance). Patients who fulfilled the inclusion criteria provided informed consent according to a protocol approved by the Institutional Review Board of our Institutions.

We administered primary chemotherapy and radiotherapy schedules consistent with those commonly used for patients with locally advanced HNSCC [9–13]. Pathological confirmation of squamous cell carcinoma was required for both the initial and subsequent (progressed or relapsed) tumor specimens. Tissue sections with cancer were selected by reviewing slides stained with hematoxylin and eosin. Tumor samples in both initial and subsequent specimens were analyzed retrospectively and compared for biological markers.

Immunohistochemical Analysis

Tumor samples were fixed in 10% formaldehyde and embedded in paraffin, before cutting 10 sections, each measuring 5 μm in thickness per tumor, and placing them on frosted glass slides. All samples were analyzed in the Stem Cell Program Laboratory of Mount Sinai Medical Center, New York, USA. In all instances, slides were reviewed by two observers and discrepancies were resolved by agreement after analyzing slides together.

Immunohistochemical stains were performed using purified mouse anti-human CD44 monoclonal antibody (G44-26) accord-
ing to the manufacturer’s protocol. For immunohistochemistry of the expression of HLA-I, we used monoclonal antibody ab52922 staining HLA-I (Abcam, Cambridge, UK). The pan-CK antigen was analyzed using the monoclonal antibody PCK. Immunohistochemistry for phosphorylated EGFR (p-EGFR) was analyzed with anti-EGFR (phospho Y1092) antibody [EP774Y] (ab40815 ab7753 Mmab; Abcam, Cambridge, UK) in paraffin-embedded sections.

Quantification of cells was determined by counting the number of tumor cells in 3 contiguous high-power fields in 3 different areas of each section, and referred to the total number of counted cancer cells. Immunoreactivity with each of the tested antibodies was considered positive when at least 1% of cells were positive per core.

Statistical Analysis
All clinical data were recorded in a computer database, and all statistical analyses were performed with IBM SPSS version 21.0 for Windows (SPSS Inc., Chicago, Ill., USA). Response was evaluated retrospectively according to the Response Evaluation Criteria in Solid Tumor (RECIST) criteria (version 1.1). We performed contingency table analysis with the Fisher exact or χ² tests for comparisons of ordinal variables, and the Student t test for paired samples was used for comparisons of quantitative variables. To analyze overall survival according to the different biological markers, we analyzed Kaplan-Meier survival curves. Multivariate Cox regression was used to analyze other factors potentially affecting overall survival. All the statistical tests were conducted at the two-sided 0.05 level of significance.

Results

Patient Characteristics
Of the 60 patients included in the study, 55 (92%) were men and 5 (8%) were women. The median age was 60 years (range 18–74 years). The primary tumor was oropharyngeal in 28 patients (47%), glottic in 8 (13%), supraglottic in 8 (13%), and hypopharyngeal in 16 (27%). At diagnosis, 15 patients (25%) had stage III disease, 22 (37%) had stage IVA disease, and 23 (38%) had stage IVB disease (table 1). In addition, 6 patients were positive for the human papillomavirus (10%), and the primary tumor was in the oropharynx in all of these patients. All but 5 patients (8.3%) were heavy smokers and drinkers.

Treatment
The following treatment regimens were employed: cisplatin-based chemotherapy in 8 patients (13%), concomitant chemotherapy and radiotherapy in 11 patients (18%), concomitant cetuximab and radiotherapy in 20 patients (34%), induction chemotherapy plus chemotherapy and radiotherapy in 13 patients (22%), and induction chemotherapy plus cetuximab and radiotherapy in 8 patients (13%).

Immunohistochemical Results
The results of the immunohistochemical analysis are summarized in table 2. The analysis of CD44 after chemotherapy treatment showed that the mean rate of positive cells in the tumor was 18.92% before treatment and 33.58% after treatment; the mean increase was therefore 14.67% (95% confidence interval, CI: 6.94 to 22.40; p < 0.001).

In contrast, there were reductions in the expressions of HLA-I and pan-CK cell differentiation markers from before to after treatment: the mean expression of HLA-I was 47.76% before treatment and 31.09% after treatment, with a mean reduction of 16.72% (95% CI: –23.87 to –9.47; p < 0.001), and the mean expression of pan-CK was 43.09% before treatment and 18.18% after treatment, with a mean reduction of 24.91% (95% CI: –32.08 to –17.74; p < 0.001).

The mean expression of p-EGFR also decreased from 35.16% before treatment to 22.86% after treatment, with a mean reduction of 12.30% (95% CI: –20.61 to –3.98; p = 0.005). In figure 1, we show representative images of the different immunohistochemical staining patterns by marker.

These results have also been analyzed separately for patients treated with radiotherapy plus chemotherapy or...
radiotherapy plus cetuximab. However, no significant differences were found between the two subgroups.

**Survival Analysis**

After a median follow-up of 60 months, the median survival for all patients was 36 months (95% CI: 23.6–48.3). In the univariate analysis, although there were some differences in survival by changes in expression of cell differentiation markers, only patients with a HLA-I expression of 31% or lower in the second specimen had statistically significantly poorer survival compared with patients with an expression over 31% (p = 0.003; fig. 2). In the Cox multivariate regression analysis, comparing gender, age (≤60 vs. >60 years), primary site (oropharynx vs. other sites), stage (IVB vs. IVA or III), and HLA-I expression (≤31 vs. >31%), we observed that overall survival was only significantly poorer in patients with HLA-I expression ≤31% (hazard ratio 2.75; 95% CI: 1.35–5.35; p = 0.005). Both univariate and multivariate analysis showed no significant differences in survival according to the expression of CD44 or pan-CK.

**Discussion**

Compared with primary tumors, relapsed or progressed tumors showed increased expression of CD44 and reduced expression of HLA-I and pan-CK. Together

---

Fig. 1. Immunohistochemical expression of different markers before and after treatment of the primary tumor.
with the observed resistance after first-line radiochemotherapy, these findings suggest that there is an enrichment of cells with CSC characteristics in resistant or relapsed tumors. This is the first time that this finding has been demonstrated in a clinical study of patients with HNSCC.

Preclinical studies have indicated that CD44 could be a CSC marker in tumor xenografts [2] and in other tumors such as breast, ovarian, prostatic, and pancreatic cancer [14]. In head and neck carcinoma, the CD44 protein has also been considered a CSC marker both in vitro and in vivo [15–17]. It has even been obtained in stem-like cells of freshly resected tumor in patients with laryngeal squamous cell carcinoma [18]. In general, clinical studies have shown that increased CD44 expression in the cells of a primary squamous cell tumor is associated with a poor prognosis [19]. Our results in relapsed patients suggest that primary chemotherapy selects a sub-population of resistant cells with the characteristics of CSCs.

Reduced expressions of HLA-I and pan-CK in relapsed tumors compared with the corresponding primaries supports the idea of an enrichment of stem-like cells. A reduction in the HLA-I histocompatibility antigen on the surface of stem cells has been reported to be a necessary step for escaping from T-cell immunity and is a typical characteristic of those cells [20].

Pan-CK and other high-molecular-weight cytokeratins are expressed in the basal and intermediate cell layers of the mucosa, suggesting that it has a role in cell differentiation [21]. Since the expression of pan-CK is reduced in stem cells, we can assume that the expression of this differentiation protein is also reduced in CSCs [22].

The results for the change in expression of p-EGFR are controversial because EGFR overexpression has been associated with increased tumor aggressiveness. Nevertheless, p-EGFR has also been shown to be decreased in tumor tissues after chemotherapy [23–25]. It is possible that our results can be explained, at least in part, by this phenomenon. Until now, p-EGFR has not been considered a stem cell-like marker in tumors.

We observed significantly poorer survival in patients with a low expression of the HLA-I surface marker than in those with a high expression, suggesting that losses in the expression of this cell surface antigen were associated with shorter survival. Multivariate Cox analysis also showed that tumors with a lower expression of the HLA-I surface antigen had a higher quantity of radiochemotherapy-resistant tumor cells, suggesting that this could be used as a prognostic factor. Indeed, changes in the expressions of other cell markers as CD44 and pan-CK were not associated with differences in overall survival. This can be explained by the influence of other prognostic factors, such as the stage of disease or the different treatments used. For this reason, a subsequent study in patients treated uniformly with radiotherapy plus cetuximab is underway.

In conclusion, the analysis of chemotherapy-resistant tumors in HNSCC showed an enrichment of cells with stem cell-like markers, suggesting that there is an increased rate of CSCs. We need to bear this subpopulation of cells in mind when designing treatment strategies.
References


