Expression of nm23H1 and MMP2 in laryngeal carcinoma and its role in aggressiveness of the tumour and node metastasis
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Introduction

Squamous cell carcinomas of the head and neck are known for their aggressive growth and propensity to metastasize. The invasion is facilitated by matrix metalloproteinases (MMPs). The overexpression of many MMPs is positively associated with tumour metastasis. The nm23H1 gene has been implicated as a suppressor gene and reduced expression of its gene product has been observed in patients with positive lymph node metastasis.

Objective

The aim of this study was to examine the degree of expression of both MMP2 and nm23H1 proteins in 24 patients with primary laryngeal carcinoma using immunohistochemical technique and also to correlate the results with the clinical, radiological and histopathological data, in order to evaluate their role in predicting the local spread and lymph node metastasis.

Patients and methods

This study included 24 patients with primary laryngeal carcinoma involving various regions of the larynx and was carried out at the Department of Otolaryngology, Ain Shams University Hospitals.

The laryngeal and nodal specimens were examined histopathologically and immunohistochemical analysis was carried out for the nm23H1 and MMP2 proteins.

Results

There was a significant correlation between MMP2 expression and the site of the tumour, as a drastic reduction in MMP2 expression was mainly associated with glottic carcinoma (P < 0.05). The tumour stages were associated with an increase in MMP2 expression, but this was not statistically significant (P = 0.07). However, the presence of lymph node metastasis was significantly related to the overexpression of MMP2 (P < 0.05). As regards nm23H1, a statistically significant correlation was found between nm23H1 expression and the tumour stage (P < 0.05). Moreover, the presence of lymph node metastasis was significantly correlated to the loss of nm23H1 expression and vice versa (P < 0.05). However, the correlation between the expression levels of MMP2 and nm23H1 was found to be statistically insignificant (P > 0.05).

Conclusion

The increased expression of MMP2 was related to the occurrence of nodal metastasis of the tumour and also to the tumour sites characterized by frequent metastasis (supraglottic and transglottic). In contrast, nm23H1 expression was inversely related to the advancement of the tumour stage and the nodal metastatic state.

Keywords:
cancer larynx, matrix metalloproteinase-2, nm23H1

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subgroups [5]. Several studies have proven that MMP2 may play an important role in the development of acute lymphoblastic leukaemia [6], breast cancer, renal cancer and head and neck carcinoma [7]. The development of nodal metastasis is a major adverse event in patients with squamous cell carcinoma of the upper aerodigestive tract, reducing the survival by about 50% [8]. The metastatic cells undergo alteration in the genes and their products, including overexpression of the metastasis-promoting factors or loss of expression of suppressing factors [9].

Steeg et al. [10] reported a family of closely related tumour suppressor genes (nm23) that is associated with the inhibition of the metastatic process through an unknown molecular mechanism. Metastasis suppressor proteins regulate multiple steps in the metastatic cascade, including cancer cell invasion, survival in the vascular and lymphatic circulation and colonization of the distant organ sites. Tumour suppressor genes may positively or negatively regulate the subsequent development of primary tumours [11].

Two closely related genes namely nm23H1 and nm23H2 have identical protein products and similar activities and shown to be identical to the human nucleotide diphosphate kinase (NDP3). Therefore, the nm23H1 gene has been demonstrated to correlate inversely with the metastatic potential in several tumours, as the data suggests that the normal expression of nm23H1 contributes to normal cell growth, whereas altered or lowered levels of nm23H1 expression aid in cancer metastasis [12].

**Aim of the study**
The aim of the study was to examine the degree of expression of both MMP2 and nm23H1 in primary laryngeal carcinoma using immunohistochemical techniques and correlating it with the findings of the clinical, radiological and conventional histopathological examination.

**Patients and methods**

**Patients**

A total of 24 patients with LSCC involving various regions of the larynx (and at various stages) were included in this study, which was carried out at the Departments of Otolaryngology and Pathology of Ain Shams University Hospitals, between March 2007 and April 2009.

**Methods**

Preoperative clinical evaluation of these patients included a full history taking, especially for hoarseness of the voice, stridor and neck masses. A thorough ENT and neck examination was performed for each patient.

Direct laryngoscopy was performed to evaluate the extent of the tumour for provisional staging and obtaining samples for biopsy.

Radiological assessment through a computed tomography (CT) scan of the neck comprised thin axial cuts at a soft tissue window. The CT scan was performed to study the site of the lesion and the extent of tumour spread; the staging was confirmed depending on the findings of the CT scan.

The specimens obtained from partial or total laryngectomy and the removed lymph nodes underwent fixation to prepare paraffin-embedded tissue blocks, from which sections of 5μm thickness were cut and stained with haematoxylin and eosin.

A routine histopathological examination of the specimens was performed and the tumour type, grade, freedom or involvement of surgical margins, depth of invasion and lymph nodal status were determined.

For immunohistochemical staining, 5μm sections were cut and placed on coated slides and then deparaffinized. The endogenous peroxidase activity was blocked using 0.3% H₂O₂. The sections were then incubated for 10 min after which the non-specific-binding sites were blocked by treatment with 3% normal goat serum for 30 min. The slides were incubated with the primary antibodies for nm23H1 (mouse monoclonal anti-nm23H1 antibody diluted to 1:100) and MMP2 proteins (rabbit monoclonal anti-MMP2 antibody diluted to 1:50).

Each primary antibody was incubated for 1 h at 37°C. After washing with PBS, the biotinylated secondary antibodies, which were anti-mouse immunoglobulin G for nm23H1 and anti-rabbit immunoglobulin G for MMP2, were added, followed by an incubation of 45 min. After rinsing with PBS, peroxidise-conjugated streptavidin was added for 20 min, followed by another rinse with PBS. The immunohistochemical reactions were developed in 20mg of freshly prepared 3,3′diaminobenzidine tetrahydrochloride for 10 min and were then rinsed with distilled water. After counterstaining with haematoxylin for 1 min, the specimens were observed under a light microscope. The sections were scanned at low power (× 40) to assess the heterogeneity of the stain distribution, and multiple fields with diffused staining were examined at high power (× 200) for scoring. As regards the scoring of the nm23H1 staining, the tissues were scored as follows:

0 = very light or no staining.
1 = for widespread faint staining or localized strong staining.
2 = for widespread strong staining.

As regards the MMP2 protein expression, a semiquantitative analysis based on a 4-point scale was used depending on the percentage of positively stained cells:

0 = no staining.
1 = low expression (<10% positive cells).
2 = moderate expression (10–50% positive cells).
3 = strong expression (>50% positive cells).
After scoring both MMP2 and nm23H1 staining, correlations were drawn on the basis of the clinical, radiological and histopathological data of the patients. Statistical analysis of the results was performed using the χ² test, one-way analysis of variance test and Student t-test by using the SPSS software version 10.0 for windows.

**Results**

A total of 24 patients with LSCCs were involved in this study that was carried at Ain Shams University Hospitals between March 2007 and April 2009. The age of the patients ranged from 42 to 89 years (with an average of 58 ± 5 years). There was a strong male predominance with 21 men and three women.

The results were classified into two groups (a) clinical and radiological results and (b) pathological and immunohistochemical results.

**Clinical and radiological results**

In all patients, the clinical evaluation and CT scan staging revealed the exact site of the tumour, its extent and presence or absence of lymph node metastasis.

Eight patients (33.3%) had supraglottic carcinoma of various T stages (one patient with T1, three with T2, two with T3 and two with T4), 11 patients (45.9%) had transglottic carcinoma involving the ventricle and the glottis areas, with subglottic extension in two patients only (three patients with T2, seven with T3 and only one with T4), five patients (20.8%) had glottic carcinoma of T1 or T2 stages only (three patients with T1 and two with T2).

The tumour was localized within the larynx in 21 patients (87.5%) but extended beyond this limit, through the thyroid cartilage and into the prelaryngeal muscles in two patients (8.3%) and into the pharyngeal mucosa in one patient (4.1%).

As regards the T stage, nine patients (37.5%) were classified under the T3 stage, eight (33.3%) under T2, four (16.7%) under T1 and three (12.5%) under T4.

As regards the N stage, nine patients (37.5%) had positively enlarged lymph nodes and 15 (62.5%) showed no lymph node enlargement.

Based on the American Joint Committee on Cancer staging system, we staged the patients as follow: three patients (12.5%) with stage I, seven (29.1%) with stage II, six (25%) with stage III and eight (33.3%) with stage IV.

**Histopathological and immunohistochemical results**

The histopathological examination revealed that squamous cell carcinomas were present in all patients and were graded (according to Broder’s classification) into: well-differentiated in 10 patients (41.1%), moderately differentiated in 10 (41.6%) and poorly differentiated in four (16.7%).

Metastatic deposits in the cervical lymph nodes were seen in nine patients (37.5%) with radiologically enlarged lymph nodes, whereas the remaining 15 patients showed no malignant deposits within their lymph node specimens (Table 1).

The immunohistochemical results were as follows: MMP2 staining was localized to the cytoplasm; it was detected in 12 patients (50%), of whom seven (29.1%) showed moderate expression and the remaining five (20.8%) showed strong expression. More intensive staining was seen at the advanced edge of the tumour. Figure 1 shows high MMP2 expression in a

**Table 1 Clinicopathologic features and immunohistochemical results**

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Main site</th>
<th>TNM status</th>
<th>Stage</th>
<th>Grade</th>
<th>Lymph node</th>
<th>MMP2</th>
<th>nm23H1</th>
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<td>M</td>
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<td>T1N3M0</td>
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<td>II</td>
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<td>M</td>
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<td>T4N1M0</td>
<td>IV</td>
<td>II</td>
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<td>T2N0M0</td>
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<td>I</td>
<td>Negative</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
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<td>T4N3M0</td>
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<td>III</td>
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</tr>
<tr>
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<td>III</td>
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<td>Positive</td>
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<td>70</td>
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<td>I</td>
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<td>Positive</td>
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<td>IV</td>
<td>III</td>
<td>Negative</td>
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</table>

F, female; Grade differentiation: I, well; II, moderate; III, poor; M, male; MMP2, matrix metalloproteinase-2.
tumour with lymph node metastasis and Fig. 2 shows low MMP2 expression in a tumour without lymph node metastasis.

For statistical analysis, the patients were divided into two groups according to the expression of MMP2: those with absent or low expression and those with moderate or strong expression. The correlation of MMP2 expression with the clinicopathological data is shown in Table 2.

There was no statistically significant correlation between the level of MMP2 expression and the mean age and sex of the patients (P > 0.05). Meanwhile, a significant correlation was found between MMP2 expression and the site of the tumour, as drastically low expression of MMP2 was mainly associated with glottic carcinoma (P < 0.05).

As regards the tumour stage, advanced tumour stages were associated with an increase in MMP2 expression; however, this was not statistically significant (P = 0.07).

However, the presence of lymph node metastasis was significantly related to overexpression of MMP2 (P < 0.05).

The expression of the nm23H1 protein was localized to the cytoplasm, with staining of the lymphocytes serving as an internal positive control. Seven patients showed negative staining (0), nine showed reduced staining (1) and eight showed increased level of expression (2).

Correlation of the level of expression of the nm23H1 protein with the clinicopathological data is shown in Table 3. There was no statistically significant correlation between nm23H1 expression and the mean age, sex and tumour site (P > 0.05).

Moreover, as regards the tumour grade, no statistically significant correlation was found between nm23H1 expression and the tumour grade (P > 0.05), whereas with respect to the tumour stage, a statistically significant correlation was found (P < 0.05), such that in more advanced tumour stages, the expression of nm23H1 was lost.

The presence of lymph node metastasis was significantly correlated to the loss of nm23H1 expression and vice versa (P < 0.05).

Figure 3 shows a tumour with lymph node metastasis accompanied by marked reduction of nm23H1 expression, whereas Fig. 4 shows a tumour without lymph node metastasis associated with increased nm23H1 expression. The correlation between the level of expression of MMP2 and nm23H1 was statistically insignificant (P > 0.05).

Discussion

Lymph node metastasis is an important factor in the treatment and prognosis of patients with head and neck cancer.
Successful metastasis is dependent on the promoters and suppressors involved in each step [14]. The proteolysis of the ECM macromolecules is related to tumour progression, including invasion, growth and metastasis [15]. The MMPs are a family of proteolytic zinc-containing enzymes that are responsible for the breakdown of the ECM components in pathological conditions; they are involved in the disruption of the basement membrane, penetration of the stroma and blood vessels and metastasis [16]. The production of large amounts of these enzymes is an important factor in predicting the aggressive behaviour and hence, the prognosis of patients with malignant tumours. Moreover, such enzymes have been demonstrated to correlate well with the metastatic potential of tumour cells [17].

Répássy et al. [18] found that laryngeal cancers lacking MMP2 expression belonged to low TNM stages, whereas those expressing MMP2 belonged to the advanced stages. Moreover, Sariolu et al. [19] concluded that an altered expression of MMP2 was seen during early neoplastic transformation, with a consecutive increase during progression of the laryngeal carcinomas. On studying the expression of MMP2 in squamous cell carcinoma [13], successful metastasis is dependent on the promoters and suppressors involved in each step [14]. The proteolysis of the ECM macromolecules is related to tumour progression, including invasion, growth and metastasis [15]. The MMPs are a family of proteolytic zinc-containing enzymes that are responsible for the breakdown of the ECM components in pathological conditions; they are involved in the disruption of the basement membrane, penetration of the stroma and blood vessels and metastasis [16]. The production of large amounts of these enzymes is an important factor in predicting the aggressive behaviour and hence, the prognosis of patients with malignant tumours. Moreover, such enzymes have been demonstrated to correlate well with the metastatic potential of tumour cells [17].

MMP2, matrix metalloproteinase-2; S, significant.

*One-way analysis of variance test, the rest by χ² test.
and it was detected in only 25% of patients without nodal metastasis. There was a positive correlation between a high level expression of MMP2 and the clinical stage and status of lymph node metastasis [21]. Wang et al. [22] found significant differences in the expression of MMP2 among the different pathological degrees of cancer (I, II, III), but the age of the patient and the clinical stage of the cancer had no effect on the expression of MMP2.

Our results support the previously recorded results that present a strong correlation between the level of MMP2 expression and the presence of lymph node metastasis. We reported MMP2 overexpression in 83% of patients with pathologically metastatic lymph nodes and in only 41% of those without lymph node metastasis, suggesting the increased metastatic tendency of tumour cells to express high values of MMP2. Nevertheless, the percentage of patients without lymph node metastasis showing overexpression of MMP2 (41%) is not as low as expected; this could be attributed to the probability that the tumours in some of these patients without nodal metastasis are already passing through the nodal metastatic process but have not yet reached the stage of pathological detection. Similarly, Bogusiewicz et al. [23] concluded that MMP2 might play a role in the lymphatic spread of laryngeal carcinoma.

As previously reported, the incidence of nodal metastasis varies with the primary site and size of the laryngeal carcinoma, as in the case of supraglottic carcinoma that shows a higher incidence of nodal metastasis [8]. The results of the present study agree with such oncological findings by demonstrating a strong association between MMP2 expression and supraglottic carcinoma, suggesting that MMP2 expression might be one of the factors facilitating the nodal metastatic spread of tumours arising in a particular region of the larynx. In contrast, a drastic reduction in the expression of MMP2 was associated with increased occurrence of glottic carcinoma, which is classically known to have a lower rate of nodal metastasis.

One of the putative metastatic suppressor genes is nm23H1 that encodes nucleotide diphosphatase kinase [12]. The nm23 gene has been implicated as a suppressor gene involved in the control of the metastatic process of malignant cells. Reduced levels of the nm23 gene product have been found in tumour cells with high metastatic potential, such as rodent ulcers and human breast, colorectal and lung carcinoma. Reduced expression of the tumour suppressor gene nm23 was significant (P < 0.05), to the extent that in the most advanced stages, the tumour lost the expression of nm23H1. In other words, downregulation of nm23H1 expression was associated with increased staging of the T stage and N stage, both of which were statistically significant (P = 0.04); this suggests that the loss of the tumour metastatic suppressor effect of the nm23H1 gene might be partially responsible for the aggressive biological behaviour of the primary laryngeal carcinoma and hence increasing its metastatic potential.

Although the expression of both MMP2 and nm23H1 was correlated to the aggressiveness of the malignancy, the statistical correlation of expression of MMP2 with that of nm23H1 was insignificant (P > 0.05). Collectively, increased expression of MMP2 was related to the occurrence of nodal metastasis of the tumour and also to the primary tumour and hence characterized by frequent metastasis (supraglottic and transglottic). In contrast, nm23H1 expression was inversely related to the advancement of the tumour stage and the nodal metastatic state.

Conclusion

Many factors are responsible for the aggressive biological behaviour of laryngeal carcinoma. Increased production of ECM-degrading enzymes (MMP2) and reduction in the expression of the tumour suppressor gene nm23H1 may be among the factors that increase the metastatic tendency in laryngeal carcinoma.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References