Original Research

Cytokeratin 8 depicts nodal metastasis in head and neck squamous cell carcinoma

Snega Thamilselvan[1], Deepak Pandiar[1], Reshma P. Krishnan[1], Chitra S.[2]

[1]Department of Oral Pathology and Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, [2]Department of Pathology, Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India

Abstract

Background: Nodal involvement in squamous cell carcinoma is an important feature directly associated with the poor prognosis in patients with head and neck squamous cell carcinoma (HNSCC). There are no clear cut indicators available currently to identify the lymph node metastases and overall prognosis in HNSCC. Thus, the current study was conducted to correlate the immunoexpression of cytokeratins (CK) 8, 10, and 14 with lymph node metastases and tumour differentiation in patients with HNSCC.

Material and Methods: The study population included 61 retrospective cases of HNSCC with lymph node metastases (n = 31) and without lymph node metastases (n = 30). Expression of CK 8, 10, and 14 was assessed by immunohistochemical staining procedure. Using Pearson's Chi-square test and Spearman's correlation coefficient, the correlation of these markers with lymph node metastases and tumour differentiation was statistically analysed.

Results: The expression of CKs in HNSCC cases was higher than in controls. In nodal metastasis cases, CK 8 expression was noted in >50% of the tumour cells at the invasive tumour front (ITF) (*P* value 0.008), and in cases without nodal metastasis, <1% or negative expression was noted. CK 10 expression gradually decreased as the tumour grade increased. Association of CK 10 expression and tumour differentiation exhibited statistically significant results (*P* value 0.03). CK 14 expression was noted in the entire epithelium and at the ITF, strongly in most cases; however, CK 14 did not correlate with the lymph node metastasis and tumour differentiation as well.

Conclusion: We found a strong correlation of CK 8 expression with nodal metastasis in HNSCC, and it can be utilised as a reliable prognostic indicator.

Keywords: Cytokeratin 8, 10, 14, nodal metastasis, squamous cell carcinoma

Address for correspondence: Dr. Deepak Pandiar, Department of Oral Pathology and Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai - 600 077, Tamil Nadu, India.
E-mail: deepakpandiar1923@yahoo.com

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) are the heterogeneous group of tumours that arise from the squamous epithelium of the oral cavity, pharynx, and larynx, and it is the sixth most prevalent cancer.[1] HNSCCs are hypothesized to initiate and progress as a result of a series of genetic changes. Furthermore, genetic and

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epigenetic changes cause dysregulation of multiple cellular signalling pathways.[2] Many HNSCC patients present with locally progressive disease, with substantial nodal involvement. Metastasis to the cervical lymph nodes is an important parameter directly linked with poor prognosis in patients with HNSCC which may further progress to distant metastasis.[3]

The cytokeratins (CK) in the epithelial cells generally provide protective structural and mechanical stability; along with it, CK 8 exhibit added regulatory functions, which include modulation of protein localization, protein targeting and protein synthesis.[4] These characteristics of CK 8 play a major role in carcinogenesis. In head and neck carcinomas, the keratin profile 8 is linked to a variety of seemingly disparate factors, including differentiation state, proliferative rate and histogenesis.[5,6] Under varied pathophysiological circumstances, posttranslational alterations influence the stability of CK 8 filament formation. These alterations in keratin filament assembly appear to be one of the determinants of the altered behaviour of the cell.[7]

Suprabasal keratinocyte expresses low molecular weight CK 10. CK 10 is hypothesized to be a specific marker for keratinocyte terminal differentiation.[8] Despite the fact that CK 10 expression was observed in terminal keratinocyte differentiation regions, it was not significantly correlated with the differentiation grade of the tumours.[9] Hence, the loss of expression in malignant stratified squamous epithelium could be considered as an initial stage of malignant transformation.

Likewise, CK 14 is an acidic keratin that is expressed in mitotically active basal cells of stratified epithelium, where it promotes proliferation and differentiation while also supporting structural integrity,[10] whereas in dysplastic epithelium, it begins as irregular expression among the epithelial layers and becomes abundant in carcinomas. The upregulation of CK 14 in carcinogenesis may be associated with increased cell proliferation, failure of terminal differentiation and oncogenic aberration.[11,12]

CK 8, 10, and 14 markers have been analysed in various tumours, but its correlation with the lymph node metastasis in HNSCC has still not yet been dealt with. Currently, there are no definitive markers available to identify the lymph node metastasis which has direct influence on the prognosis. Hence, prediction of lymph node metastasis is important to decrease the local recurrence and second primary malignancies.

This current study was thus designed to quantitatively analyse the expression of CK 8, 10, and 14 in HNSCC patients and to correlate the histopathological parameters with immunoexpression of CK 8, 10, and 14, thus predicting the lymph node metastasis in HNSCC patients. The characterization of these markers may aid in prior treatment decisions, which is critical for the patient's disease-free survival.

MATERIALS AND METHODS

Sample selection

The present retrospective study was conducted in the Department of Oral Pathology and Microbiology in conjunction with the Department of General Pathology over a period of 1 year (August 2021–August 2022) and aimed to evaluate and correlate the expression of CK 8, 10, and 14 with lymph node metastasis and tumour differentiation in HNSCC. The study consisted of a total of 71 samples categorized into three groups— Group I: apparently normal oral mucosa as controls (n = 10), Group II: HNSCC with lymph node metastasis (n = 31) and Group III: HNSCC without lymph node metastasis (n = 30). The sample size was calculated using the results obtained via the pilot study. Prior approval from Ethical committee was obtained from Institutional Ethical Committee-Scientific Review Board (SRB/SDC/OPATH-1901/22/TH-042), and all the cases and controls gave informed consents. Only histologically proven cases of HNSCC were included who were treated with wide excision and radical neck dissection. Patients who received prior chemotherapy or radiotherapy were excluded. Recurrent cases were also not included in the study. The histological grading of OSCC cases was done according to Bryne's classification (1992),[13] and grading for laryngeal and pharyngeal SCCs was done with WHO classification (2017). Of the 61 cases, 30 cases were WDSCC, 24 were MDSCC and 7 were PDSCC. All 61 HNSCC cases and 10 apparently normal oral mucosa were subjected to IHC staining with CK 8, CK 10, and CK 14. 3 μm thick sections were cut from all the selected blocks for immunostaining. A similar immunohistochemical procedure was performed as described elsewhere.[14] The CKs immunohistochemical kit consisted of mouse monoclonal—anti-CK 8, anti-CK 10, anti-CK 14 as the primary antibodies (Vitro Master Diagnostica, Sevilla, Spain) and the Super Sensitive Polymer-HRP/DAB system as the secondary antibody and detection kit (DAKO, Denmark).

Evaluation of IHC-stained slides

Brown-stained malignant epithelial cells (cytoplasmic localization), individual cells, islands or nests were

observed and assessed at the ITFs. A quantitative scale of +/++/+++ was used for indicating the positive expression of CK 8, 10, and 14. The cases with + were considered as low expression of the cytokeratins, and the cases with +++ were considered intense positivity. The scoring was based on the location and percentage of positive staining [Table 1].[8]

For evaluation of CK 8, 10, and 14 expressions in HNSCCs, the slides were examined under light microscope at 10X and 40X magnifications. Two pathologists independently analysed and evaluated the IHC-stained slides (ST and DP). Any discordance was resolved by discussion with a third evaluator to reach an unequivocal conclusion (RPK).

Statistical analysis

All the parameters were tabulated in Microsoft Excel spreadsheet and assessed for statistical significance using IBM SPSS statistics 23.0 version, US. Statistical analysis for correlation of CK 8, 10, and 14 expression markers with lymph node metastasis and tumour differentiation in HNSCC patients were performed using Pearson's Chi-square test and Spearman's correlation coefficient. The *P* value <0.05 was considered to be statistically significant.

RESULTS

The present study included 71 samples including 10 apparently normal oral mucosa samples as controls and 61 HNSCC cases. Among 61 HNSCC patients, 16 (26.2%) were women and 45 (73.8%) were men. The peak incidence was seen in 5th–6th decade. Out of n = 61 HNSCC cases, 41 (62%) included cases were OSCC—24.6% buccal mucosa, 16.4% tongue, 9.8% gingivobuccal sulcus, 6.5% alveolus, 1.6% maxilla and 1.6% floor of the mouth; 10 cases (19% each) of laryngeal and pharyngeal SCC constitutes the remaining 20 cases. Patient and tumour characteristics of HNSCC patients included in this study are listed in Table 2 and photomicrographs from the most representative sites are shown in Figure 1.

Table 1: Scoring criteria for the IHC stained slides – CK 8, 10, and 14 with respect to staining intensity and proportionality index in tumour cells

Staining Intensity	Proportionality Index
Negative (-)	0% of tumour cells
Mild (+)	1-25% of tumour cells
Moderate (++)	25-50% of tumour cells
Intense (+++)	>50% of tumour cells

Table 2: List of demographic data and tumour characteristics of HNSCC patients included in this study

Characteristics	Group	Sample *n* (%)
Gender	Male	45 (73.8%)
	Female	16 (26.2%)
Age (decades)	31-40	4 (6.55%)
	41-50	20 (32.78%)
	51-60	21 (34.42%)
	61-70	12 (19.67%)
	71-80	4 (6.55%)
Laterality	Left	21 (34.4%)
	Right	18 (29.5%)
	NA	22 (36.1%)
Anatomical location	Oral cavity	41 (62%)
	Buccal mucosa	15/41
	Tongue	11/41
	Gingivobuccal sulcus	7/41
	Alveolus	5/41
	Maxilla	2/41
	Floor of the mouth	1/41
	Larynx	10 (19%)
	Pharynx	10 (19%)
Tumour differentiation	WDSCC	30 (49%)
	MDSCC	24 (39.34%)
	PDSCC	7 (11.57%)
Tumour staging	pT1	6 (9.83%)
	pT2	16 (26.22%)
	pT3	12 (19.67%)
	pT4	27 (44.28%)
Lymph node metastasis	Yes	31 (50.81%)
	No	30 (49.19%)

Lymph node metastasis and its correlation with cytokeratin expression

Cytokeratin 8: When the CK 8 expression was correlated with the lymph node metastasis, the expression levels were significantly increased in cases with nodal metastasis. Statistical significance was noticed on correlation of CK 8 expression with nodal metastasis (Pearson's Chi square, *P* value 0.008; Spearman's correlation, 0.179). Out of 31 cases with positive nodal metastasis; 38.7% cases showed intense expression, 22.6% cases showed moderate expression, 19.4% cases showed mild expression and 19.4% cases showed negative expression. Of 30 cases with no lymph node metastasis, 10%, 10%, 26.7% and 53.3% of cases showed intense, moderate, mild and negative expression, respectively.

Cytokeratin 10: The expression levels of CK 10 were found to be variable when correlated with the lymph node metastasis. Positive lymph node metastasis: Intense expression was seen in 12.9% cases, moderate expression in 29% cases, mild expression in 32.3% cases and negative expression was seen in 25.8% cases. 30 cases of OSCC with no nodal metastasis, 3.3%, 36.7%, 33.3% and 26.7% of cases showed intense, moderate, mild and negative expression, respectively. No statistical significance was noted between CK 10 expression and lymph node metastasis (Pearson's Chi-square test, *P* value 0.576; Spearman's correlation, *P* value: 0.036).

Cytokeratin 14: The entire thickness of the epithelium displayed a positive expression for CK 14. In our study,

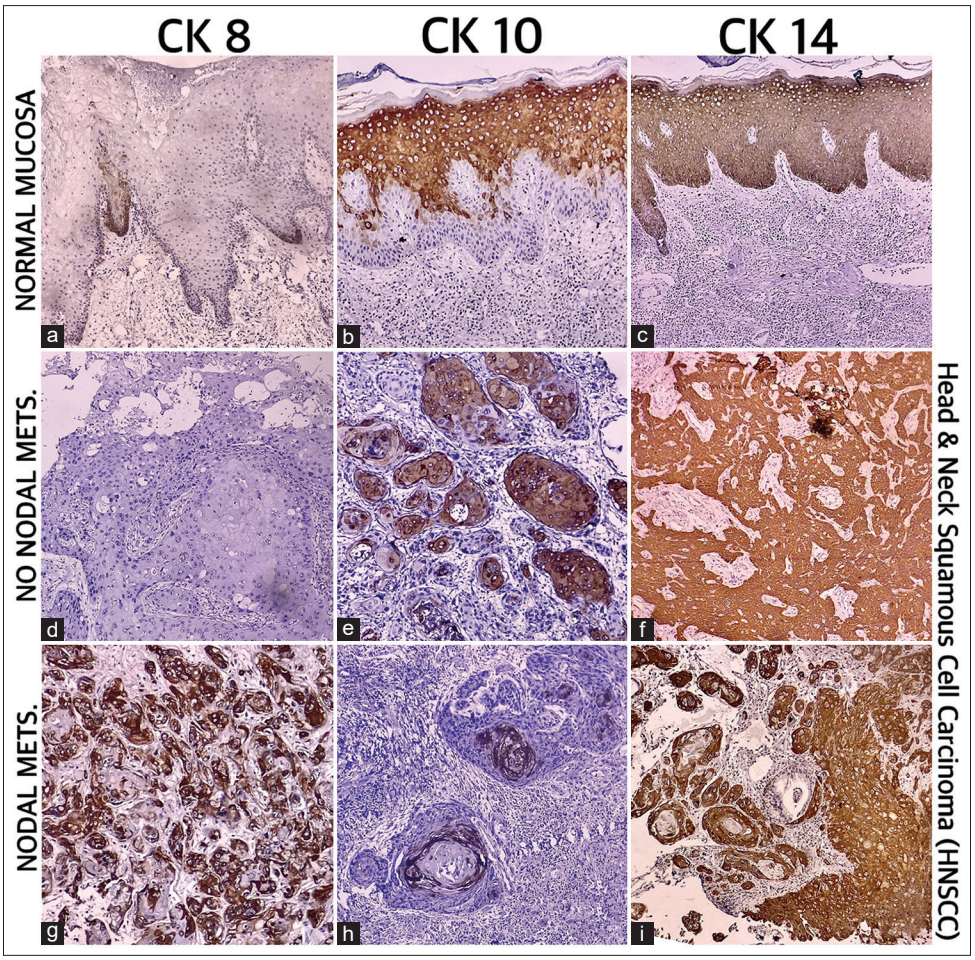

Figure 1: Photomicrographs of IHC-stained sections under 4X magnification. (a) Expression of CK 8 in epithelium of normal mucosa; the expression was limited to the basal and parabasal layer where the maturation of the epithelial cells begins. (b) CK 10 was expressed in the superficial layers of the keratinized epithelium in the normal mucosa, whereas (c) CK 14 was expressed throughout the epithelial layers. (d–f) Expression of CK 8, 10, and 14, respectively, in HNSCC cases without lymph node metastasis and pictures (g–i) CK 8, 10, and 14 expression, respectively, in HNSCC cases with lymph node metastasis

no association was noted between CK 14 expression and HNSCC cases with lymph node metastases. 83.9% of cases with positive metastasis, and 83.3% of cases without metastasis showed strong expression of CK 14. Association between CK 14 expression and lymph node metastasis did not show any statistical significance (Pearson's Chi-square test = *P* value 0.469; Spearman's correlation = *P* value -0.168).

Tumour differentiation and its correlation with cytokeratin expression

Cytokeratin 8: On correlation with tumour differentiation, the expression of CK 8 was variable. The expression patterns of intense, moderate, mild and negative in 30 cases of WDSCC were seen in 23.3%, 13.3%, 13.3% and 50% of cases, respectively. 24 cases of MDSCC were included, 20.8% of cases showed intense expression; 16.7% of cases demonstrated moderate expression; 33.3% of cases showed mild expression and 29.2% of cases showed negative expression. In 7 cases of PDSCC, 42.9%, 28.6%, 28.6% and 0% of cases showed intense, moderate, mild and negative expression, respectively. There was no statistical significance between the percentage expression of CK 8 and the tumour differentiation (Pearson's chi square: *P* value 0.185; Spearman's correlation: 0.325).

Cytokeratin 10: The expression of CK 10 showed a definitive correlation with the tumour differentiation rather than nodal metastasis. On correlation with tumour differentiation, the expression levels of CK 10 gradually decreased as the tumour grade increased. Association of CK 10 expression and tumour differentiation exhibited statistically significant results (Pearson's Chi-square test, *P* value 0.03; Spearman's correlation, *P* value 0.65). In 30 cases of WDSCC, intense, moderate, mild and negative expression patterns were found in 16.7%, 46.7%, 20.0% and 16.7% of cases, respectively. There were 24 cases of MDSCC out of which 20.8% exhibited moderate

expression, 45.8% showed mild expression and 33.3% showed negative expression. 14.2%, 42.9% and 42.9% of PDSCC patients, respectively, displayed moderate, mild and negative expression. No intense positivity of CK 10 positivity was seen in PDSCC.

Cytokeratin 14: 96.7% of the WDSCC cases; 70.80% of MDSCC cases and 71.40% PDSCC cases showed strong positivity for CK 14 among the tumour cells at the ITF. There was no statistical significance identified between CK 14 and tumour differentiation (Pearson Chi-square test *P* value 0.064; Spearman's correlation coefficient *P* value: 0.191). On correlation with tumour differentiation, CK 14 was consistent with no major decrease in the levels of expression with regards to the increasing tumour grade. No significant association was noted with CK 14 and the tumour differentiation.

DISCUSSION

Nodal metastasis dictates the disease-free survival in HNSCC and thus has been proven to be an important prognostic indicator. There was a noticeable difference in the pattern of expression of CK 8 in HNSCC patients with and without nodal metastasis. 80.6% of HNSCC cases with nodal metastasis showed increased expression but with different intensities of staining, whereas 53.3% of HNSCC cases without nodal metastasis showed negative expression in the ITF (*P* value = 0.008). CK 8 was seen positive mainly in individual cells and small nests (<5 cells) at ITF. This expression pattern was irrespective of the age, gender, anatomical location as well as tumour staging of HNSCC. No previous studies have assessed the correlation between CK 8 expression and lymph node metastasis in HNSCC. However, studies have shown correlation with grades of tumour and overall survival in different SCCs. This correlation of CK 8 with the lymph node metastasis is plausible. Progression of a tumour is dependent on the migratory ability. The role of CK 8 in normal cells is to provide mechanical stability, but in tumours, it is involved in the phosphorylation of keratin. Phosphorylation of keratin filaments is an incredibly crucial mechanism that regulates keratin filament organization.[7] The loss of phosphorylation may affect the dynamics of the filament assembly in an epithelial cell. As a result, CK 8 is expressed in dephosphorylated cells with greater mobility in order to provide the cell with more stability. When the expression is seen to increase, the patient might have high propensity for nodal metastasis in the future.

CK 10 and 14 markers exhibited variable results when correlated with nodal metastasis in HNSCC. In instances with and without nodal metastases in HNSCC, there was no change in the expression pattern of CK 10. There was a difference in the staining proportionality according to cases but that was noncontributory to the prognostic role. CK 10 is involved in the terminal keratinization and hence expressed in keratinized epithelium normally. In a tumour, initially, the malignant epithelial cell loses its structure, and as the tumour progresses, the original function of cells to produce keratin gets disturbed and disrupted. Despite the fact that tumour cells lose their function, they are not involved in the metastatic process.

Regardless of the status of the nodal involvement the expression of CK 14 was consistently high (>80% HNSCC cases). CK 14 is generally seen in the epithelial cells which undergo proliferation and differentiation. Every tumour cell is constantly differentiating and undergoing proliferation at different phases of cell cycle. Hence, independent of tumour differentiation, stage or metastatic status, it is not surprising to detect a strong expression of CK 14. This is the first study which has attempted to correlate CK 10 and CK 14 expression with nodal metastasis in HNSCC.

CK 8, 10, and 14 were also correlated with the tumour differentiation in this study, but CK 8 and CK 14 did not show a positive association, whereas CK 10 expression correlated significantly with tumour differentiation. CK 10 expression decreased as tumour grade increased, and there was a discernible change in the pattern of CK 10 when correlated with tumour differentiation. Furthermore, CK 10 was robustly expressed in the squamous cells in both normal and hyperplastic epithelium, but it was negative in poorly differentiated SCC (*P* value = 0.031). These findings were consistent with the previous studies which have exhibited similar results with gradual decrease in expression of CK 10 in HNSCC and OSCC patients[8,15,16] and lung SCC patients.[17] Contradictory results were revealed in another study performed on hepatocellular carcinoma (HCC). Instead of declination of CK 10 expression, they noticed an increase in CK 10 expression in metastatic HCC cells and 10 times higher in recurrent HCC patients than in nonrecurrent HCC cases. Additionally, the authors discovered that CK 10-positive patients' survival rates were considerably lower than CK 10-negative patients', and it was opined that the heterogeneous expression of cytokeratin was related to tissue development, proliferation and differentiation.[18] The authors justified this by commenting about the association of molecular phenotypes of CK 10 with cell transformation and invasive potential which is questionable. Rapid tumour cell proliferation and differentiation eventually result in losing the ability to synthesize keratin, rendering them functionally inoperable.

The tumour cells do not get enough time to mature fully. This might have an impact on CK 10 expression levels on complete malignant transformation. The results of the present study need validation with a larger sample size among various ethnic group.

Limitations

Although the present study yielded some significant results, few limitations need to be addressed. First, site-wise segregation and correlation were not done with immunoexpression of the markers studied. This would have further diluted the data yielding heterogeneous results. Similarly, gender-wise correlation could not be done, as the number of females included was much lesser than the males owing to the fact that head and neck cancers show preponderance for the male gender.

Clinical significance

Immunoexpression of CK8 could be employed as an important indicator of cervical lymph node metastases in HNSCC. Thus, any case, particularly in small incisional biopsies showing expression of CK 8 may be considered a potential candidate for predicting nodal metastasis and may be used as early diagnostic and prognostic marker for appropriate theragnostics in HNSCC.

CONCLUSION

CK 8 seems to be an important marker to depict nodal metastases, while CK 10 correlated with tumour differentiation. As aforementioned, CK 8 could be a fruitful marker in depicting the nodal metastasis in advance so that the treatment may be planned in advance particularly in developing countries where the cancer burden is more. However, the results of the present study need validation with future studies comprising of larger sample size.

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Conflicts of interest

There are no conflicts of interest.

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