Immunohistochemistry, A Valuable Tool in Detection of Cervical Lymph Node Micrometastases in Head and Neck Squamous Cell Carcinoma: A Prospective Study

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Abstract The regional failure after comprehensive clearance of neck metastasis and consequent pathological report of N0 disease has been reported fairly frequently. The role of recurrence of disease in the neck in the cases has been variously reported by different authors. The light microscopy does not detect the micrometastasis and the specimen is reported negative for metastasis. The presence of micrometastasis (the reason for neck failure) has been reported by many studies as 5–58 % (mean 19.6 %). These figures are significantly high. The present study was done to ascertain the micrometastasis after comprehensive neck clearance (pN₀ report). Two groups of patients were included in this study. (1) Group I included patients with N0 necks (80 patients). (2) Group II included Patients with N+ necks (107 patients). We found that 20 % case were reported N0 (Group I) in light microscopy but on immunohistochemistry these were positive for disease. 15 % upstaging was reported in N+ cases (Group 2). Immunohistochemistry has been more sensitive for cancer detection and has significantly changed the tumor staging and its consequent management.

Keywords Micrometastasis · Immunohistochemistry · Cytokeratin · Cervical metastases

Introduction

Squamous cell carcinoma of the head and neck is one of the most common cancers with a global incidence of 7, 80,000 new cases per year [1]. The presence of cervical lymph node metastasis in head and neck cancers is known to be the single most adverse prognostic factor [2]. The number of lymph nodes, the levels in the neck involved, the size of the metastases, and the presence of macroscopic or microscopic extra capsular spread affect the final outcome of neck metastasis (for both long term survival and recurrence free survival) in patients with HNSC [3, 4].

The presence of lymph node metastasis can be detected clinically, radiologically and by ultrasound guided fine needle aspiration cytology. Current methods such as computed tomography (CT), magnetic resonance imaging (MRI) and position emission tomography (PET) are widely used for preoperative detection of metastasis [5]. In fact none of the imaging modalities used for the extent of nodal disease has 100 % sensitivity or specificity [6].

Histopathological examination of neck dissection specimens is highly sensitive and specific test for detection of metastasis [7, 8]. Immunohistochemistry is the gold standard test to detect occult metastasis, micrometastasis or isolated tumor cells in the dissected lymph nodes which show negative metastasis on histopathology.

Immunohistochemistry is the most common method used to detect cytokeratin in metastatic cells in lymph nodes. This technique offers the advantage of preserving cellular and tissue morphological factors.

Thereby, the present study was done to find out the rate and the characteristics of micrometastasis in negative nodes with routine H&E stain using the immunohistochemistry method.

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Material and Method

Eighty patients with $T_{1-2}N_0M_0$ and 109 patients with $T_{1-3}N_{1-3}M_0$ squamous cell carcinoma of Head and Neck region were admitted to the Department of ENT and Head and Neck Surgery, SMS Medical College and Hospital Jaipur from June 2006 to Dec. 2010. No $T_3/T_4 N_0$ cases were reported. Eight patients of T_4N+ were seen who refused surgery and were subsequently referred for radiotherapy.

Site wise there were 86 cases of oral cavity, 31 cases of oropharynx, 43 cases of larynx and 29 cases of pyriform fossa. Staging of the primary tumor and nodal metastasis was done according to AJCC and UICC. Assessment of primary tumor was accomplished by using telescopic endovisual assessment, direct endoscopy, computerized tomography, palpation under general anaesthesia and macroscopic and microscopic evaluation of the surgical specimen.

Nodal assessment was done clinically and radiologically. N_0 disease was decided on palpation, ultrasonography and computerized tomography (CT). All patients underwent both USG and CT. If a patient had no palpable nodes clinically, but the USG and CT scan indicated abnormal lymph node, the patients were treated as clinically $N+$. If USG and CT scan revealed no abnormal lymph nodes, then the patient was marked clinically N_0 . After complete diagnostic evaluation (imaging, FNAC) all these patients were operated (wide primary excision with SOHND/Extended SOHND/JND/MRND-I) and followed up periodically till date. All patients were operated upon by the same team of surgeons.

We send each level of node in different container. Sides and different levels were delineated with the help of landmark stitches placed just after the removal of the specimens. The classification of the American otolaryngology—head and neck surgery was used to determine the level of nodal involvement. All surgical specimens were evaluated by same group of pathologist who were uninformed about the study. The face of the block of the bivalved lymph node served as the first section was obtained (5 μ m). The next ten sections from the block were discarded. The second consecutive section was obtained. This process was repeated three times. Every node was examined on six levels. All of the sections were stained with hematoxylin and eosin and examined under light microscopy. Immunohistochemical staining was performed.

To perform immunohistochemical stain, formalin fixed paraffin-embedded tissues were cut into 4- μ m-thick pieces and mounted on the cationic slides. The specimen was then incubated at 60 °C for 1 h, deparaffinized with xylene for 5 min three times, rehydrated in graded alcohol series (100–70 %), and then washed with distilled water. The 3 %

hydrogen peroxide was applied for 10 min so as to block the activation of endogenous peroxidase and the specimen was then cleansed with Tris buffer solution (Tris 3.025 g, 1 M NaCl 40 g, 1 M HCl 22 ml in H_2O 5 l, pH 7.4). To recover the antigenicity, the citrate buffer solution (sodium citrate 14.7 g, 1 M HCl 27 ml in H_2O 5 l, pH 6.0) was filled into a pressure chamber and heated. At the start of boiling, the slides were inserted into the solution and heated for two additional minutes after reaching the maximum pressure (103 kPa). The slides were then immersed into cold water to lower the pressure and reinserted into the buffer solution. The primary antibodies 2 % AE1/AE3 solution, which is monoclonal antibodies to cytokeratin, was incubated for 1 h at room temperature after dilution in the Tris buffer solution. After three rinses in the Tris buffer solution, sections were incubated with prediluted biotinylated link anti-mouse and anti-rabbit immunoglobulin buffer, as the secondary antibodies, for 30 min at room temperature. After three rinses in the Tris buffer solution, the streptavidin horseradish peroxidase conjugate, which is produced by a combination of horseradish peroxidase with streptavidin, was applied for 30 min at room temperature. Antigenic sites were revealed by incubating sections in 0.05 % 3,3'-diaminobenzidine (DAB) in Tris buffer with 0.1 % hydrogen peroxide. After washing in distilled water, the sections were counterstained with Harris hematoxylin and mounted with Permount. The positive control slide was prepared from tonsil tissues. The negative control slide was prepared from the same tissue block but, instead of the primary antibody, a normal, nonimmune serum supernatant was used.

Fine needle aspiration cytology, original slides of H&E stain and new preparation of cytokeratin stain were reviewed by same pathologist. The cytology slides were interpreted in a blind fashion as negative or positive for the presence of squamous cell carcinoma by a pathologist. Postoperative irradiation was given in cases with histopathologically positive lymph nodes with capsule invasion and/or multiple positive lymph nodes in the specimen.

Observation

The patients were divided in two groups, the first group was $T_{1-2} N_0M_0$ Patients (80) and the second was $T_{1-3} N+M_0$ patients (109).

In first group of 80 patients the primary tumors were T_1-T_2 with clinically N_0 neck. All these patients were treated by an elective neck dissection of level I, II and III (Supraomohyoid neck dissection) for cases of oral cavity and oropharynx and level II, III and IV (Jugular neck dissection) for cases of larynx and pyriform fossa. All these cases have clinically N_0 neck so FNAC could not be performed.

In the second group of 109 patients, the primary tumors were T₁–T₃ with N+ neck. FNAC was performed in all these cases. All these patients underwent radical excision of the primary tumor with adequate oncological clearance and therapeutic neck dissection including level I–V (Modified neck dissection—Type-I).

Neck dissection specimens of the both groups were sent for HPE and IHC for the evaluation of neck metastasis (occult or micrometastasis) to decide the post operative treatment plan. Occult metastases was considered if lymph nodes show metastases by H&E and/or IHC but were undetected by clinical or radiological examinations. Occult metastasis was further divided in two groups. In the first group metastases can be detected by H&E stain, was termed 'established occult metastases'. The Term micrometastasis was coined for the second group in which tumor cells were detected only by IHC method and not by H&E staining (Figs. 1, 2) [9–14].

Results

Status of Nodal Metastasis in N₀ Cases

1,136 Lymph nodes were dissected in 81 patient of N₀ neck (average 14.2 lymph nodes per patient). All these lymph nodes underwent H&E and IHC staining. In H&E stained samples, metastasis were observed in 23/80 patients (28.7 %). The prevalence of site specific nodal metastasis in N₀ neck was 27, 20, 33, 33 %, in malignancy oral cavity, oropharynx, larynx and pyriform fossa respectively (Table 1).

In immunohistochemistry micrometastases were observed in 39 patients (48.7 %) and were mostly found in first echelon

node. Micro metastases were found in 50 % cases (24/48) of oral cavity and 40 % (2/5) cases of oropharynx. Larynx and pyriform fossa also showed micrometastasis in 47 % (10/21) and 50 % cases (3/6) respectively (Table 1).

Status of Nodal Metastasis in N+ Cases

FNAC showed metastasis in 69.7 % case (76/109) of N+ neck. H&E staining and IHC performed over 2,507 lymph nodes which were dissected in 109 N+ patients (mean per patient 23).

In 109 cases of N+ neck, lymph node metastasis was found in 84 patients (77 %) on H&E staining. Oral and Oropharyngeal malignancy had metastasis in 73.6 and 80.7 % cases respectively. 77.2 % patient of laryngeal malignancy and 78 % cases of Orophageal carcinoma showed nodal metastases on H&E staining (Table 2).

Out of 109 (92.6 %) 101 patients showed tumor cells positive to pancytokeratin antibody. Oral cavity, oropharynx, larynx, and pyriform fosse malignancy showed micrometastasis in 94.7, 92.3, 90.9 and 91.3 % respectively (Table 2).

Discussion

Lymph node metastasis is an important prognostic factor for a patient with HNSCC. The clinical significance of neck node metastasis in head and neck cancer has long been well appreciated. The cure rate drops to nearly half, with involvement of regional lymph nodes [15]. The lymph node metastasis is, the important factor in the management (surgical/adjuvant therapy) of HNSCC.

Fig. 1 An algorithm for classification of lymph node metastasis [9–14]

Fig. 2 Micrometastasis from tongue carcinoma in a lymph node. **a** Routine histological examination using H&E failed to detect metastatic foci. Bar 100 mm. **b** Immunohistochemical staining using

an anticytokeratin antibody (AE1/AE3) identified micrometastatic tumor cells (arrows). Bar 100 mm

Table 1 Comparison of HPE and IHC in Clinically N₀ Neck

Site	Total N ₀ neck	HPE + neck	IHC + neck
Oral cavity	48	13 (27.08)	24 (50 %)
Oropharynx	5	1 (20.7)	2 (4 %)
Larynx	21	7 (33.33 %)	10 (47.6 %)
Pyriform fossa	6	2 (33.33 %)	3 (50 %)
Total	80	23 (28.75)	39 (48.75 %)

Table 2 Comparison of FNAC, Histopathology and IHC in clinically N+ cases

Site	Total clinical N+ neck	FNAC + neck	HPE + neck	IHC + neck
Oral cavity	38	26 (68.4 %)	28 (73.6 %)	36 (94.7)
Oropharynx	26	18 (69.2 %)	21 (80.7 %)	24 (92.3)
Larynx	22	15 (68.18 %)	17 (77.2 %)	20 (90.9)
Pyriform fossa	23	17 (73.9 %)	18 (78.2 %)	21 (91.3)
Total	109	76 (69.7 %)	84 (77 %)	101 (92.6)

Histopathological examination is the highly sensitive and specific test for detection of metastasis but the earliest stage of metastasis to neck can be difficult to identify by light microscopy [7, 8]. Small foci of metastatic cancer called micrometastases are often missed because of sampling problems. If a single 5 µm section is required, the 1-cm lymph node has to be sectioned 2,000 times [7, 8].

Cytokeratin is a 7–11 nm intermediate filament that forms a major component of the mammalian cytoskeleton. CK expression in a certain type of epithelium has been

found to depend on various factors, such as the stage of embryonal development [16], inflammation [17], and environmental influences such as vitamin A [18], growth condition [19] and (pre) malignant transformation [20].

Lindeman et al. [21] demonstrated the absence of cytokeratins in any types of cells of a normal lymph node except the metastatic cells from epithelial primary. He observed the CK expression on metastatic cells only in a lymph node which was secondary to epithelial primary tumors. The cytokeratin expression has been detected in non-neoplastic inflammatory lymphadenopathy also, by Gould et al. [22]. Many epithelial inclusions in lymph node such as ectopic salivary inclusions, mesothelial inclusions, and metastatic thyroid follicles may be CK positive too. These observations do dilute the specificity of CK expression as a tumor marker. Thus the authenticity of CK as a tumor marker needed to be corroborated with other cyto-neoplastic features. The present study included the observations like increased nucleus/cytoplasmic ratio, pleomorphism and mitotic figures etc. to a CK positive cell to substantiate the diagnosis.

In the present study, metastasis was found on H&E staining in 28 % of cN₀ necks and 84 % of cN+ necks. Observations of Jatin P. Shah are comparable to us (33 % for N₀ neck and 82 % for N+ necks) [15]. Becker MT reported the Recurrence rate approximately 10 % in patients who had histopathologically negative neck dissection specimen, suggesting a significant failure rate of light microscopy for cancer detection. Sampling problem was the reason attributed to the failure of light microscopy [23]. The micrometastasis are not detected by clinical examination or by modern imaging techniques but some time also missed by routine HPE [24, 25].

Many authors have found micrometastasis in 5–58 % (mean 19.6 %) of patients who were reported to be negative for metastasis on routine histopathological examination [26, 27]. In the present study we observed 84 pts (77 %) were positive for metastasis on histopathological examination out of 109 cases of cN+ necks. This figure raised to 101 pts (90 %) positive for metastasis, on immunohistochemistry of those 109 cases of cN+ necks. The observation was no different for cN₀ necks. Occult metastasis found in 28 % cases on HPE and 48 % case on IHC in clinically N₀ cases.

The pathological upstaging was observed by 20 % in N₀ necks and 13 % in N+ necks, in the present study. Barrera et al. [25] reported the upstaging of 29 % of N₀ cases by IHC. Rhee et al. [28] observed cervical micrometastasis in half of their patients with clinically N₀ head and neck cancer and Hamakawa et al. [29] found micrometastasis in 15.9 % of N₀ necks, in oral cancer patients. Yoshida et al. [30] had even better upstaging to report. They observed 20–30 % occult metastasis with H&E staining in cervical lymph nodes, in patients of cN₀ tongue cancers. He went on to use CK staining and occult metastases rate rose to 58 %. His observations are comparable with our findings.

Different authors have reported variable figures claiming, IHC a more sensitive method than serial sectioning. Kwon et al. [31] reported 13.8 % micrometastasis by IHC staining with AE1/E3, in cases which were negative on HPE in clinically N+ necks. Enepekides et al. [32] reported 9.3 % micrometastasis in oral cancers. Several researchers have claimed 9–37 % micrometastasis rate in cases of breast cancer [33, 34]. The present study was inspired by these tall claims and observed the comparable results.

FNAC is very useful, simple and available at almost every centre in the world. We also did FNAC to all the N+ pts. The specificity of FNAC was 100 % and false positivity was 0 % in the present study. Haque et al. [35] have reported high specificity too [97 %]. High specificity of FNAC, makes it a valuable tool in the diagnosis and management of Head and Neck Cancers, specifically so in occult primary cases.

The IHC has proved to be of high significant value, when compared with FNAC and HPE. The present study also showed that the cases which were positive on HPE, all turned out to be positive on IHC also in both N₀ and N+ necks. We did not find false positive case on HPE. The specificity of HPE was also 100 %. The likelihood of missing a malignant area with serial sectioning in HPE, is fairly above the acceptable limits and has affected the appropriate management of cancer. A cN₀ neck managed with elective neck dissection and is mistakenly assumed to be sterile after negative HPE report. The N+ necks, reported to be negative after therapeutic neck dissection or positive for a single node, are assumed to be adequately

treated with single modality management, have resulted in gross mismanagement of cancer. In the follow up, such assumptions and illusions are soon realized, when the pt reports with recurrence in the neck. Such disastrous outcomes are often attributed to inadequate and inefficient surgery. Thus the deficiencies of HPE perpetuate. The IHC has come in a very handy and useful tool by unearthing the metastasis in those lesions which were decided to be nonmalignant by HPE. With the information of inadequacy of treatment, the IHC has changed the line of management to just and appropriate approach with better oncological results. The sensitivity and specificity of IHC is the highest achieved by any other method thus far. The hitherto low staged cancers are up staged and are appropriately managed with better disease free survival. We advocate IHC, to be included in the routine diagnostic methods and should be done before deciding a lesion as non-malignant.

Conclusion

IHC has proved to be of immense value for diagnostic/prognostic assessment and judicious surgical/adjunct management of metastatic neck disease in HNSCC.

Conflict of interest Authors do not have any financial relationship with the organization that sponsored the research. The authors declare that they have no conflict of interest.

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