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Expression of biomarkers vimentin and Metastasis Associated 1(MTA1) protein in different stages of laryngeal cancer and their impacts on survival

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Abstract

Laryngeal cancer is the second most common cancer after the lip and oral cavity cancer ofhead and neck region[1]. Laryngeal malignancy is the seventh most common malignancy in males in India. Total number of new laryngeal cancer and death from laryngeal cancer have been estimated 12410 and 3760 respectively in 2019[2]. As per last survey available the number of new cases diagnosed and total number of deaths from laryngeal malignancy were 25,460 and 17,560 respectively in 2012 in India[3] . The incidence of cancer larynx is 1.26-8.18 per 100,000 populations in different regions of India. Carcinoma larynx constitutes40% of all Head and Neck cancers. Squamous cell carcinoma constitutes 95% of all malignancies of larynx. Glottis being the most common subsite followed by supraglottis and the least common site is the subglottis. There is an increasing incidence rate of this malignancy among middle aged and elderly men and women throughout the world. Expression of Biomarkers at different stages of laryngeal cancers and their impact on survival can play a crucial role in future management of laryngeal malignancies. In spite of having different modalities of treatments like surgery, radiotherapy, chemoradiation, the prognosis of laryngeal malignancies is still poor. The most common adverse factor for laryngeal cancer has been found lymph node metastasis. The process of progression of LSCC is complex and difficult to know it completely. Despite multi-modality therapeutic advances in recent decades, improvements in overall survival and disease free survival is very less. In this study we emphasized the role of expression of biomarkers vimentin and metastasis associated 1(MTA1) protein in different stages of laryngeal cancer and their impacts on survival.

Keywords: Laryngeal cancer, Biomarker, MTA1, Vimentin

1. Vimentin

Vimentin type III intermediate filament protein is present in all mesenchymal cells. Its main function is to maintain cellular integrity as well as shape and resistance against stress. It is a biomarker of epithelial-to-mesenchymal transition (EMT)[4] . Vimentin is being found elevated in many epithelial cancers such as prostate cancer, gastrointestinal tumors, CNS tumors, Head &Neck cancer, breast cancer and malignant melanoma. Elevated level of vimentin is associated with lymph node metastasis. The role of vimentin expression in OSCC and metastases is controversial. VE-cadherin, CD44,

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and vimentin are related to angiogenesis and VM (vasculogenic mimicry) formation in OSCC, therefore, in tumor progression and metastasis.

2. MTA1

MTA (metastasis-associated gene) is a newly discovered family of cancer progression-related genes and their encoded products. MTA1 is the first member of this MTA gene family. MTA1 of the MTA (metastasis-associated) gene family is found mainly in nucleus but also present in extra-nuclear compartment. It's an integral part of nucleosome remodeling and histone deacetylation (NuRD) complex[5]. MTA1 is elevated in many solid tumors like gastric, breast, ovarian, colon, prostrate and head and neck cancer. MTA1 is one of the most important prognostic oncogenepresent in human cancers. Elevated level of MTA1 is directly related to the aggressiveness and poor prognosis of different human cancers. Wang H et al suggested that the overexpression of the MTA1 gene correlates with lymph node metastasis in carcinomas of larynx[6]. Laryngeal squamous cell carcinoma did not respond evenly to either radiotherapy or chemoradiotherapy due to some resistance against them. This causes residual disease or early recurrence or death in spite of radical treatment for organ sharing therapy for laryngeal carcinoma. Different biomarkers have been identified in laryngeal cancer and their prognostic role in this malignancy to overcome resistance mechanism. Biomarkers like p53, Bcl-xL, CD4, EGFR and MDM2 are evaluated as predictive and prognostic factors in laryngeal cancers. In the past however Vimetin and MTA1 are thought to be novel biomarkers. Cancer cells cause genetic alterations that lead to alteration in the molecular pathways, uncontrolled proliferation, and down-regulation of apoptosis, increased vascularisation, and an altered metabolism. According to Tang et al MTA1 gene is strongly related to LSCC with lymph node metastasis. Predictive biomarkers found in advanced LSCC may be future deciding factor for treatment. With this hypothesis in consideration, we have studied the over expression profile of vimentin and MTA1 and their impact on survival in laryngeal cancers.

3. Material and Methods

This is a prospective cohort study of 90 laryngeal squamous cell carcinoma patients who were suitable for radical treatment presenting to outpatients department of Otolaryngology & HNS at PGIMER, Chandigarh from October 2019 to June 2021.

3.1. Histopathological examination:

The slides (hematoxylin and eosin: H&E-stained) of all the cases were reviewed by histopathologist, and the original histopathological diagnosis of squamous cell carcinoma was reconfirmed.

3.2. Immunohistochemistry

3.2.1. Principle of immunohistochemistry

Immunohistochemistry is a technique to detect the antigens in cells within a tissue section with the help of labelled antibody based on antigen-antibody reactions. The primary antibody binds to specific tissue antigens. The biotinylated secondary antibody is directed to the primary antibody. The streptavidin/ HRP complex is then applied. Streptavidin then attaches to the biotin on the secondary antibody and horse radish peroxide (HRP) acts as the indicator enzyme. On addition of a 3, 3- diaminobenzidine (DAB) chromogen, a colored precipitate develops at the tissue antigen sites.

3.2.2. Procedure

The biopsied tissue was fixed in 10% buffered formaldehyde and paraffin embedded. IHC was carried out at least on one representative block in all the cases. For immunohistochemistry, 4-µm sections of a representative block were obtained. The primary antibodies used were: anti- Vimentin (Cell Marque, dilution 1:100) and anti- Metastasis-associated 1(MTA) (Santa Cruz Biotechnology; dilution 1:100) Immunohistochemistry was performed on Ventana, Biotek System with appropriate positiveand negative controls run concurrently 12 Briefly, paraffin sections were mounted on charged glass slides, air-dried over-night, and then deparaffinized. To enhance the immunostaining, a heat-induced epitope-retrieval procedure was performed. After incubation with blocking serum, sections were incubated with primary antibodies, followed by a biotinylated polyvalent secondary antibody solution (Ultra View from Roche Ventana). Sections were then incubated with horse radish peroxidase conjugated avidin-biotin complex, followed by 3,3diaminobenzidine (DAB) andhydrogen peroxidase. Sections were counter stained with hematoxylin, cleared and mounted with Dibutylphthalate Polystyrene Xylene (DPX). Appropriate positive controls and negative controls were also used following the guidelines of the manufacturer of each antibody.

3.2.3. Evaluation

A visual semi-quantitative grading scale was applied to assess the immunoreactivity. Vimentin:

The evaluation of vimentin immunostain in the tumor cells was considered positive as cytoplamic immuno expression.

3.3. MTA 1

The evaluation of MTA1 was recorded as nuclear positivity in tumor cells of alllaryngeal carcinomas. The evaluation and semi-quantitative scoring of vimentin and MTA1was done as detailed in table 1. For scoring, the intensity of staining and the percentage positivity in the tumor cells was taken into account. Percentage positivity score and intensity score were multiplied to achieve a final score that ranged from 0-9. Score less than 3 was taken as weak positivity and above 3 was taken as strong positivity.

Table 1 Evaluation and scoring of Vimentin and MTA1

Proportion Score	Positive cells (%)	Intensity	Intensity Score
0	0	None	0
1	<25	Weak	1
2	26 to 50	Intermediate	2
3	>50	Strong	3

4. Results

This prospective study had taken 90 patients of laryngeal cancer of different stages and different subsites of larynx and novel biomarkers MTA1 and Vimentin were studied in laryngeal squamous cell carcinoma tissues. The salient characters of the study are shown in table 2.

Table 2 Salient characteristics of the study cohort

Sl No.	character	Number	%age
1	Total number of patients	90	100
2	**Age(in yrs)**	**Median**	**Std. Deviation**
	35-77	61yrs	8.48
3	**sex**	**Number**	**%age**
	Male	84	93.33
	Female	6	6.67
4	**addiction**	**Number**	**%age**
	smoking	79	87.78
	alcohol	64	71,11
	Smoking +Alcohol	64	71.11
5	**Comorbidities**	**Number**	**%age**
	Diabetic mallitus	7	7.78
	Hypertension	9	10.00
	Dm+htn	5	5.56
	Coronary artery disease	3	3.33
6	**Disease subsitesof larynx**	**Number**	**%age**
	Supraglottis	45	50
	Glottis	45	50

	Subglottis	00	00
7	**Histopathological tumour grade**	**Number**	**%age**
	Well differentiated	00	00
	Moderately differentiated	88	97.78
	Poorly differentiated	2	2.22
8	**Tumor Stage distribution**	**Number**	**%age**
	I	9	10
	II	17	18.89
	III	35	38.89
	IVA	25	27.78
	IVB	4	4.44
9	**PRESENT STATUS**	**Number**	**%age**
	ALIVE	78	86.67
	DEAD	12	13.33
10	**TREATMENT GIVEN**	**Number**	**%age**
	SURGERY & POST-OP RT	12	13.33
	RADIOTHERAPY(RT) ALONE	45	50.00
	CHEMORADIOTHERAPY(CTRT) ALONE	28	31.11
	RT F/B SALVAGE SURGERY	3	3.33
	CTRTF/BSALVAGE SURGERY	2	2.22
11	**Recurrence in laryngeal cancer at 21 months**	**Number**	
	Recurrence	31	34.44
	Norecurrence	59	65.56

The pattern of tumor Biomarkers positivity MTA1and Vimentin in laryngeal cancer is shown in details in table 3. In this study mean age was 60.05 with a SD of 8.48, majority were male (93.3%) and smoking association was present in 87.78%, alcohol was in 71.11 % and both together was in 71.11%.Diabetes association was seen in 7.7 %, hypertension was in 10 %, both together was in 5.56%. 50 % of diagnosed cases were glottis carcinoma and other 50% were supra-glottic carcinoma and majority of them were in advanced stage T3 40%, T4 27.78%. Tumor Biomarkers positivity shows that MTA1was strongly positive in all the 90 cases where as Vimentin was found positive in 29 cases only. Regarding nodal status, supraglottic carcinomas were commonly presented with regional lymph node metastasis with incidence of 44.40%whereas glottis cancers were 11.10% and none of the cases in our study showed distant metastasis. Histolopathologically mostly were moderately differentiated SCC i.e. 97.78% and 2.22% poorly differentiated. Supraglottic carcinomas were seen at advanced stages at the time of presentation whereas glottic cancer was presented early as well as advanced stages at the time of diagnosis. Stage III was more common (38.89%), followed by stage IV A (27.78%).Majority of laryngeal cancers in our study were treated with radical radiotherapy (50%), followed by chemoradiation (31.11%).After treatment 78 cases (86.66%) are surviving (13.33%) & 12 cases had expired and recurrence was found in 31 cases (34.4%) at 21 months follow up. Overall survival was better in glottic cancer than supraglottic cancer at 21 months. Disease free survival was better in glottic cancer than supraglottic cancer at 21 months. Mean of overall survival in months is 10.66 and the standard deviation is 4.45.When the recurrence status was compared with T, N category and staging only N category shows a significant difference though statistically was not significant.There was no significant difference when present live/ dead status is compared with Surgery, Radiotherapy and Chemoradiotherapy.

Fig.1a, 1b are showing Vimentin negative staining and positive staining and 1c and 1d pictures are showing different strength of MTA1staining.Fig.1a slide shows cytoplastic vascular componentis stained with vimentin on IHC with clear cytoplasm on IHC Fig.1b slide shows cytoplasm is densely stained vimentin on IHC. Fig. 1c slide shows nuclear staining with MTA1 biomarker with approximately 60% positive cell with intensity score 3 i.e., strong intensity.Fig.1d slide shows nuclear staining with MTA1 biomarker with approximately 80% positive cell with i intensity score 3 i.e. strong intensity.

Table 3 Patterns of tumor Biomarkers positivity MTA1and Vimentin in laryngeal cancer

(n=90)	**Early stage (Stage 1+2) n=27**		**Advance stage (Stage 3+4) n=63**		**Chi-Sq.**	**p-Value**
Vimentin	n	%	n	%		
None	20	74.07	41	65.08	3.72	0.294
Weak	0	0.00	6	9.52		
Intermediate	5	18.52	8	12.70		
Strong	2	7.41	8	12.70		
Total	7	25.97%	22	34.92%		
MTA1						
None	0	0.00	0	0.00	0.43	0.510
Weak	0	0.00	0	0.00		
Intermediate	0	0.00	1	1.59		
Strong	27	100.00	62	98.41		
Total	27	100.00%	63.00%	100.00%		

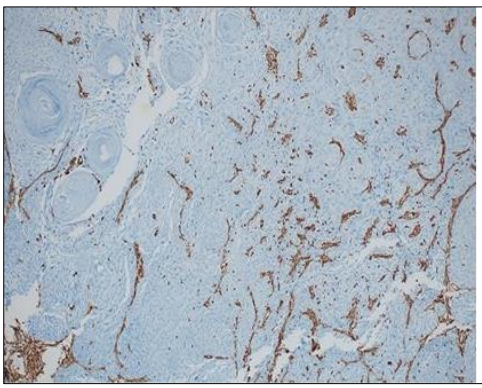

Figure1a: vimentin negative staining

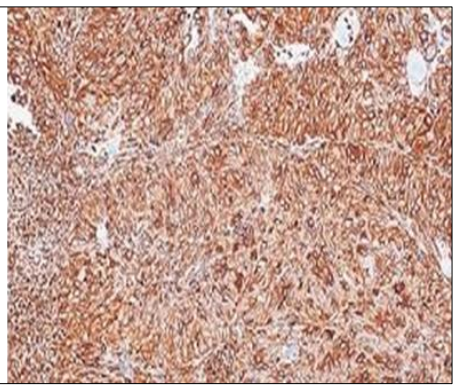

Figure1b: vimentin positive staining

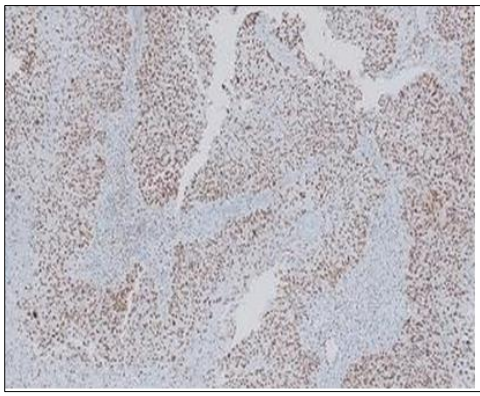

Figure 1c: MTA1 staining strongly positive

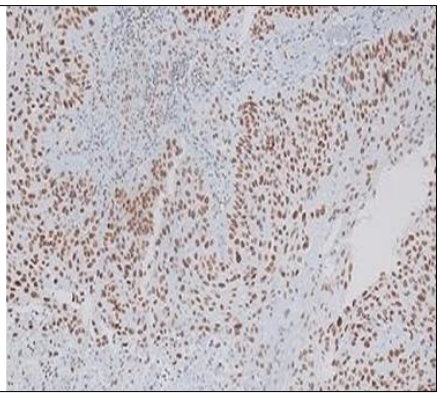

Figure 1d: MTA1 staining strongly positive

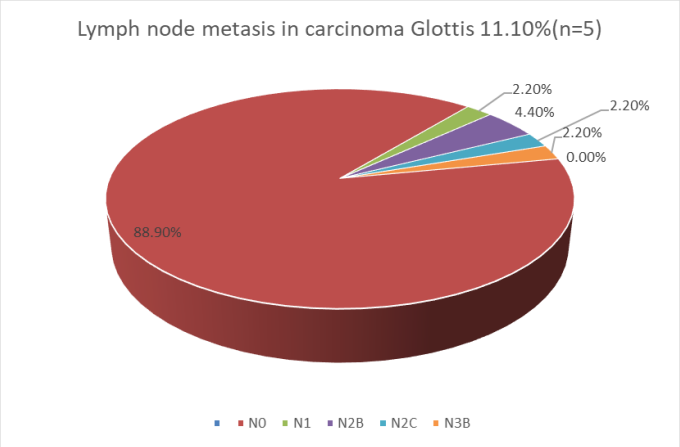

Figure 2 Lymph node metastasis in carcinoma Glottis

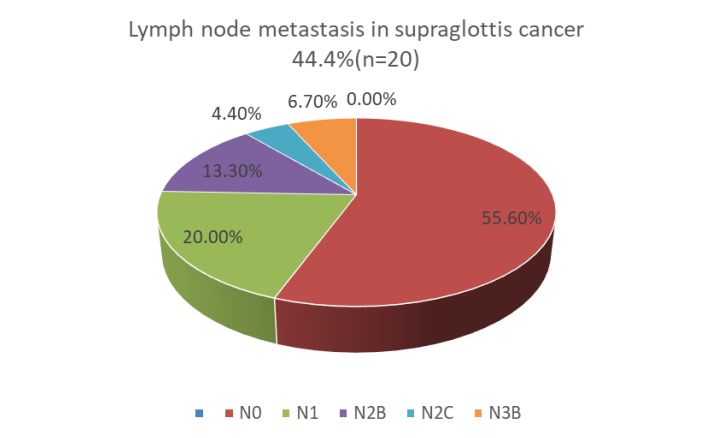

Figure 3 Lymph node metastasis in carcinoma Supraglottis

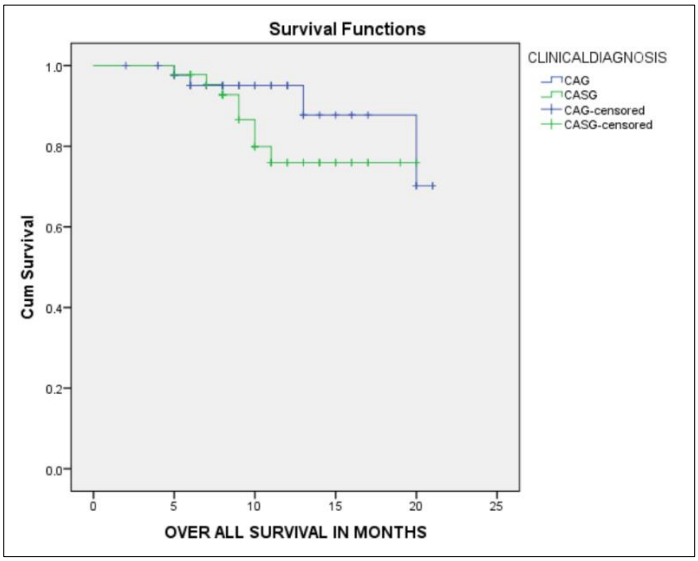

Figure 4 This Kaplan Meier curve shows that mean overall survival of different stages of laryngealcarcinoma in glottis subsite was 19.473± .759 (SE) months and in supraglotticsubsite was 17.334 ± .833 (SE) months. This difference was statistically was not significant. Log Rank (Mantel-Cox) p value 0.239

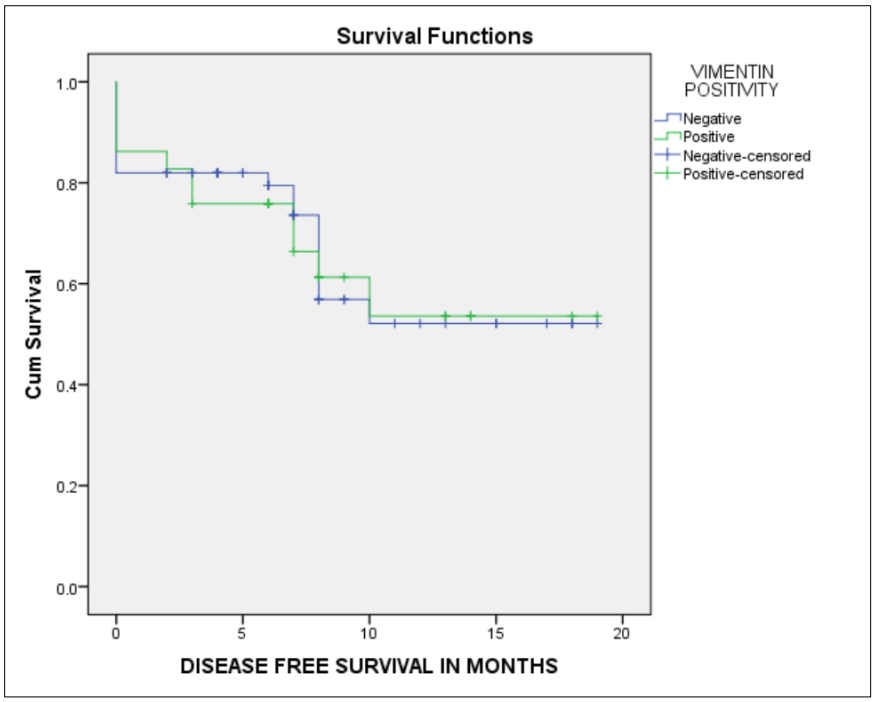

Figure 5 This Kaplan Meier curve shows that mean disease free survival of Vimentin positive laryngeal carcinoma was 12.301±1.560 (SE) months and in Vimentin negative laryngeal carcinoma was12.278± 1.159(SE) months. This difference was statistically not significant.Log Rank (Mantel- Cox) p value 0.926

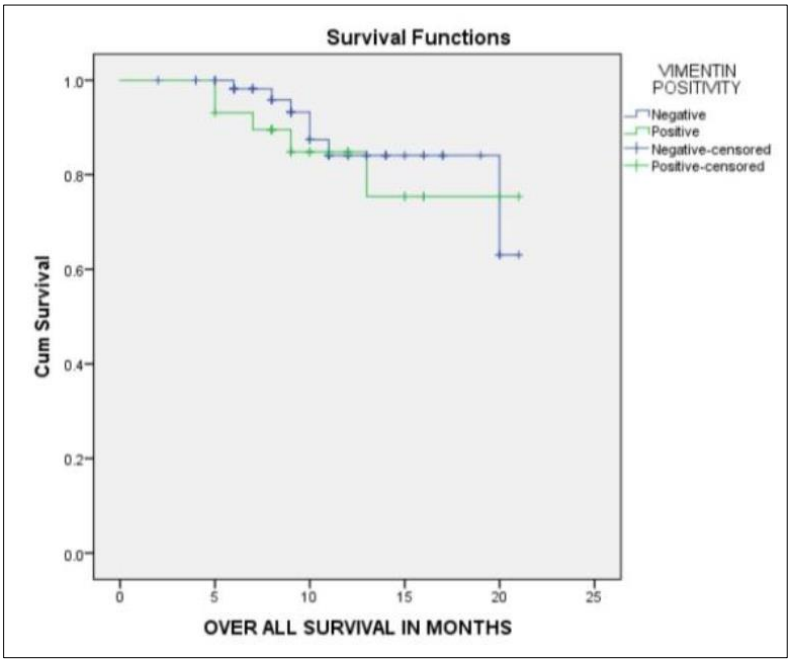

Figure 6 This Kaplan Meier curve shows that mean overall survival of Vimentin positive laryngealcarcinoma was 12.113± 0.842 (SE) months and in Vimentin negative laryngeal carcinoma was11.005± .635 (SE) months. This difference was statistically not significant. Log Rank (Mantel-Cox) pvalue 0 g.29.422

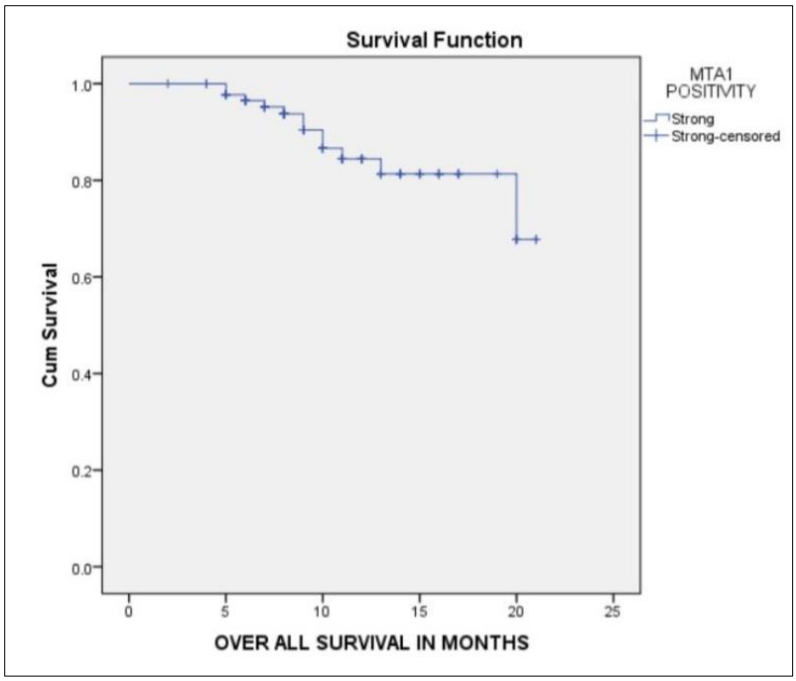

Figure 7 This Kaplan Meier curve shows that mean DFS of MTA1 positive laryngeal carcinoma was 12.326± .928 (SE) months and overall DFSl of MTA1 positive laryngeal carcinoma was also12.326±.928 (SE)because there was no negative MTA1 laryngeal carcinoma case was found

Laryngeal carcinoma shows high rates of locoregional recurrence in some patients even after radical treatment modality either surgery or radical radiotherapy / chemoradiotherapy In literature many tumor biomarkers had been explored to predict the outcome and prognosis of early as well as advanced laryngeal cancer. Tumor biomarkers for laryngeal cancers studied so far are BAK Bcl2, BclxL, CD24, EGFR, MDM2, NFκB, p53, PCNA, ,RhoC ,Survivin, Vimentin, MTA1 but none of them were proven to be significant in predicting LARYNGEAL CANCER outcome. Role of Tumor Biomarkers like vimentin and MTA1 in predicting overall survival and disease-free survival in case of different stages of laryngeal cancer have not been studied in detail in literature till now while the other biomarkers have been studied by many authors. Bradford et alhad studied various biomarkers (cyclin D1, CD24, EGFR, MDM2, PCNA, p53, survivin, Bcl-xL, Bcl-2, BAK, rhoC, and NFκB) in 58,47 cases of advanced laryngeal cancer .They recommended that biomarkers expressions in pretreatment biopsies may predict prognosis of laryngeal cancer.Yice Xu et alhad studied that SOX18 (SRY-related HMG-box 18) plays an important role in laryngeal cancer.SOX18 is responsible for cell proliferation, migration, and invasion in laryngeal cancer and also regulates different biomarkers like cyclinD1, caspase-3 ,MTA1,MMP-2 and MMP-7 in laryngeal cancer. MTA1 of the MTA (metastasis-associated) gene family is found mainly in nucleus but also present in extra nuclear compartment. It's an integral part of nucleosome remodelling and histone deacetylation (NuRD) complex. It is found in all tissues and cell lines, normal as well cancer cells, embryonic and adult cells, but its expression is lower in normal cells thancancerous cells. In our study it has been seen that MTA1 over expression is much higher in advanced disease than early disease. MTA1 biomarkers over expression was seen in all cases (100% overexpression) of our study. It was also seen that MTA1 was found highly over expressed in advanced laryngeal cancer and also in LARYNGEAL CANCER with lymph node metastasis. Yahong Wu et al had stated in their research article that MTA1 is found in many human malignancies and their over expression is related to invasion andmetastasis.MTA1 also express epitope which may help for developing new targeted drug or vaccine in human malignancies which are resistant to conventional treatment. It was also studied in literature the role of tumor biomarkers in predicting outcome of premalignant lesions of mouth, larynx and nasopharynx, however, there have not been any valid biomarker that has been studied for predicting outcome of laryngeal premalignant lesion. It was found in our study that 50 % of diagnosed cases were glottis carcinoma and other 50% were supraglottic carcinoma as it is more or less similar to Indian subcontinent data. Vimentin expression was strongly positive in 29 cases (32.22%). High expression of Vimentin and Metastasis associated 1 protein (MTA1) was seen in squamous cell carcinoma of the larynx in this study. MTA 1 expression was seen in all 90/90 (100%) laryngeal carcinoma patients irrespective of the stage. But according to recent study by Karamagkiolas S et al. showed that MTA1 immunopositivity predicts longer survival in early-stage laryngeal carcinoma and our study could not predict overall survival on the basis of MTA1histoimmunopositivity because of 100% MTA1 positivity in laryngeal cancer .Though very strong expression of MTA 1 was seen in patients with lymph node metastases thus signifying poor prognosis in such patients contradict the recent study done by Karamagkiolas S et al.

Andishehtadbiretal studied 44 cases of oral squamous cell carcinoma (OSCC) and 15 normal epithelium with IHC staining for MTA1 CD105 and foundin about 97.7% cases higher value of MTA1 in OSCC and linked them with angiogenesis andlymph node metastasis. MTA1 over expression remains to be a poor prognostic biomarker in OSCC. Vimentin over expression was found in 29/ 90 (32.22%) patients of laryngeal carcinoma. On univariate and multivariate analysis, expression of both Vimentin and MTA 1 markers showed strong correlation with disease free survival in laryngeal cancer patient but statistically was not significant. No significant association of tumor markers expression was found with smoking, alcohol and tobacco chewing. When the recurrence status is compared with T, N category and staging only N category shows a significant difference. High recurrence rate at primary laryngeal site was seen in patients with strong positivity of tumor markers.Overall survival and Disease-free survival were found to decline over long term follow up in patients with Vimentin positivity as compared to patients with vimentin negative laryngeal cancer. Overall survival and DFS was found to decline in patients with MTA1 positive laryngeal cancer as compared to MTA 1 negative patients. Lothaire P et al tried to review recent development of biomarkers in HNSCC and their role. They had also felt the need of further prospective research and hypothesis in relation to biomarkers and tumorigenesis and their clinical applicability.

5. Conclusion

High recurrence rate at primary laryngeal site was seen in patients with strong positivity of tumor biomarkers MTA1 and Vimentin. Overall survival and Disease free survival were found to decline over long term follow up in patients with Vimentin positivity as compared to patients with vimentin negative laryngeal cancer. .Limited follow up and limited number of patients operated in this study are the limitations as long term survival could not be assessed due to proposed study duration. More number of operated cases would have been better for follow up data to see the expression of these biomarkers in operated patients over long term follow-up. More patients couldn't be operated in this study due to a smaller number of operation theaters available during the covid-19 pandemic.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest among the authors who have contributed in this manuscript.

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