EXPRESSION OF CD44S CELL ADHESION MOLECULE IN MAXILLOFACIAL SQUAMOUS CELL CARCINOMAS AND ITS IMAGING SIGNIFICANCE

Mihaela Hedeşiu¹, Lumița Leluțițu², Sorana D. Bolboacă³, V. Popiță³,
T. Guttmann³, Mihaela Baciț⁴

"Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca
1 Department of Dental Radiology
2 Cancer Institute Cluj-Napoca
2 Department of Medical Informatics and Biostatistics Cluj-Napoca, Romania
4 Department of Maxillofacial Surgery

EXPRESSION OF CD44S CELL ADHESION MOLECULE IN MAXILLOFACIAL SQUAMOUS CELL CARCINOMAS AND ITS IMAGING SIGNIFICANCE (Abstract): Objectives: CD44 is a cell adhesion molecule which has been described to play a role in tumor progression and in promotion of metastasis. The aim of this study was to establish whether there is a significant correlation between CD44 expression in tumor cells in maxillofacial squamous cell carcinomas and the imaging diagnosis, the histopathological diagnosis, respectively, of the tumor stage. Material and methods: The study included 43 cases of maxillofacial squamous cell carcinomas with preoperative computed tomography assessment. The immunohistochemical analysis was performed in all cases used paraffin embedded tissue sections. CD44 expression in tumor tissue was quantitatively expressed by the percentage of positive malignant cells and qualitatively, by the intensity of the immunopositive reaction to CD44 antibodies. Results: The expression of the CD44 molecule in tumor cells was present in 74.41% of cases. Both the percentage of CD44 positive cells and the intensity of staining were statistically significantly correlated with the histopathological tumor stage (pT) (p<0.05), but not with the imaging tumor stage (iT). A statistically significant difference was found between histopathological and imaging staging for both the N0 stage (p=0.041) and the N2 stage (p=0.009). The number of CD44 positive tumor cells was significantly correlated with the pN stage (p<0.05), but there was no correlation between this and the N stage determined by computed tomography. Conclusions: The determination of CD44 in tumor cells is a valid molecular marker for diagnosis and tumor extension in maxillofacial carcinomas. Keywords: HEAD AND NECK CARCINOMA, CD44, TUMOR STAGE, COMPUTED TOMOGRAPHY

INTRODUCTION

An essential characteristic of carcinomas is the invasion of malignant cells into the adjacent connective tissues and the migration of tumor cells, with the development of metastases at a distance from the site of origin. These processes involve the alteration of interactions between cells, as well as between cells and the extracellular matrix. Cell adhesion molecules play an important role in the alteration of these interactions, being involved in tumor invasion and metastatic processes.

CD44 is a cell adhesion molecule with a role in the binding of hyaluronic acid (HA), extracellular matrix proteins and growth factors[1]. The genomic structure of this molecule includes 20 exons. The first and last 5 exons are constant, but the 10 exons of the intermediate region may present a variability that generates multiple isoforms with different molecular sizes. The smallest CD44 molecule described lacks the whole variable region and is considered the standard CD44 molecule (CD44s). The CD44 isoform
molecules that contain the last 3 exons of the variable region (CD44v3-10) are preferentially expressed by epithelial cells.

In head and neck carcinomas, the expression of the CD44s molecule and its isoform variants CD44v3, CD44v5-9 has made the object of many researches [2,3,4,5]. Some studies show that in head and neck carcinomas, the expression of the CD44 molecule and its isoforms is altered. At the same time, there seems to be a significant correlation between the expression of the CD44s molecule or of certain isoforms of this (CD44v6, CD44v9) and tumor invasion capacity[6].

Imaging TNM (iTNM) staging can be different from histopathological TNM (pTNM) staging, depending on the accuracy of the imaging technique used. In maxillofacial carcinomas, the imaging assessment of both tumor extension and lymphatic invasion is most frequently performed by computed tomography (CT).

The aim of this study was to establish whether there is a significant correlation between CD44 expression in tumor cells in maxillofacial squamous cell carcinomas and the imaging diagnosis, the histopathological diagnosis, respectively, of the tumor stage.

MATERIAL AND METHOD

The study included the cases of maxillofacial squamous cell carcinomas diagnosed histopathologically at the Cancer Institute Cluj-Napoca, in the period January 2005-December 2006. Only the cases investigated by preoperative computed tomography (CT), whose image records were stored in DICOM format were included.

Computed tomographic evaluation: Computed tomography was performed using a Philips CT device. The examination protocol included the performance of continuous, 5 mm thick axial sections parallel to the inferior orbitomeatal plane, situated between the skull base and the supraclavicular fossa. The computed tomographic images were retrospectively examined by 2 examiners (specialist radiologists) who assessed the tumor stage (it) and the node stage (in) according to the TMN staging of American Joint Committee on cancer staging for epithelial tumors of the oropharynx (AJCC, 1999)7. The computed tomographic evaluation of the cases was retrospectively performed, by a specialist in maxillofacial imaging.

Histopathological examination: The operative specimens were used for histopathological evaluation. The histopathological staging of the cases was performed by the macroscopic and microscopic examination of the operative specimens and by the determination of tumor extension, infiltration of resection margins and lymph node invasion. When the histopathological examination of the operative specimen was not possible, histopathological biopsy diagnosis was used.

Immunohistochemical analysis: The immunohistochemical analysis used paraffin embedded tissue sections (paraffin blocks). The blocks were sectioned with a microtome, the sections obtained being 4 μm thick. Subsequently, these sections were placed on silane coated slides and kept in the incubator (37° C) for a period of up to 24 hours. Then, the slides were deparaffinized by the usual technique (xylene and alcohol in decreasing concentrations), washed with distilled water and left for a few minutes in the buffer solution (Tris Buffer Saline-TBS).

The antigen was revealed by introducing the slides into the water bath at a temperature of 98° C, which were subsequently left to cool at room temperature. The sections to be analyzed immunohistochemically were encircled with a special pencil, used for this technique. The antigen was blocked with hydrogen peroxide (peroxidase).

In the next stage, the sections were incubated with the primary CD44 antibody (1:25 dilution). In order to obtain an optimal dilution in the case of the primary antibody,
this was tested in control sections recommended by the protocols accompanying the antibody (optimal dilution – dilution at which target cells are labeled and there is no background).

The tissue sections were incubated with secondary antibodies (LSAB+ system – HRP: Biotinylated link universal and streptavidin – HRP). The chromogen (Diaminobenzidine – DAB – DAK) was applied and the sections were subsequently stained with hematoxylin. Finally, the slides were introduced in increasing alcohol concentrations and were subsequently mounted.

The microscopic examination of the sections stained with hematoxylin-eosin (HE) and of immunohistochemical stainings was carried out using a system consisting of a Nikon E1000 fluorescence microscope and a Sonny digital video camera. The images were captured and analyzed using the Axio Vision software program.

The immunohistochemical evaluation of the CD44 expression in tumor tissue was quantitatively expressed by the percentage of positive malignant cells and qualitatively, by the intensity of the immunopositive reaction to CD44 antibodies. Taking into account the percentage of immunohistochemically positive tumor cells, 4 distinct groups were constituted: group 1 with 0-24% positive cells, group 2: 25-49%, group 3: 50-74% and group 4: >75%. The intensity of the reaction was assessed on a 3 grade scale: low, moderate, and high.

The association of the quantitative and qualitative expression of the CD44 molecule in tumor tissue was correlated with the computed tomographic imaging diagnosis (iT, iN) and the histopathological diagnosis (pT, pN) of tumor extension. The data were processed and summarized using the Microsoft Excel and Statistica 6.0 software programs. For the quantification of the relationship between two qualitative variables, the Spearman rank correlation method was used for a 5% significance threshold. The confidence

**RESULTS**

Forty-three cases met the inclusion and exclusion criteria. Only 27 of the 43 cases included in the study were staged histopathologically based on operative specimens, while other 16 cases were histopathologically diagnosed only by biopsy.

The origin of the maxillofacial tumors included in the study is shown in Table I.

The presence of the CD44 molecule in tumor cells was identified in 32 of the 43 studied cases (74.41%, 95% CI [58.19 – 85.99]).

The distribution depending on the intensity of reaction is shown in Table II.

The distribution of cases depending on the percentage of positive cells is shown in Table III.

The distribution depending on tumor staging: histopathological (pT) compared to imaging (iT) staging is shown in Table IV.

The distribution of histopathological N staging (pN) and computed tomographic N staging (pN) in the studied sample is shown in Table V.

The analysis of the bivariate correlation of CD44 expression with tumor staging (pT, iT) and lymph node staging (pN, iN) is shown in Table VI.

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification of maxillofacial tumors depending on their origin</td>
</tr>
<tr>
<td>Tumor location</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Base of the tongue</td>
</tr>
<tr>
<td>Mobile tongue</td>
</tr>
<tr>
<td>Oropharynx</td>
</tr>
<tr>
<td>Oral cavity</td>
</tr>
<tr>
<td>Retromolar trigone</td>
</tr>
<tr>
<td>TOTAL NO.</td>
</tr>
</tbody>
</table>

f₁ = absolute frequency  
fᵣ = relative frequency  
95% Clᵣ = 95% confidence interval
**TABLE II**

<table>
<thead>
<tr>
<th>Intensity of reaction in the studied sample</th>
<th>(f_a)</th>
<th>(f_r)</th>
<th>95% CI (f_a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>absent CD44</td>
<td>9</td>
<td>20.93</td>
<td>[9.36 - 34.83]</td>
</tr>
<tr>
<td>low</td>
<td>7</td>
<td>16.28</td>
<td>[7.03 - 30.18]</td>
</tr>
<tr>
<td>moderate</td>
<td>15</td>
<td>34.88</td>
<td>[20.98 - 51.11]</td>
</tr>
<tr>
<td>high</td>
<td>12</td>
<td>27.91</td>
<td>[16.33 - 44.13]</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

\(f_a\) = absolute frequency  
\(f_r\) = relative frequency  
95% CI \(f\_a\) = 95% confidence interval

**TABLE III**

| Distribution by tumor cell groups presenting the expression of CD44 molecule |
|--------------------------------------------------|--------|--------|----------------|
| CD44 group (positive cell %)                      | \(f_a\) | \(f_r\) | 95% CI \(f\_a\) |
| GR1 (0-24%)                                         | 15     | 34.88  | [20.98 - 51.11] |
| GR2 (25-49%)                                        | 5      | 11.63  | [4.71 - 25.53]  |
| GR3 (50-74%)                                        | 10     | 23.26  | [11.68 - 39.48] |
| GR4 (>74%)                                          | 12     | 27.91  | [16.33 - 44.13] |
| Missing data                                        | 1      | 2.36   | [0.05 - 11.57]  |
| Total                                               | 43     | 100    |                |

\(f_a\) = absolute frequency  
\(f_r\) = relative frequency  
95% CI \(f\_a\) = 95% confidence interval

**DISCUSSION**

The results of the study show that the expression of the CD44 molecule in tumor cells was present in 74.41% of cases. This finding supports the idea that the decrease in CD44 receptors and the expression of the CD44s molecule or its variants in tumor cells might be a diagnostic molecular marker for maxillofacial carcinomas.

However, the literature reports numerous contradictory results related to the expression of the CD44 molecule in head and neck cancer and to its correlation with clinical-pathological factors. Some authors believe that although the CD44s molecule and especially the v5, v6 exon variants are expressed by a significant percentage of tumor cells of head and neck squamous cell carcinomas [10,11], the expression of these molecules is not significantly different from that in the normal epithelium of the oral cavity and the oropharynx [12,13,4].

Other results have demonstrated a decrease in CD44v6 expression in oral cavity dysplasias14 and maintain that the abnormal expression of the CD44 molecule confers the cell an increased susceptibility to oncogenic transformation15.

Another recent report of a group of researchers from Florida shows that the ELISA test for the identification of salivary soluble CD44 was positive in 79% of patients with invasive squamous cell carcinoma of the oral mucosa; a larger number of patients is required for the validation of results with a view to a possible screening modality for the early diagnosis of oral cancer16.

There are also literature studies on the correlation between serum soluble CD44 (CD44s, v5 si v6) levels and clinical-pathological factors: age, sex, smoking, tumor volume, TNM staging and tumor differentiation degree. Kawano T. et al. show a significant correlation between pretreatment levels of soluble CD44 molecules and TNM staging (CD44s p = .0017; CD44v5 p = .0005; CD44v6 p = .0046), and a decrease in their levels after treatment administration. According to other authors, serum soluble CD44v6 cannot be considered a valid marker for the evolution of oral carcinomas because there are no differences in the expression of this molecule in smoking patients and its serum levels do not correlate with the TNM stage and the tumor differentiation degree17.

In our study, there was a statistically significant difference between histopathological tumor staging (pT) and imaging staging (iT) for the T4 tumor extension stage (p = .04). This is why we considered important the comparative assessment of the correlation of CD44...
expression in tumor cells with histopathological and imaging TNM staging.

As regards the percentage of tumor cells presenting the expression of the CD44 molecule, a uniform distribution of the cases was found. Group 1 (0-24% CD44 positive cells) included 34.88% of the studied tumors, group 2 (25-49% CD44 positive cells) – 11.63%, group 3 (50-74% CD44 positive cells) and group 4 (>75% CD44 positive cells). The intensity of the immunohistochemical reaction was low in 16.28%, moderate in 34.88% and high in 27.91% of the studied cases.

Both the percentage of CD44 positive cells and the intensity of staining were statistically significantly correlated with the histopathological tumor stage (pT) (p<0.05), but not with the imaging tumor stage (iT). This proves that the determination of the CD44 expression in tumor cells is a marker of the real tumor volume and tumor extension as shown by histopathological and not by imaging staging.

The results obtained also show that CD44 is a marker of lymphatic metastasis. A statistically significant difference was found between histopathological and imaging staging for both the N0 stage (p=0.041) and the N2 stage (p=0.009). The number of CD44 positive tumor cells was significantly correlated with the pN stage (p<0.05), but there was no correlation between this and the N stage determined by computed tomography. It is known that the accuracy of computed tomographic examination in the detection of adenopathies is low18, this is why the association of CD44 expression only with the histopathological pN stage supports the fact that this is a valid marker for the assessment of the risk of lymphatic metastasis.

Our study only included the determination of CD44s expression in tumor cells, without determining the expression of exon variants. However, other researches show that the increased expression of the CD44v6 [24] and CD44v9 exon variants is associated with metastatic capacity [2,19,20,21]. Sato S. et al.[22] demonstrated in a number of 120 biopsies from tongue epidermoid carcinomas that metastases were more frequent in the group of tumors presenting an increased expression of the CD44v9 molecule. Moreover, the determination of CD44v9 expression in adjacent non-tumor epithelium showed a significant association between this and extracapsular invasion in lymph nodes (p=0.02)[23].

At the same time, it was found that the injection of specific monoclonal CD44s or CD44v antibodies inhibits local tumor growth and metastasis. These results suggest that CD44 could be used not only as a marker of lymphatic metastasis, but also as a target for antineoplastic therapy. The dosage of plasma-soluble CD44 (CD44s, CD44v5, CD44v6) in patients with head and neck carcinomas shows a decrease in its expression in circulating cells and an increase in its serum levels after treatment24.

The current contradictory results of the literature studies regarding the expression of the CD44 molecule in head and neck tumors could also be explained by the presence of exon variants and the different expression of these molecules in tumor tissue, plasma and saliva.

CONCLUSIONS

In conclusion, the determination of CD44 in tumor cells is a valid molecular marker for diagnosis and tumor extension in maxillofacial carcinomas. The number of CD44 positive cells and the intensity of the immunohistochemical reaction are significantly correlated with histopathological T and N staging, but there is no significant correlation between these and imaging T and N staging. This might explain the many controversies in the literature regarding the correlation of CD44 expression and clinical-pathological factors in head and neck squamous cell carcinomas.
### TABLE IV

**T staging: histopathological versus computed-tomographic staging**

<table>
<thead>
<tr>
<th>T</th>
<th>( f_s )</th>
<th>( f_r )</th>
<th>( p_T )</th>
<th>( i_T )</th>
<th>( p_{(f_{s&gt;T} - f_{s&lt;i&gt;T})} )</th>
<th>( 95% CI_{f_r} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>4</td>
<td>2</td>
<td>9.30</td>
<td>4.65</td>
<td>0.4693</td>
<td>[2.38 – 20.87]</td>
</tr>
<tr>
<td>T2</td>
<td>4</td>
<td>10</td>
<td>9.30</td>
<td>23.26</td>
<td>0.0802</td>
<td>[2.38 – 20.87]</td>
</tr>
<tr>
<td>T3</td>
<td>5</td>
<td>9</td>
<td>11.63</td>
<td>20.93</td>
<td>0.2641</td>
<td>[4.71 – 25.53]</td>
</tr>
<tr>
<td>T4</td>
<td>12</td>
<td>21</td>
<td>27.91</td>
<td>48.84</td>
<td>0.0486</td>
<td>[16.33 – 44.13]</td>
</tr>
<tr>
<td>Tx</td>
<td>18</td>
<td>1</td>
<td>41.86</td>
<td>2.33</td>
<td>&lt;0.0001</td>
<td>[27.96 – 58.09]</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>43</td>
<td>100</td>
<td>100</td>
<td></td>
<td>[0.05 – 11.57]</td>
</tr>
</tbody>
</table>

\( f_s \) = absolute frequency  
\( f_r \) = relative frequency  
\( 95\% CI_{f_r} \) = 95\% confidence interval  
\( p_T \) = histopathological T staging  
\( i_T \) = computed tomographic T staging

### TABLE V

**N staging: histopathological versus computed-tomographic staging**

<table>
<thead>
<tr>
<th>N</th>
<th>( f_s )</th>
<th>( f_r )</th>
<th>( p_N )</th>
<th>( i_N )</th>
<th>( p_{(f_{s&gt;N} - f_{s&lt;i&gt;N})} )</th>
<th>( 95% CI_{f_r} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>10</td>
<td>13</td>
<td>23.26</td>
<td>54.08</td>
<td>0.0041</td>
<td>[11.68 – 39.48]</td>
</tr>
<tr>
<td>N1</td>
<td>3</td>
<td>5</td>
<td>6.98</td>
<td>16.22</td>
<td>0.1944</td>
<td>[2.38 – 18.55]</td>
</tr>
<tr>
<td>N2</td>
<td>12</td>
<td>25</td>
<td>27.91</td>
<td>64.90</td>
<td>0.0009</td>
<td>[16.33 – 44.13]</td>
</tr>
<tr>
<td>N3</td>
<td>18</td>
<td>0</td>
<td>41.86</td>
<td>0</td>
<td>&lt;0.0001</td>
<td>[27.96 – 58.08]</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>43</td>
<td>100</td>
<td>100</td>
<td></td>
<td>n.a.</td>
</tr>
</tbody>
</table>

\( f_s \) = absolute frequency  
\( f_r \) = relative frequency  
\( 95\% CI_{f_r} \) = 95\% confidence interval  
\( p_N \) = histopathological N staging  
\( i_N \) = computed tomographic N staging  
n.a. = not applicable

### TABLE VI

**Quantification of correlation between CD44 expression and tumor and lymph node staging**

<table>
<thead>
<tr>
<th>CD44</th>
<th>( p_T )</th>
<th>( p_N )</th>
<th>( i_T )</th>
<th>( i_N )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (tumor cell %)</td>
<td>-0.3243*</td>
<td>-0.3278*</td>
<td>-0.0531</td>
<td>-0.0175</td>
</tr>
<tr>
<td>Intensity of immunohistochemical reaction</td>
<td>-0.3390*</td>
<td>-0.2660</td>
<td>-0.0622</td>
<td>-0.0496</td>
</tr>
</tbody>
</table>

\( p_T \) = histopathological T staging  
\( p_N \) = histopathological N staging  
\( i_T \) = computed tomographic T staging  
\( i_N \) = computed tomographic N staging  
* \( p < 0.05 \)
Expression of CD44s cell adhesion molecule in maxillofacial squamous cell carcinomas and its imaging significance

BIBLIOGRAPHY


