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Research Article

Expression of Matrix Metalloproteinases 2 and 9 in Laryngeal Carcinoma and it's Correlation to Short Term Outcome

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Abstract

Objectives: To study the expression of Matrix metalloproteinases 2 and 9 in laryngeal squamous cell carcinoma and their correlation with short term outcome.

Study Design: In a prospective study of 15 patients with LSCC hospitalized in a tertiary center, RT-PCR was used to examine the expression of MMP-2 and MMP-9 in tissue samples post-surgery and results were compared to clinicopathological features and prognosis.

Results: The positive expression of MMP-2 and MMP-9 in patients with LSCC was 86.66% whereas 13.33% showed decrease. MMP-2 and MMP-9 were increased in patient with Stage IV A (1 patient) and 85.7% of patients (12 patients) in Stage III showed increase in MMP-2 and MMP-9 and 14.3% (2 patients) showed decrease. Patients with metastatic lymph nodes showed 100% increase in MMP-2 and MMP-9 whereas patients with non-metastatic lymph nodes showed 92.3% increase and 7.7% decrease in MMP-2 and MMP-9. No significant association was found between nodal disease and stage and mmp-2 and mmp-9 ($p = 0.551$ and 0.685 resp.). The association between decrease in MMP-2 and MMP-9 to well differentiated and increase in MMP-2 and MMP-9 to poor differentiation was found to be significant ($p = 0.03$). The association between MMP-2 and MMP-9 upregulation and recurrence in follow up was not found to be statistically significant ($p = 0.685$).

Conclusion: The results of the present study indicate that the expression of both MMP-2 and MMP-9 are up-regulated in laryngeal cancers with 100% survival in shorter outcome of 6 months.

Keywords: MMP-2; MMP-9; Laryngeal Carcinoma

Introduction

Laryngeal carcinoma is frequent in Head and Neck cancers. Laryngeal carcinoma is still a problem worldwide with nearly 1.7 percent comprising new cancer diagnosis, 25 percent comprising all Head and Neck malignancies and out of which nearly 90 percent being squamous cell carcinoma. According to the data of National Cancer Institute's surveillance epidemiology and end results (SEER) programmes between 2009 - 2013, 3.2 per 1,00,000 men and women per year were the new cases of laryngeal carcinoma and the deaths were 1.1 per 1,00,000 men and women per year. At some point during their lifetime, nearly 0.4% of men and women will be diagnosed with carcinoma larynx based on 2010 - 2012 data.

According to Hospital Based Cancer Registry (HBCR) of the Regional Cancer Center (RCC) PGIMER, Chandigarh, from 2011 - 2013, the number of cancer patients recorded was 18068 (male: 10019; female: 8049). The ten leading cancer sites altogether contributed to 67.4% of all cancers among males and 74% of all cancers among females. Among males, cancer of the lung (10.9%) was the leading site followed by the cancer of oral cavity (10.7%) and carcinoma larynx being the 6th leading cancer site (6.2%). Among females, cancer of the breast (20.3%) was the leading site followed by the cancer of cervix (15.4%). Many factors are associated with laryngeal carcinoma such as tobacco use being the most important factor and other factors such as alcohol consumption, infection by virus, and genetic susceptibility are also associated with it.

Studies have also demonstrated that elevated matrix metalloproteinases [1] is associated with poor prognosis in a variety of cancers, and therefore, these MMPs can serve as a marker of tumor progression. MMPs are a family of zinc-dependent proteinases which are present in stromal cells and tumor cells and are capable of degrading essentially all extracellular matrix components, which is an important event in the invasion and also metastasis of many different malignancies. MMP-2 and MMP-9, among more than 23 members of the MMPs family have been found to degrade elements of extracellular matrix and basement membrane. MMP-2 (gelatinase-A) is located on chromosome 16q13-q21, causes digestion of gelatin, type IV collagen, and other bioactive molecules, such as growth factor binding proteins and other growth factor receptors [2,3]. MMP-9 *(*gelatinase B), among the MMPs is the most complex

MMP and is capable of degrading elastin, fibrillin, decorin, gelatin, laminin and collagens of types IV, V, XI and XVI. Over expression of MMP-2 and MMP-9 has been found to be associated with the development of cancer, including Head and Neck cancer, suggesting role of MMPs in Head and Neck cancer development.

Several epidemiologic studies of the association of MMP-2 and MMP-9 have been carried out [4-7]; the results show strong association between the endopeptidases and laryngeal carcinoma with prognostic outcome still needed to be explored.

Materials and Methods

The study was conducted in the Department of Otolaryngology and Head and Neck Surgery, Post Graduate Institute of Medical Education and Research, Chandigarh in collaboration with the Department of Experimental medicine and Biotechnology, Department of Histopathology and Department of Radiation Oncology. The study was prospective study from January 2015 to June 2016 and with 15 patients of resectable laryngeal squamous cell carcinoma taken for the study.

The following patients were included in the study: All Carcinoma larynx patients up to Stage IV A were enrolled in this study including patients with neck nodes; Both male and female sex; Age range between 25 years and 75 years; Patients willing to participate in the study. The following patients were excluded from the study: Patients with unresectable (more than stage IV A) laryngeal carcinoma; Patients less than 25 years and more than 75 years; Patients with recurrent disease; Patients already treated outside; Patients having any synchronous disease in any of the other head and neck subsite; Patients with chronic inflammatory diseases like TB, Leprosy, Sarcoidosis, etc.

Study plan: The study was a prospective analysis of 15 patients with resectable laryngeal carcinoma after the diagnosis and staging workup was completed.

15 patients with biopsy proven laryngeal cancer underwent total laryngectomy + neopharynx formation (+/- total/hemithyroidectomy) (+/- TEP insertion). Tumor tissue from laryngectomy specimen was subjected to RT-PCR for detection of MMP-2 and MMP-9. Similarly, normal tissue from 15 subjects were subjected to RT-PCR for detection of MMP 2 and 9 from supraglottis and the mean value was taken as control value.

RT-PCR for MMP-2 and MMP-9

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is an important tool to measure gene expression in biological samples. Biopsies were typically small and was transported in RNA latter and stored in -80°C and were snap-frozen immediately after removal from the body. Test was performed in two-steps of qRT-PCR, in which one RT tube per sample produced enough cDNA to perform 12 qPCR reactions, which allows for one endogenous control reference gene and triplicate analysis of three genes of interest. Optimization was done in present protocol for bias reduction.

SybrGreen employs one pair of specific primers and during amplification the fluorescent dye incorporates into the DNA. qPCR inhibitory agents may be present in tissue samples. OCT is a major factor in qPCR inhibition in therefore it is important that prior to qRT-PCR analysis RNA is extracted and isolated from the microdissected sample. The sample is subjected to DNase to ensure total RNA to be free of large DNA fragments because when using glass filter RNA extraction methods DNA is often a contaminant. The quantitation method to be chosen is appropriate for the range of RNA obtained which is on the order of 5 - 20 pg per cell. Bioanalyzer system and RNA integrity number (RIN) was used to provide an adequate assessment of total RNA quality.

Total RNA was extracted with mirVana™ miRNA isolation kit (Ambion, USA). Total RNA (1microgram) was used for synthesis of cDNA with RevertAid™ reverse transcriptase by using RevertAid first strand cDNA synthesis kit. Real-Time PCR 7500 (Applied Biosystem) was used for performing qRT-PCR. Then, 20 microlitre final reaction volume was prepared with the following reagents: 10 microliter SYBR Green Taq ReadyMix (Applied Biosystem), 0.5 microliter of each forward and reverse primers (MMP-2 and MMP-9), 8 microliter dH_2O and 1 microliter cDNA. Melting curve analysis was used in determining the melting temperature (Tm) of specific amplification products and primer dimers. The data was normalized to 18S RNA to account for differences in reverse-transcription efficiencies and amount of template in the reaction.

Figure: Showing CT value of MMP-2 (blue), MMP-9 (green) and 18s (endogenous control) (pink).

CT value i.e. the cycle threshold is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level) which shows the number of cycles required to detect a real signal of gene from the samples.

Relative quantitation

All biopsy proven laryngeal cancer patients underwent detailed history taking, physical examination and routine haematological and biochemical tests before surgery. The diagnosis of laryngeal cancer was done with laryngeal biopsy in all cases. CECT BOS to T4 was done in all patients prior to surgery for assessing the disease

Steps	Formula	Definition
1. Gene expression normalization	$\Delta C_T = X - EC$	For a given sample (diseased or normal), X is C_T value of gene of interest and EC is the endogenous control C_T value.
2. Comparison of gene expression	$\Delta\Delta C_T = C_T x - C_T cb$	For a given case: x is the gene of interest and cb is the calibrator (normal tissues). (comparing diseased (D) and normal (N) tissues from the same organism)
3. Biologic fold change	$2^{-\Delta\Delta CT}$	Translates the C_T value in terms of biologic fold change to show biological significance. If C_T is a positive number after calculating 2^{-CT} transform by 1/x to obtain the under expression value.

Table 1

extent and staging. After admission of the patients in the Otolaryngology Inpatient Department, surgery was done as planned according to disease extent. Post-operative radiotherapy was given to all patients with positive risk factors for disease recurrence.

All patients were followed up at 3 months and 6 months after treatment completion by clinical assessment for local/loco-regional control and recurrence.

Statistical analysis

The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS for Windows). All the measurable data was checked for their Normality by Kolmogorov Smirnov test. For normally distributed data means were compared using paired student t-test. Quantitative data was measured using mean ± standard deviation. Qualitative data (Classified/ categorical) was described as frequencies and percentage and was analyzed for its association using Chi-square test or Fisher's exact test. Using Pearson's correlation coefficient, correlation between the variables were found out. Survival was assessed by Kaplan Meier method. The data is also presented graphically by Bar Diagrams. A 'P' value of < 0.05 was considered significant in all the tests.

Results

The study consisted of 15 patients with laryngeal squamous cell carcinoma who underwent surgery in Department of Otolaryngology and Head and Neck Surgery, PGIMER, Chandigarh. RNA was isolated from tumor tissue and expression of MMP-2 and MMP-9 were analysed. Similarly, normal tissue obtained from supraglottis were also analysed using qRT-PCR. The mean value of control samples (n = 15) was used as cut-off to determine change in expression of MMP-2 and MMP-9 in patient samples (n = 15). Those having higher relative quantification values (RQ values) were considered increased expression and vice-versa. The cases were also analysed for a number of parameters which included age, gender, addictions, site of larynx involved, histopathology, lymph node status, MMP-2 expression, MMP-9 expression, post-operative recurrence at 3 months and 6 months and survival.

Gender: All the 15 patients in the study were males.

Age: The average age of the patients was 55.53 years (range = 40 - 66 years).

Out of 15 patients 86.66% (n = 13) showed overall increase in MMP-2 and MMP-9 expression whereas 13.33% (n = 2) showed decrease in expression of MMP-2 and MMP-9 as compared to mean RQ value of control.

Relative quantitation of MMP-2

Sample	X	EC	ΔC_T = X - EC	$C_T cb$	$\Delta\Delta C_T = C_T x - C_T cb$	$2^{-\Delta\Delta CT}$
1	27.62	12.33	15.29	14.256	1.033636	0.488477
2	24.83	14.62	10.20	14.256	-4.04886	16.5512
3	26.91	14.0	12.9	14.256	-1.34886	2.547114
4	24.29	11.28	13.01	14.256	-1.24136	2.364219
5	24.67	13.22	11.4	14.256	-2.80886	7.007324
6	26.49	12.88	13.61	14.256	-0.64386	1.562508
7	33.94	20.38	13.56	14.256	-0.69636	1.620415
8	25.94	13.43	12.5	14.256	-1.75136	3.366766
9	25.13	11.59	13.5	14.256	-0.72136	1.64874
10	26.43	12.87	13.56	14.256	-0.68886	1.612013
11	27.66	19.32	8.34	14.256	-5.91636	60.39527
12	25.66	13.32	12.34	14.256	-1.92386	3.794379
13	30.31	18.64	11.67	14.256	-2.58386	5.995432
14	26.47	10.86	15.6	14.256	1.351136	0.391983
15	29.1	18.12	10.9	14.256	-3.27886	9.705911

Table 2

Relative quantitation of MMP-9

X = C_T value of gene of interest (MMP-2 or MMP-9),

EC = C_T value of Endogenous Control,

ΔC_T = Gene expression normalization within a sample,

$C_T cb$ = Mean of C_T value of MMP-2 or MMP-9 of the 15 control samples

$\Delta\Delta C_T$ = Gene expression comparison between cases and controls

$2^{-\Delta\Delta CT}$ = Expression of C_T value in terms of biological fold change.

Addictions

Out of 15 patients enrolled in study, 14 were smokers, 8 were alcoholics and 1 had no history of addiction. A significant association was found between smoking and increased expression of MMP-2 and MMP-9 (p = 0.008). However, no significant association was observed between alcohol and increased expression of MMP-2 and MMP-9 (p = 0.104).

Sample	X	EC	$\Delta C_T = X - EC$	$C_T cb$	$\Delta\Delta C_T = C_T x - C_T cb$	$2^{-\Delta\Delta CT}$
1	26.67	12.33	14.34	12.80227	1.545227	0.342642
2	23.91	14.62	9.29	12.80227	-3.50977	11.39061
3	24.82	14.0	10.8	12.80227	-1.98477	3.958003
4	22.71	11.28	11.4	12.80227	-1.37477	2.593271
5	22.78	13.22	9.56	12.80227	-3.24227	9.462837
6	24.73	12.88	11.85	12.80227	-0.94477	1.924886
7	30.71	20.38	10.33	12.80227	-2.47227	5.549173
8	25.24	13.43	11.8	12.80227	-0.99477	1.992767
9	23.2	11.59	11.6	12.80227	-1.19477	2.289088
10	24.94	12.87	12.07	12.80227	-0.73227	1.661254
11	24.78	19.32	5.4	12.80227	-7.34727	162.8356
12	25.1	13.32	11.7	12.80227	-1.02977	2.041703
13	25.54	18.64	6.9	12.80227	-5.89977	59.70471
14	24.98	10.86	14.12	12.80227	1.317727	0.401166
15	25.98	18.12	7.8	12.80227	-4.94477	30.79817

Table 3

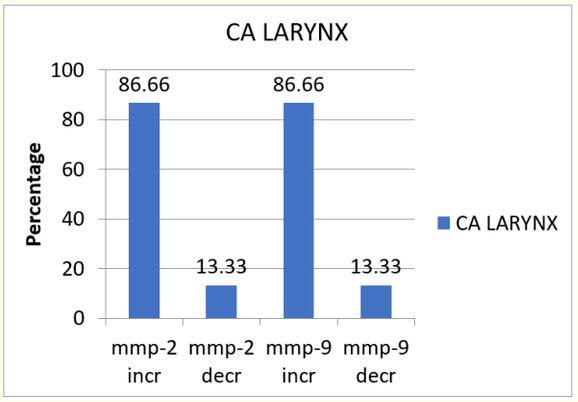

Figure 1: Bar diagram showing MMP-2 and MMP-9 expression among laryngeal carcinoma patients (incr: Increased Expression; decr: Decreased Expression).

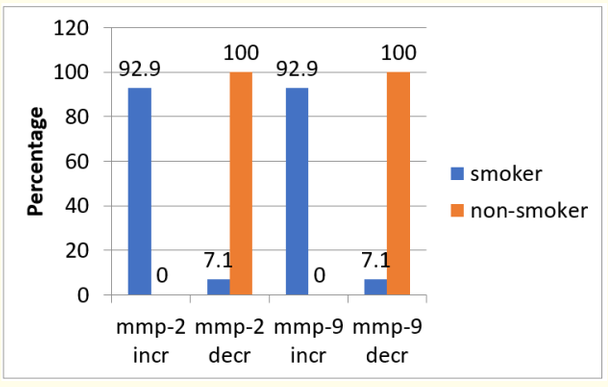

Figure 2: Bar diagram showing positive association between smoking and MMP-2 and MMP-9 levels (incr: Increased Expression; decr: Decreased Expression).

Disease factors

Out of 15 patients enrolled in the study, 14 were in stage III and 1 patient was in Stage IV A. MMP-2 and MMP-9 expression were increased in 1 patient with Stage IV A. Among patients in stage III, 85.7% (n = 12) of patients showed increased expression of MMP-2 and MMP-9 and 14.3% (n = 2) showed decreased expression of MMP-2 and MMP-9. However, the association of stage and increased expression of MMP 2 and MMP 9 was not statistically significant (p = 0.685).

Tumour differentiation

On histopathological analysis of 15 enrolled patients, 13 had moderately differentiated squamous cell carcinoma and 1 each had well differentiated and poorly differentiated squamous cell carcinoma respectively. Among moderately differentiated, 92.3% (n = 12) had increase in both MMP-2 and MMP-9 and 7.7% (n = 1) had decreased expression. In case of well differentiated patient sample, decreased expression of MMP-2 and MMP-9 was observed. Poorly differentiated patient sample had increased expression of both MMP-2 and MMP-9. A significant association was observed between decreased expression of MMP-2 and MMP-9 in well differentiated and increased expression of MMP-2 and MMP-9 in poor differentiation (p = 0.03).

Nodal disease

On histopathological analysis of 15 enrolled patients post-surgery, 2 had metastatic lymph nodes whereas 13 patients had non-metastatic lymph nodes. All patients with metastatic lymph nodes (n = 2) had increased MMP-2 and MMP-9 expression. Among the patients with non-metastatic lymph nodes 84.6% (n = 11) had increased expression of MMP-2 and MMP-9 and 15.4% (n = 2) had decreased expression of MMP-2 and MMP-9. No statistically significant association was observed between nodal disease and expression of MMP-2 and MMP-9 (p = 0.551).

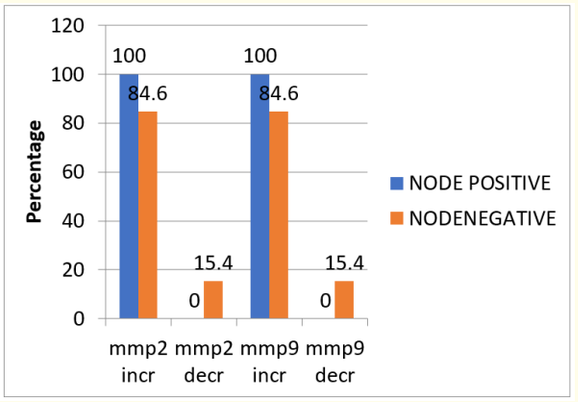

Figure 3: Bar diagram showing lymph node association with MMP-2 and MMP-9 expression. (Biological fold change observed in 2 samples with node positive were - MMP-2: 3.79 and 9.7 and MMP-9: 2.04 and 30.79) (incr: Increased Expression; decr: Decreased Expression).

Follow-up

Out of 15 enrolled patients, 1 had recurrence of disease in 3 months and no recurrence was observed in 14 patients. Patient with recurrence had increased expression of MMP-2 and MMP-9 whereas out of 14 patients with no recurrence, 85.7% (n = 12) showed increased expression of MMP-2 and MMP-9 and 14.3% (n = 2) showed decreased expression of MMP-2 and MMP-9. Survival in patients was 100% during 3 months and 6 months follow up. No statistically significant association was observed between MMP-2 and MMP-9 expression and follow up survival (p = 0.685).

Discussion

Head and Neck cancer comprises 5 - 10 percent of all the tumours and is the 8th and 16th most common malignancy in males and females respectively. The variation in the incidence is seen with the change in the geographical location as high rates are being reported in India, France, South America and Eastern Europe [8]. The different nature of the disease course in carcinoma larynx has been observed among the patients. Many factors are associated with increased risk of carcinoma larynx such as tobacco use, alcohol consumption, viral infection, and genetic susceptibility with tobacco being the most important risk factor.

Studies have also demonstrated that elevated matrix metalloproteinases in a variety of cancers are associated with poor prognosis and can serve as a surrogate marker of tumor metastasis and progression [1]. MMPs are a family of zinc-dependent proteinases present in stromal cells and tumor cells and are found to be playing role in degradation of extracellular matrix components, which is a thought to be an important event in the tumor invasion and metastasis. More than 23 members of the MMPs family have been described and out of which MMP-2 and MMP-9 as described being able to degrade extracellular matrix and basement membrane have specific roles in tumor progression, invasion and metastasis. Lymph node metastasis and tumor invasion has been associated to MMP-2 and MMP-9, by activating specific growth factors has been found to be controller of neovascularization.

Conclusion

- The present study suggests that both MMP-2 and MMP-9 had increased expression in laryngeal cancers with 100% survival in shorter outcome of 3 and 6 months.
- High expression of MMP-2 and MMP-9 can cause early recurrence in laryngeal carcinoma patients, thus highlighting the need for early adjuvant therapy following surgery.
- MMP-2 and MMP-9 can be used as reliable markers in laryngeal carcinoma patients for early detection of recurrence and metastatic disease.
- However, further investigation with larger sample size and longer follow-up is necessary to explore the expression and role of MMP-2 and MMP-9 in relation to prognostic outcome in laryngeal cancer patient.

Bibliography

1. Brinckerhoff CE., *et al*. "Interstitial collagenases as markers of tumor progression". *Clinical Cancer Research* 12 (2000): 4823-4830.

2. Stokes A., *et al*. "Expression profiles and clinical correlations of degradome components in the tumor microenvironment of head and neck squamous cell carcinoma". *Clinical Cancer Research* 16.7 (2010): 2022-2035.

3. Price SJ., *et al*. "Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation". *Journal of Biological Chemistry* 276.10 (2001): 7549-7458.

4. Bellayr IH., *et al*. "Biochemical insights into the role of matrix metalloproteinases in regeneration: challenges and recent developments". *Future Medicinal Chemistry* 1.6 (2009): 1095-1111.

5. VanSaun MN and Matrisian LM. "Matrix metalloproteinases and cellular motility in development and disease". *Birth Defects Research Part C Embryo Today* 78.1 (2006): 69-79.

6. Stetler-Stevenson WG., *et al*. "Extracellular matrix 6: role of matrix metalloproteinases in tumor invasion and metastasis". *FASEB Journal* 7.15 (1993): 1434-1441.

7. Nelson AR., *et al*. "Matrix metalloproteinases: biologic activity and clinical implications". *Journal of Clinical Oncology* 18.5 (2000): 1135-1149.

8. Moore SR., *et al*. "The epidemiology of mouth cancer: a review of global incidence". *Oral Diseases* 6.2 (2000): 65-74.