

Correlation Between the Expression of Matrix Metalloproteinase-9, Matrix Metalloproteinase-13, Tissue Inhibitor of Metalloproteinases-1, p16 and Differentiation of Head and Neck Squamous Cell Carcinoma: A Prospective Observational Study

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Abstract

Introduction: The expression of matrix metalloproteinase-9 (MMP-9), MMP-13, and tissue inhibitor of metalloproteinases (TIMP-1) in head and neck squamous cell carcinoma (HNSCC) could be a useful predictor of tumour differentiation, nodal metastasis, and invasiveness. We conducted this study to ascertain the correlation between the expression of these markers and differentiation of tumour cells. **Materials and Methods:** A prospective observational study was conducted in a tertiary care center. Forty-three cases of proven HNSCC were recruited after obtaining informed consent. Using the surgically excised specimen, tumour differentiation and invasiveness were assessed and correlated with rates of expression of the markers. Chi-square test was done to correlate immunohistochemical (IHC) marker positivity and the degree of differentiation of the tumour, lymph node metastasis, and invasiveness. **Results:** MMP-9, MMP-13, and TIMP-1 were expressed in 72%, 34%, and 18% of cases, respectively. p16 expression was not found in any of the cases. MMP-13 expression correlated with poorer differentiation of the tumour ($p = 0.03$), and relatively younger age at diagnosis ($p = 0.01$). However, there was no correlation with lymphovascular or perineural invasion or lymph node metastasis. **Discussion:** In our study, MMP-13 expression correlated with poorer tumour differentiation and younger age at diagnosis, giving indirect evidence of tumour aggressiveness. IHC markers can provide additional information to prognosticate HNSCC. Identifying potential targets for newer biological therapy is essential in the Indian population as there are biological differences in cancer behavior. Increased expression of the proteolytic MMP-13 correlated with poorer differentiation of HNSCC.

Keywords: Immunohistochemistry, metalloproteases, metalloproteinases, neoplasm invasiveness, squamous cell carcinoma of the head and neck

INTRODUCTION

Cancer of the head and neck is recognized as the tenth-most common cancer affecting people worldwide.^[1] The trinity of therapeutic options, namely surgery, radiotherapy, and chemotherapy have their own limitations and are associated with significant morbidity. The major cause of mortality in head and neck cancer is locoregional recurrence. Current evidence suggests that a complex interplay of different molecular and genetic factors determines the clinical behavior and aggressiveness of cancer and consequently its prognosis.^[2] Thus, the response to treatment varies owing to

the biological heterogeneity of these tumours. Cancer cells invade tissue and metastasize by degrading the extracellular

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matrix (ECM).^[3] Matrix metalloproteinases (MMPs) are endopeptidases, capable of degrading most ECM components and appear to be essential for tumour invasion.^[3] MMPs are not commonly expressed in normal tissue.^[4] The role of many MMPs has been studied in the progression of various cancers as well as metastasis but with inconsistent results and limited information on the actual correlation between tumour behavior and expression of these proteins.^[5] Previous studies have shown that MMP-9 is elevated in Head and Neck Squamous Cell Carcinoma (HNSCC). MMP-13 also plays a central role in the MMP activation cascade, both activating and being activated by several MMPs.^[6] An imbalance between MMPs and tissue inhibitor of metalloproteinases (TIMPs) is also a speculated mechanism for tumour aggressiveness as TIMPs inhibit the breakdown of ECM.^[7]

The primary objective of this study was to determine the correlation between the expression of MMP-9, MMP-13, TIMP-1, p16, and histological tumour differentiation, which was used as a surrogate measure of tumour aggressiveness. Results of this study can be extrapolated and applied for prognosticating patients based on their initial biopsy and to identify targets for individualized treatment in the future as inhibitors of MMP are an upcoming therapeutic option and the biological behavior of HNSCC in Indian population may be different from that seen in the developed world.^[8]

MATERIALS AND METHODS

Study design and population

We conducted a prospective observational study from August 2016 to December 2018, in the Department of Otorhinolaryngology in collaboration with the Department of Pathology and Lab Medicine of our institute. Ethical clearance was taken from the Institutional Ethics Committee before enrolling patients and a written informed consent was obtained from all participants and all ethical standards pertaining to the Declaration of Helsinki were observed.

We recruited 43 consecutive patients with histopathologically proven squamous cell carcinoma of the head-and-neck region. Patients were eligible to be included if their primary treatment was definitive surgery as decided by the tumour board. We excluded recurrent or previously treated cases, patients who received neoadjuvant chemotherapy or prior radiation therapy, and nonsquamous cell carcinomas to reduce confounding factors.

Procedure

The patients were clinically evaluated and the disease staged according to the most recent American Joint Committee on Cancer staging system in use at the time of surgery after a complete metastatic work up. All baseline clinical data were collected using a specified data collection pro-forma. All patients underwent surgery to address the primary tumour targeting complete tumour excision with adequate margins with or without appropriate neck dissection as indicated. The excised specimens were sent to the Department of Pathology

and Lab Medicine for processing. Inclusion in this study *per se* did not have a bearing on the course of further treatment or follow-up of the patients.

Grading of tumour

The surgical specimens were fixed with buffered formalin, embedded in paraffin and slides were prepared. The slides were then stained by hematoxylin and eosin and graded based on the WHO grading system for tumour differentiation by the second author.^[9] Tumour differentiation was used as a surrogate measure for tumour aggressiveness. However, the specimens included in the current study fell into two categories only (well and moderately differentiated tumour) based on their histological characteristics. We also looked for evidence of metastasis in the regional lymph nodes and the presence of lymphovascular invasion (LVI) or perineural invasion (PNI) as indirect evidence of increased tumour aggressiveness.

Immunohistochemical staining

Three µm thick sections of the specimen were cut, dewaxed, and retrieved at a pH of 9.0. Peroxide block was done for 10 min at room temperature and rinsed in phosphate buffer saline (PBS) for 5 min and the primary antibody was applied for 1 h. Sections were then rinsed in PBS for three cycles of 5 min each. Afterward, post-primary staining was done for 10 min followed by two cycles of rinsing. The sections were then incubated in peroxidase substrate solution (DAB Sigma-Aldrich, Darmstadt, Germany) for 30 min at room temperature and then rinsed in deionized water for 5 min. Hematoxylin was applied to all sections and rinsed in deionized water for 5 min. Finally, the sections were dehydrated by washing in 95% ethanol for 1 min, 100% ethanol for two cycles of 3 min each, and cleared in xylene for two cycles of 5 min each, and then mounted. We used a negative control in which the primary antibody was not used, for quality check for each immunohistochemical (IHC) marker. After the slides were prepared, they were examined for the expression of the respective marker under light microscopy. Breast cancer slides were used as control for the antibodies. The positivity of the stained slides was documented and recorded using Microsoft Excel.

Study variables

The primary variables which were estimated were the expression of MMP-9, MMP-13, TIMP-1 and p16, the histopathological grading of the tumour, the presence of regional lymph node metastasis confirmed by pathological examination, and LVI or PNI.

Statistical analysis

Chi-square test was done to analyze the relationship between the IHC marker positivity and the degree of differentiation of the tumour, lymph node metastasis, and LVI or PNI. All statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp. USA.

RESULTS

Samples from forty-three patients who met the inclusion criteria were included in the study. Of these, 23 were males

and 20 were females. The median age of the study population was 57 years (range 32–78 years). The primary tumour site distribution and pT (pathological tumour) staging is shown in Table 1. Twenty-eight patients had moderately differentiated squamous cell carcinoma, whereas 15 had well-differentiated squamous cell carcinoma. Out of the 43 cases, 12 patients had regional lymph node metastasis based on final histopathology. LVI and/or PNI was found in 11 patients.

MMP-9, MMP-13, and TIMP-1 were expressed in tumour cells in 72%, 34%, and 18% of cases, respectively. p16 was not found to be expressed in any of the cases. Tumour marker expression was found to be higher among the moderately differentiated carcinomas as compared to well-differentiated carcinomas except for TIMP-1 as depicted in Table 2. We did not have any case with poor differentiation; hence that correlation could not be studied.

The expression of the four markers in the study population and its correlation with the degree of tumour differentiation was analyzed using Chi-square test [Table 2]. The staining patterns of MMP-9, MMP-13, and TIMP-1 in representative slides are shown in Figure 1.

The median age of the patients was 57 years. We stratified the cases as patients older than 57 years and those 57 years or younger and studied the frequency of marker positivity in both the groups. On analyzing the data, we found that there was a significantly higher expression of MMP-13 in tumours occurring in the younger group of patients possibly denoting higher tumour aggressiveness and earlier manifestation [Table 3].

Several studies have evaluated the possible role of increased expression of these markers in the development of regional

nodal metastasis and LVI or PNI. We evaluated the positivity of these four markers in patients with lymph node metastasis and those with LVI or PNI using the Chi-square test but did not find any statistically significant association [Table 3].

DISCUSSION

There is a complex interplay between the expression of various metalloproteinases in the development of HNSCC. Targeted therapy against these markers has had very limited success due to extreme adverse effect profiles of the agents currently available.^[10] This study was conducted to determine if the expression of MMP-9, MMP-13, TIMP-1, and p16 was associated with the degree of differentiation of the tumour. Most studies have attempted to predict overall survival based on the expression of these markers. However, the role of these markers in tumour differentiation will help us to identify areas of research, such as patients who require adjuvant treatment, future biological agents, and framing treatment guidelines.

There is contrasting literature regarding the source of MMP-9. Some authors have reported it to be expressed predominantly in the stroma compared to the tumour.^[11] In a study done on gastric squamous cell carcinoma, it was shown that the inflammatory neutrophils present at the tumour interphase are responsible for the production of MMP-9, which in turn was correlated to increased angiogenesis.^[12]

In our study, MMP-9 was found to be over-expressed in tumour tissues compared to MMP-13 and TIMP-1. MMP-9 expression was found in 72% of our cases. Most slides showed intense nuclear and nucleolar positivity implying that the source of this proteinase was the tumour cells rather than the stromal cells. There was no statistically significant correlation between MMP-9 expression and the grade of tumour differentiation in our study. A meta-analysis based on 419 cases, reported that MMP-9 expression was associated with poorer overall survival, higher T stage and regional metastasis.^[13] However, the comparison based on the differentiation of the tumour was not reported.

There was a strong positive correlation between the expression of MMP-13 and the differentiation of the tumour ($P = 0.03$) even though the overall positivity was low (34%) compared to MMP-9 (72%). Studies have reported up to 81% positivity of MMP-13 expression in HNSCC.^[14] Based on experiments with immortalized head and neck cancer cell lines, Kudo *et al.* demonstrated that the MMP-13 promotes angiogenesis. This was also proven in HNSCC tumour tissue by IHC markers demonstrating increased number of blood vessels at the tumour front in MMP-13-positive cases.^[14] Thus, angiogenesis appears to be the most robust explanation for the role of MMP-13 in tumour progression.

There was also an increased incidence of MMP-13 positivity in specimens from patients who had age lesser than the median (57 years). Other studies have not found such a correlation.^[6] There was significant increase in the expression

Table 1: Baseline parameters of the study population

Parameter	n (total=43)
Gender	
Male	23
Female	20
Differentiation	
Well differentiated	15
Moderately differentiated	28
Poorly differentiated	0
Site	
Buccal mucosa	26
Tongue	9
Larynx	7
Maxilla	1
pT stage	
pT1	7
pT2	16
pT3	14
pT4	6
Presence of nodal metastasis	
pN0	31
pN+	12

Table 2: Individual immunohistochemical marker expression correlated with tumour differentiation

IHC marker	MMP-9		MMP-13		p16 [#]		TIMP-1	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Moderately differentiated (n=28)	22	6	13	15	0	28	5	23
Well differentiated (n=15)	9	6	2	13	0	15	3	12
<i>p</i>	0.19		0.03*		NA		0.86	

*Significant positive correlation between expression of MMP-13 and poorer differentiation, [#]p16 was not positive in any of the cases. NA: Not applicable, IHC: Immunohistochemical, MMP: Matrix metalloproteinase, TIMP: Tissue inhibitor of metalloproteinase

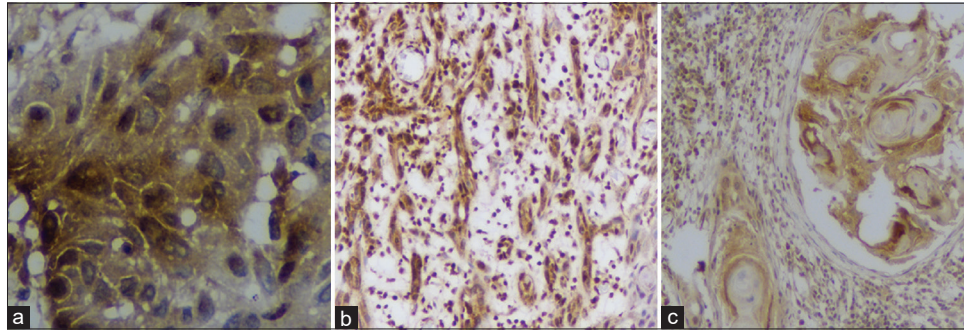


Figure 1: Immunohistochemical staining patterns of (a) tissue inhibitor of metalloproteinases-1- strong cytoplasmic positivity at 40x, (b) matrix metalloproteinase-9 nuclear and nucleolar staining pattern at 10x, and (c) matrix metalloproteinase-13-stromal cytoplasmic positivity at 10x

of MMP-13 in the moderately differentiated group compared to the well-differentiated group in our study. This indicates that MMP-13 positivity would predict poorer prognosis. Similar observations regarding the role of MMP-13 have been made based on studies done in breast cancer and esophageal cancer specimens even though the underlying mechanisms are unclear.^[15,16]

TIMP-1 expression in our study was 18% which is much lesser compared to other studies (48%–81%).^[14,17] We did not find any significant association between its expression and tumour differentiation. Carpen *et al.* demonstrated the positive correlation between elevated serum levels of TIMP-1 with poor prognosis in oropharyngeal squamous cell carcinoma. They suggested that in HPV-negative patients, TIMP-1 expression may have a greater role in oncogenic changes. However, in the same study, TIMP-1 IHC expression in tumour specimens did not reveal a significant association with prognosis.^[18]

It is well-established that p16 positive and HPV-positive squamous cell carcinomas have a better prognosis.^[19] Tumour cells need to have nuclear and cytoplasmic staining to be considered p16 positive. However, only cytoplasmic positivity was noted in our cases which is not considered true p16 positivity. We found no case with p16 expression as compared to reported levels of 3.5%–4.4% in some series. Thus, the interpretation of the role of other markers studied was not confounded by p16 positivity which is a known determinant of prognosis. Many authors do not consider p16 alone as an entirely representative surrogate marker for HPV status, especially in nonoropharyngeal tumours.^[20] Even though p16 positivity indirectly supports supposed viral infection in tumour tissue, a high percentage of HPV-positive tests in

nonoropharyngeal sites may be due to acute infection or false positives.^[21,22]

Regional lymph nodal metastasis is an indicator of significantly worse prognosis for the patient and is a clinical indicator of the aggressiveness of the tumour.^[23] We evaluated the correlation between node positivity and the expression of these four markers. Twelve patients had positive nodes on final histopathology. However, we could not find any statistically significant association between these parameters in our study. This is in contrast to recent studies which found nodal metastasis to correlate with high expression of MMPs especially MMP-9 and MMP-13.^[15,24,25]

Another parameter which suggests increased invasiveness is the presence of LVI or PNI in the tumour specimen. It has been reported that increased expression of different MMPs positively correlates with LVI and PNI.^[26,27] We could not detect any such positive correlation; possibly due to the small number of cases with LVI or PNI [Table 3].

Limitations of the study

The specimens included in the current study fell into two categories only-well differentiated and moderately differentiated tumours. None of the study participants had a poorly differentiated tumour. In addition, the sample size was limited.

To summarize, the IHC expression of MMP-9 and MMP-13 was upregulated in tumours with poorer differentiation. However, only the MMP-13 expression achieved statistically significant levels. TIMP-1, on the other hand, was not significantly overexpressed in either group. This indicates that determining a single prognostic marker may neither be possible nor be of any clinical value. There is a complex

Table 3: Correlation of the immunohistochemical marker expression with age, nodal metastasis and perineural invasion/lymphovascular invasion

Variable (n)	MMP-9		MMP-13		TIMP-1	
	Positive	Negative	Positive	Negative	Positive	Negative
IHC expression correlated with age (n)						
Age ≤57 (n=22)	16	6	11	11	4	18
Age >57 (n=21)	15	6	3	18	4	17
p	0.92		0.01*		0.92	
IHC expression correlated with node status (n)						
N0 (31)	22	9	9	22	5	26
pN+ (12)	9	3	6	6	3	9
p	0.90		0.19		0.05	
IHC expression correlated with PNI/LVI (n)						
PNI/LVI+ (11)	9	2	5	6	3	8
PNI/LVI- (32)	22	10	10	22	5	27
p	0.40		0.39		0.39	

*Statistically significant ($p < 0.05$). PNI: Perineural invasion, LVI: Lymphovascular invasion, IHC: Immunohistochemical, MMP: Matrix metalloproteinase, TIMP: Tissue inhibitor of metalloproteinase

interplay between various factors responsible for tumour behavior and prognosis. Panels of marker proteins rather than a single protein are likely to provide prognostic information for personalized and targeted treatment of head and neck cancer. While immunohistochemistry alone may not be foolproof in the identification and quantification of these markers, it certainly is a cost-effective method of providing prognostic information as compared to *in situ* hybridization and molecular techniques.^[4] Before recommending the incorporation of such tests into routine clinical work, more studies are needed to determine their actual prognostic value as well as cost-effectiveness.

CONCLUSION

Increased expression of the proteolytic MMP-13 contributes to tumour expansion by degrading components of the ECM, thereby accelerating tumour spread. Even though MMP-9 and TIMP-1 were also found to be over-expressed by tumour cells, a statistically significant correlation with the degree of differentiation could not be established. There was an increased expression of MMP-13 in patients who developed malignancy at an earlier age. We did not find any association between the presence of lymph node metastasis, LVI or PNI and the expression of any of these markers.

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Conflicts of interest

There are no conflicts of interest.

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