Abstract

Purpose: Lymph nodes estimated as pN0 in conventional morphological studies could have focuses of carcinoma cells with a diameter of ≤2 mm referred to as micrometastases (pN+). Matrix metalloproteinases (MMPs) are proteolytic family of endopeptidases which are capable to degrading components of the extracellular matrix and play an important role in cancer invasion and metastases. The aim of this study was to investigate MT1-MMP expression in carcinoma of the larynx and analyze morphological parameters to relate the expression to CKs in pN0 lymph nodes.

Material and methods: To presented the direct correlation between 6 morphological features of tumor front and the probability of micrometastases and prediction of prognosis 22 patients operated for squamous cell carcinoma of the larynx were analyzed. The total score of TFG, chosen clinicomorphological features and grade of matrix metalloproteinase membrane type 1 staining in tumor front were analyzed to predict the presence of micrometastases and prognosis. Immunohistochemical methods with a panel of CKs antigens in lymph nodes and MT1-MMP expression in tumor tissue were performed.

Results: Our study showed that the total morphologic score TFG is very useful in the prediction of micrometastases in patients with laryngeal squamous cell carcinoma. The statistical analysis has revealed a significant correlation between the total TFG score and the presence of cell carcinoma microfocuses in lymph nodes. There was no significant relationship for immunoexpression of MT1-MMP and positive poliCKs stain.

Conclusions: The results of study suggest that extended traditional pathologic evaluation by features from the TFG classification could aid in diagnosis of micrometastases. The positive expression of poliCKs in the pN0 lymph nodes appears to play an important role in determining prognosis in patients with carcinoma of the larynx.

Key words: laryngeal carcinoma, tumor front grading classification, cytokeratin filaments, matrix metalloproteinase membrane type 1, micrometastases.

Introduction

The prognosis of patients, in whom the presence of nodal metastases has not been confirmed in the conventional pathologiological examination (pN0), is still unclear. The problem is connected with the possibility of the presence of nodal micrometastases – carcinoma foci less than 2 mm in diameter (pN1), which may be the cause of nodal recurrence and shortened survival [1-13]. One of the methods to disclose occult foci of malignant cells within lymph nodes is immunohistochemical analysis of the nodes for the presence of cytokeratin antigens belonging to the family of median filaments (CKs) [1,3,6-9,13].

Matrix metalloproteinases (MMPs) are proteolytic family enzymes of endopeptidases which are capable of degrading extracellular matrix components (ECM), e.g. type I collagen, elastin, fibronectin, gelatin and the basement membrane [14,16]. MMPs including active enzymes such as collagenase, stromelysin and gelatinase [14]. MMPs are inactivated by tissue inhibitors of MMPs (TIMPs) which form complexes with them [15]. MT1-MMP degrades components of the tissue barriers thus could determine the presence of cell carcinoma metastases and microfocuses in lymph nodes [14-23]. Literature reports the prognostic value of the morphological features of the front
of the primary tumor which determines aggressive malignant invasion and increases the likelihood of micrometastases [6-9,24,25].

The aim of the study was to analyse the morphological features of the primary tumor, its stroma using the classification TFG (Tumor Front Grading) and the expression of MT1-MMP in tumor front to evaluate the relationship between these parameters and cytokeratin antigen expression within the negative lymph nodes, thus attempting to assess the probability of developing micrometastases.

Materials and methods

In study tissue samples obtained from 22 patients (17 men, 5 women; age 58-87 yrs; mean age 63±5 yrs) who had undergone surgery for laryngeal carcinoma at the Department of Laryngology and Laryngeal Oncology of the Medical University in Łódź between the years 1998-1999 were analyzed. All patients underwent total laryngectomy with uni- or bilateral radical or modified neck dissection. The lesions were assessed according to the TNM criteria (TNM Classification of WHO –2003 – International Classification of Diseases for Oncology). Directly after surgery the material was preserved in a 10% solution of buffered formaldehyde. Samples from the primary tumor designated for microscopic analysis were obtained according to standardized procedure. The depth of invasion was assessed at the point of the deepest invasion of the surrounding tissues. Two samples were obtained from every lymph node after it had been dissected along its greatest diameter. The tissue samples were embedded in paraffin, routinely cut into 4-5 μm sections (at least 3-4 from the primary tumor and each lymph node), attached to glass slides and H&E stained. Pathological analysis was performed according to classification TFG [6,9,25]. We assessed 6 pathological features of the tumor and the type of interaction between the tumor and the surrounding tissues (cytoplasmic differentiation, nuclear differentiation, mitotic figures type and stage of invasion, the presence of lymphocytes invasion). The analysis was performed under a light microscope (magn. 200X, number of mitoses magn. 400X), going by the areas of deepest invasion of the surrounding tissues. Tumor assessment has been presented as the number of scored points. Each factor was graded according to a scale ranging from 1 to 4. Immuno-histochemical reactions with polyclonal antibodies (NCL-C11, Multi-Cytokeratin 4/5/6/8/10/13/18, RTU-D Novostatin Universal Detection Kit, NCL-L-DAB Liquid DAB Substrate Kit; Novocastra UK) were performed on 2-3 sections obtained from the same lymph node (3-4 μm sections attached to polysin-covered glass slides) in accordance with the manufacturer’s directions. For analysis of metalloproteinases expression in tumor front reactions with monoclonal antibodies (Mouse Anti-Human MT1-MMP, Chemicon) were made and immunohistochemical index was used. For analysis of MT1-MMP expression the scale was used: 0 – none, 1 – low expression (<10% positive cells), 2 – medium expression (<50%), 3 – high expression (≥50%). For the sake of pathological analysis of the lymph nodes it was assumed that a micrometastasis is a focus ≤2 mm in diameter. The results of nodal cytokeratin microfilament expression were compared with the morphological features of the tumor and expression of MT1-MMP. In the course of the statistical analysis we applied a Spearman’s tests (the significance level was set at p<0.1; R=0.3) to analyze correlation of chosen features and micrometastases.

Results

In examined group 81.8% cases were diagnosed as advanced cancer involving three regions of the larynx and originating from the supraglottic area. The most numerous group of patients (20/22; 90%) had stage III and stage IV tumors. 16 patients (72.7%) underwent unilateral selective neck dissection, in 2 cases in the form of radical neck dissection. The remaining 6 patients (27.3%) underwent bilateral lymphadenectomy. Pathological analysis of the primary tumor revealed that a majority of the patients presented with squamous cell carcinoma of intermediate differentiation G2 – 12 (54.5%) and distribution of pT feature was: 4 (18.2%) with pT2 tumor, 4 (18.2%) – pT3 and 14 (63.6%) – pT4 tumor. Pathological routine study of H&E stained lymph nodes did not confirm the presence of metastases, however, immunohistochemical evaluation with polyclonal anti-CK antibodies confirmed the presence of cancer cells (pN1) within the lymph nodes of 11 (50%) patients. In remaining lymph nodes immunological reactivity was revealed (pN0). The total score obtained in the course of primary tumor assessment acc. to the TFG classification oscillated between 9 and 20 (mean: 15.4 points) and for CKpoly positive tumors and between 12 and 20 (mean: 16.5 points). Distribution of 6 pathological features of the tumor front was estimated. Positive MT1-MMP expression in 15 (68.2%) cases was observed and distribution of matrix metalloproteinases expression acc. to proposed scale was performed (Fig. 1, 2). In study group 12 patients (54.4%) achieved 3-year survival, and 7 (31.8%) 5-year survival. Statistical analysis aimed at the assessment of the correlation of developing nodal micrometastases in relation to the results of pathological analysis revealed the statistical significance for TFG total score (p=0.09). Analysis of each morphological feature used for morphologic grading revealed that depth of invasion (p<0.08) and number of mitoses per high-power field in cells of laryngeal carcinoma (p<0.09) had significant influence on the presence of micrometastases. The statistical analysis showed no significant correlation between expression of matrix metalloproteinases type I in tumor front and the presence of cell carcinoma microfociuses in lymph nodes. Our study showed that the positive polyclontkeratines stain in the routine pN0 lymph nodes is very useful in prediction of 3- and 5-year survival in patients with laryngeal squamous cell carcinoma as well (p<0.09 and p<0.02) (Tab. 1).

Discussion

On the basis of the several studies performed recently, biologic factors or host-related factors probably play the most important role in determining the eventual disease outcome and maybe thus the presence of occult micrometastases.
in lymph nodes [1-13]. TFG is the technique, which assesses the dynamics of the tumor growth and provides multifactorial morphologic information about the carcinoma tissue. This classification includes assorted pathological features of the tumor and the types of interactions between the cancerous tissue and the front of the tumor [6-9,24,25]. The results presented here, obtained from 22 laryngeal cancer patients confirm the value of this classification in prognosis of the development of nodal micrometastases. A high total score relates to an increased probability of the presence of foci of single cancer cells within lymph nodes which were originally, in the course of routine assessment, pronounced non-metastatic (pN0). Important factors influencing the probability of nodal micrometastases, and affecting patient prognosis, include the depth of laryngeal wall invasion [6,8,9,24]. Tumors characterized by diffuse invasion with small foci of malignancy were found to be more commonly associated with treatment failure and nodal recurrence. Our studies have shown the value of the depth of invasion for assessing the probability of the presence of occult foci of cancer cells within the lymph nodes. While studying the prognostic factors of laryngeal cancer, pathologists also assess the interactions between the tumor and the surrounding tissues, however, there exist no reports as to the independent prognostic value of these morphological features. Nevertheless, they begin to appear as elements of numerous classifications and scores for the assessment of the primary tumor [9,24,25]. Our own studies performed on the postoperative material obtained from the patients which were aimed at assessing the correlation between the total score acc. to the modified classification of Anneroth, Batsakis and Luna and risk of micrometastases have shown that the achieved score relates to prognosis. A more detailed morphological assessment of laryngeal cancer specimens based on this classification might improve the diagnosis of nodal micrometastases. The risk of nodal micrometastases in laryngeal cancer increases with a high total Anneroth, Batsakis and Luna score (>16 points), diffuse cancerous invasion with single cells and deep invasion of the laryngeal wall involving the cartilages [6,8]. Many authors have undertaken multifactorial morphologic valuation of the tumor in squamous cell carcinoma of other sites introduced similar conclusion [26].

Matrix metalloproteinases type I (MT1-MMP) play an important role in destruction of ECM thus effect on tumor...

Table 1. Analysis of correlation positive expression for CKs (micrometastases) and chosen features of tumor front, MT1-MMp expression and survival

<table>
<thead>
<tr>
<th>Feature</th>
<th>R Spearman’s</th>
<th>p [&lt;0.1]</th>
<th>t(N-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cT</td>
<td>-0.39</td>
<td>0.06</td>
<td>-1.93</td>
</tr>
<tr>
<td>cN</td>
<td>-0.39</td>
<td>0.06</td>
<td>-1.93</td>
</tr>
<tr>
<td>Number of nodes</td>
<td>-0.41</td>
<td>0.05</td>
<td>-2.06</td>
</tr>
<tr>
<td>G</td>
<td>-0.12</td>
<td>0.57</td>
<td>-0.57</td>
</tr>
<tr>
<td>TFG</td>
<td>0.36</td>
<td>0.09</td>
<td>1.77</td>
</tr>
<tr>
<td>MT1-MMP expression</td>
<td>-0.09</td>
<td>0.73</td>
<td>-0.34</td>
</tr>
<tr>
<td>Nuclear differentiation</td>
<td>-0.11</td>
<td>0.60</td>
<td>-0.52</td>
</tr>
<tr>
<td>Number of mitoses</td>
<td>0.36</td>
<td>0.09</td>
<td>1.73</td>
</tr>
<tr>
<td>Mode of invasion</td>
<td>0.32</td>
<td>0.14</td>
<td>1.52</td>
</tr>
<tr>
<td>Stage of invasion (depth)</td>
<td>0.37</td>
<td>0.08</td>
<td>1.81</td>
</tr>
<tr>
<td>Plasmalymphocytic invasion</td>
<td>0.23</td>
<td>0.29</td>
<td>1.08</td>
</tr>
<tr>
<td>3-year survival</td>
<td>-0.36</td>
<td>0.09</td>
<td>-1.75</td>
</tr>
<tr>
<td>5-year survival</td>
<td>-0.48</td>
<td>0.02</td>
<td>-2.50</td>
</tr>
</tbody>
</table>

Statistically significant correlation (R=0.3; p<0.1) was marked with bold faced type
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