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ROLE OF IMMUNOHISTOCHEMIMICAL MARKERS IN METASTATIC ORAL SQUAMOUS CELL CARCINOMA: EXPERIENCE AT A TERTIARY CARE CENTRE

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ABSTRACT

Aim: This study aims to find out any association between expressions of a panel of immunohistochemistry (IHC) markers including Ki 67, Cyclin D1, p53, bcl2, C-KIT, and Her2/neu and metastatic disease in oral squamous cell carcinoma (OSCC).

Materials and methods: A total of 236 cases of OSCC presenting to our centre between Jan 2013-Dec 2016 with available clinical details were included in the study. Forty cases each of non metastatic disease and metastatic disease at presentation were selected randomly for evaluation of IHC marker's expression as enumerated above.IHC expression of the markers were interpreted as positive ,negative or indeterminate or non-contributory based on standard practice in clinical use. Other important clinical and pathological features were also noted for further evaluation and analysis.

Results: MIB 1(Ki 67) index as a percentage value, and diffuse p53 expression were found to be independently associated with metastatic OSCC at presentation (p values <0.001, confidence interval 95%).Cyclin D1,bcl2, C-KIT, and Her2/neu expressions did not show any association with metastatic/ non-metastatic disease at presentation.

Conclusion: MIB 1 mitotic index and p53 positivity are significantly associated with metastatic OSCC. Further studies on this subject are needed to substantiate this important finding which may be used to analyze the role of these IHC markers in possible prediction of metastatic potential and therefore prognosis in the cases of OSCC.

KEYWORDS : Immunohistochemistry; Metastasis; Oral squamous cell carcinoma

Introduction

Oral Squamous cell carcinoma (OSCC) contributes to roughly one third of all newly detected cancers in South East Asian countries including India, Sri Lanka, Bangladesh and Pakistan [1, 2].A significant number of cases of OSCC presents at an early stage without any evidence of nodal or distant metastasis, especially so in India [2]. Though, no single factor has been found to have a good prognostic value; metastatic disease generally portends a poor prognosis with about 30-55% 5 year survival rates as against > 68% 5 year survival for non-metastatic cases [3]. However, we do not have a reliable predictor of metastatic disease in OSCC and therefore, this study is imperative in this context.

OSCC, like most other malignancies, progresses from multistep accumulation of heterogeneous genetic aberrations in keratinocytes. The heterogeneity of these changes possibly lies at the heart of different clinical outcomes and treatment responses in the cases of OSCC [3,4]. Although, in recent years, the number of molecular- based assays has increased, yet histopathology remains the gold standard for most diagnostic and therapeutic decisions. Immunohistochemistry (IHC) complements histopathological analysis by detecting gene expression at the protein level thus providing an affordable surrogate for molecular changes. An immunohistochemical panel using multiple molecular biomarkers can provide useful information about clinico-biologic attributes of OSCC thereby, helping in management and prognosis of cases. Metastatic disease in OSCC is almost universally accepted as a poor prognostic indicator and therefore predicting metastatic potential of tumor in a given case would provide valuable information to guide the therapy and prognosis. This hypothesis underlies the core of this study.

Candidate biomarkers do exist. Cyclin D1 is an oncogene that drives cell cycle progression, and the critical decision for cell growth or arrest may depend on the concentration of Cyclin D17[4]. It is widely used as an IHC marker in diagnosis of Mantle cell lymphoma and as a predictive marker in multiple myeloma and estrogen receptor (ER) positive breast cancer. It has also been found to be over expressed in 50-60% of OSCC though its role as a marker to aid treatment or prognosis is not well established. MIB1 (Ki67) is a proliferation marker expressed by nuclei of cells that have entered the cell cycle. It is widely used in ER positive breast cancer and neuroendocrine tumors but its prognostic role in OSCC remains controversial [4].

P53 is one of the most commonly mutated or lost gene in many of human cancers with robust diagnostic and predictive values in gastrointestinal cancers and genitourinary carcinomas. The over expression of p53 has been associated with a poor prognosis in OSCC in a number of studies.Bcl-2 is an anti-apoptotic nuclear protein encoded by bcl2 gene. Studies in OSCC have largely been inconclusive in establishing any prognostic or predictive role for bcl2 IHC [4].

C-kit is a proto-oncogene whose product, a trans-membrane tyrosine kinase protein, is implicated in causation of various malignancies including, gastro intestinal stromal tumor, melanoma, seminoma and some acute myeloid leukemia [4]. Its role in OSCC has not been well studied so far.HER2/neu is a member of epidermal growth factor receptor (EGRF 2) family of transmembrane tyrosine kinase proteins. Its role as an important biomarker in breast carcinoma is well established and is used routinely in therapy decision of almost one third of the breast cancer patients. Over expression of Her2/neu has been variably reported between 0-88% in OSCC, though its clinical use remains controversial [4, 5].

The primary objective of this study is to find out any association between the expression of a panel of IHC markers (Cyclin D1, Ki67,

p53, bcl 2, CD117(C-KIT) and HER2/neu) by the tumor cells in cases of OSCC and metastatic disease .This may help in therapeutic decisions and prognostication in an overwhelming number of OSCC cases especially in countries with high disease burden. Molecular studies are available at limited centres in India and are expensive for the majority of the poor patient population. Therefore, finding a cheaper and relatively cost-effective alternative in widely available IHC markers is our secondary objective.

MATERIALS AND METHODS

Patients

All consecutive cases of oral squamous cells carcinoma diagnosed and treated at our center between January 2013 and December 2016 and with available clinical details were considered for inclusion in this study. A total of 236 cases with adequate archival tissue were finally included in our study. Diagnoses were confirmed from hematoxylin and eosin-stained sections by experienced pathologists at this center. Tumor Node Metastasis (TNM) status was based on clinical examination and radiological evaluation of the case. All cases were treatment naïve and the primary treatment consisted of surgical resection of the tumor with or without nodal dissection and followed by radiation and or chemotherapy in selected patients. Clinical outcome was retrospectively traced from the hospital's records in all cases. Metastatic disease at presentation was defined as regional lymph nodal spread and or spread to another organ in the body. Local contiguous extension of the tumor in surrounding soft tissue of the oral cavity or invasion of the underlying bone was not considered as metastasis. Based on these criteria 102 cases were assigned to the non-metastatic category whereas the rest 134 cases were included in the metastatic category. Selection of cases for IHC study Forty cases each from non-metastatic and metastatic categories were randomly selected for IHC analysis on the primary tumor tissue.03 cases in the metastatic category had either unidentifiable primary or minimal primary tumor tissue; in these cases the metastatic tumor focus was submitted for IHC evaluation.

Antibodies

Monoclonal primary antibodies used in this study were Dako, USA, mouse antibody concentrates. All of the antibodies were diluted in phosphate-buffered saline (PBS) at the concentration titrated to optimal staining in a previous institutional standardization. All antibody clones were of the mouse antihuman immunoglobulin G (IgG) subtype.

Immunohistochemistry

IHC was performed at our centre by manual method as under:

1. Samples were fixed in formalin, embedded in paraffin, and cut into 3 µm sections.
2. Deparaffinization was performed with xylene and rehydration done with descending concentrations of alcohol.
3. Antigens were retrieved by boiling in 0.01% citric acid at pH 8.0 in a microwave oven for 6-8 minutes.
4. Hydrogen peroxide (0.3%) in PBS was used to block endogenous peroxidase activity. Samples were washed, and non specific binding was blocked with 1.5% normal serum in PBS for 60 minutes.
5. Primary antibody was added to the samples and incubated at room temperature for 60 minutes. 6. Slides were washed in PBS, and secondary antibody (Elite anti-mouse IgG ABC kit; Vector Laboratories, Burlingame, CA) was used according to the manufacturer's recommendations.
7. Diaminobenzidine was used for 3 minutes at room temperature for visualization, and Mayer's hematoxylin was used for the background staining.

Microscopic Evaluation

1. All H& E and IHC slides were evaluated by a single blind approach by two independent observers (PS & DSH) under Olympus BX- 40 (Olympus, Tokyo, Japan) microscope. In cases with difference of opinions, the slides were reviewed together until a consensus was reached between the two observers.
2. On histopathologic evaluation cases were assigned to well, moderately and poorly differentiated category based on conventional criteria (a mix of Broder's and Anneroth's criteria).
3. A total of 40 randomly selected cases from the two categories were evaluated for IHC staining of Cyclin D1, Ki 67; p53, bcl2; C-KIT(CD117) and HER2/neu.
4. IHC staining results for all antibodies were evaluated by standard method as described in the literature and cases were termed 'positive', 'negative' or 'indeterminate or non-contributory' respectively for a particular antibody. Ki 67 index was calculated by counting at least 200 cells in the highest proliferating areas of tumors.
5. A high mitotic index (KI 67 index) was taken as >20% of tumor cells showing nuclear positivity for this stain.
6. Positivity of any of the above IHC stains was defined as diffuse (>50% cells) and at least moderately intense staining of tumor cells in their respective parts of the tumor cells as described in standard textbooks.
7. Appropriate positive and negative control tissues were put for comparison with every batch of IHC stain.

Recording of data

Associations between IHC marker expression and tumor category were meticulously recorded for further interpretation and finalization of results. Important clinical and pathologic variables which were also recorded in the study included; age, sex, smoking history, tumor location grade of the tumor and depth of tumor invasion.

RESULTS

Out of the total 236 cases, 66 were females with a male to female ratio of 3.6:1.A history of tobacco use was present in 189/236(65.4%) cases. Fifty-one (21.6%), 118 (50%) & 67 (28.4%) cases were assigned to well, moderately and poorly differentiated grades, respectively.

Definitive follow up records were available in 109 cases; 61(56%) of these had died or were lost to follow up during the study, 33(30.3%) cases showed recurrence or metastasis after the initial diagnosis and treatment.

The important clinical and pathological features seen in the study are presented in Table1.

Thirty-one out of 40 metastatic OSCC cases at presentation revealed a MIB index of > 20 % (Mean=32%, Range, 21-60%) whereas MIB index in non-metastatic cases averaged at approximately 15% (Range, 8-30%). p53 nuclear protein was diffusely positive in 27/40 (67.5%) of metastatic cases whereas positivity for p53 was seen only in 8 of 40 (20%) non-metastatic cases. (Fig1, Table 2).

Table 1

Parameter	N=236	
Mean Age	**47**	
Sex	**M**	**170**
	F	**66**
Tobacco use	**Never**	**47**
	Past	**44**
	Present	**145**
Metastatic Disease	**Present**	
	Absent	
Grade	**Well differentiated**	**51**
	Moderately differentiated	**118**
	Poorly differentiated	**67**
Tumor Location	**Lip**	**41**
	Buccal mucosa	**103**
	Tongue	**52**
	RMT	**17**
	Others	**23**
Depth of invasion	**≤1 cm**	**112**
	>1cm	**124**

On IHC evaluation of 40 cases each from randomly selected non-metastatic and metastatic tumor categories, high Ki 67 index and

p53 positivity were found to be significantly associated with metastatic disease at presentation (p<0.001, CI=0.95).Other markers including Cyclin D1,bcl2,C-KIT & HER2/Neu did not show any association with metastatic or non-metastatic disease at presentation.

Table 2

Disease at presentation	Ki 67 >20%	Cyclin D1	P53	C-KIT	Bcl2	Her2/neu
Non-metastatic disease (n=40)	3	14	8	17	13	23
	33	20	21	17	19	14
Positive	4	6	11	6	8	3
Negative						
NC						
Metastatic disease (n=40)	31	22	27	18	13	15
	6	10	8	20	20	24
Positive	3	8	5	2	7	1
Negative						
NC						

IHC evaluation results of forty non-metastatic and metastatic disease cases each (NC; non contributory)

Discussion:

OSCC is a public health problem in many developing countries. The situation in India is very grim due to prevalence of tobacco use and areca nut chewing. The majority of patients presents at an advanced stage, leading to increased mortality and morbidity. An abysmal primary health care system in our country remains an impediment for early diagnosis and management of OSCC cases.

OSCC is generally a disease of the late adulthood and sporadic cases in childhood are extremely rare. The average age of cases in our study is 47 years which is in line with most similar studies from this part of the world. Tobacco use was reported in 65.4% of our cases which only reiterates the role of tobacco in causation of OSCC. Other clinical and pathological findings as depicted in Table 1 are similar to findings reported by numerous researchers in their studies and therefore do not warrant further discussion or elaboration.

Search for an easily available and reliable predictor of prognosis in OSCC has largely remained elusive. Numerous IHC markers have been studied in context with OSCC. But, none has found favor with the clinicians and therefore quest for a reliable set of markers is still ongoing [2, 7]. Most researchers have included a single or a few IHC markers in their study. We have tried to include a set of commonly available IHC markers and studied them in context with possibly the most relevant clinical tumor attribute –its metastatic potential which is a reliable surrogate for its biologic or clinical behavior.

Cyclin D1 is a cell proliferation marker which has been variously proved to be of use in OSCC by different workers [4, 6]. Shikari et al found a significant relation between the adverse overall survival (OS) in OSCC and over-expression of cyclin D1 along with p53 in their study [18]. Jayasurya et al reported a similar finding when cyclin D1over-expression coupled with reduced IHC expression of p16 were evaluated in a cohort of OSCC patients [5,19]. In our study, however, we could not find any association between IHC expression of cyclin D1 and metastasis of OSCC.

Ki 67 is strongly associated with cell proliferation and is not expressed by cells in G0 of the cell cycle. Most of the studies including one by Myoung et al have found an association between adverse prognosis and IHC over-expression of Ki 67 in OSCC patients [8, 20]. Lee et al, on the other hand, reported no independent association between its IHC expression and survival in OSCC cases [9, 21].In this study we found a significant association between metastatic disease at presentation and over-expression of Ki 67 (p value <0.001, CI 95%).This only reiterates the belief that highly proliferating tumors have more propensity to metastasize and therefore are likely to show markers of high mitosis. This is an important finding of our study because of the potential use of this IHC marker in predicting metastasis in OSCC cases can be of immense value in therapeutic decision making as well as better prognostication of the cases. Similar findings are reported by most of the studies so far in the literature. One of the important limitations of our study is non-inclusion of IHC for p16 protein expression in these tumors. The author/s is presently evaluating p16 protein over-expression in OSCC in conjunction with a set of common IHC markers and the results and interpretation of the findings are under evaluation.

p53 is a cellular protein regulating a number of critical cellular activities, the most important being apoptosis of cells with irreversible DNA injuries. Loss or mutation of p53 is one of the commonest occurrences in many human malignancies. The high expression of p53 has been associated with a poor prognosis, and the combined expression of p53, cyclin D1, and EGFR has been correlated with an unfavourable overall survival (OS) in OSCC patients by Shikari et.al.[10, 19].In our study, there was strong independent co-relation seen between metastatic disease and p53 positivity. This is in line with the long held belief that mutation of p53 confers invasive potential to OSCC. Though, no clear-cut relation has been conclusively established between metastatic potential in OSCC and p53 over expression, our study is one of the few to demonstrate such relation. More studies are required to verify this finding. This again is an important result of our study as IHC with p53 antibody is widely available in most parts of the world and is easy to employ in routine clinical settings.

Bcl2 is an anti-apoptotic cellular protein and has found immense clinical application as an IHC marker in a number of human malignancies [11]. Camisasca et al. and dE Vicente et al. found two completely contradictory results that Bcl-2 protein expression was associated with favorable and poor prognosis in OSCC, respectively [12]. In our study, the authors could not find any association between bcl2 expression and metastatic OSCC. This possibly supports the widely held view that blc2 gene has limited role to play in carcinogenesis and tumor evolution in cases of OSCC.

Her2/neu is one of the earliest IHC marker to be used in clinical practice in breast carcinomas and is now found to be useful in gastric and other carcinomas as well [13]. In some studies, like Agra e.t al., C-erb2 (Her2/neu) over expression has been correlated with poor prognosis in OSCC patients but these results were not confirmed in other investigations [14,15,16,17,18,22].In this study, there was no association found between IHC expression of Her2/neu and metastasis in OSCC. This is possibly because of involvement of different driver pathways leading to metastatic potential in OSCC [23].

The stem cell factor C-KIT (CD117) is an important cellular protein which is seen to be over expressed in a wide variety of mesenchymal and few epithelial tumors [2, 5, 10]. Its association in cases of OSCC has not been well studied and that is one of the main reasons why we included this IHC marker in our study. The hypothesis behind selecting this marker was its potential role in replenishing or maintaining of adequate number of stem cell pools to support and sustain the rapidly dividing tumor cells or their progenitors. However, we did not find remarkable association between C-KIT IHC expression and metastatic OSCC. Further studies are needed to establish or refute any association with OSCC and C-KIT IHC expression because of very limited data on this subject.

Conclusion:

The authors conclude that Ki 67 (MIB1) mitotic index and p53 positive staining by IHC are significantly associated with metastatic disease at presentation in cases of OSCC. This finding may be use as a surrogate marker for prediction of metastatic potential in OSCC especially in resource-poor countries with limited molecular diagnostic wherewithal. However, further studies are required to establish this finding before it may be accepted for clinical use by the medical community.

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Compliance with Ethical Standards:

Funding: This study did not receive any funding from any agency.

Conflict of interest: All authors declare that they have no conflict of interest.

Ethical Approval: No animals or humans were involved in this study. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Legends:

Figure1

Hematoxylin and eosin (H&E) photomicrographs showing A; 10X view of a moderately differentiated squamous cell carcinoma; B&C; 10 X and 40X views of poorly differentiated squamous cell carcinoma respectively. D; IHC staining with KI67 (MIB1) antibody showing a low mitotic index (<20%) in a non-metastatic case. E; High mitotic index (>20%) with Ki 67 in a metastatic OSCC. F; Diffuse positivity for p53 IHC antibody in a metastatic OSCC.

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