ORIGINAL RESEARCH PAPER

Pathology

USE OF IMMUNOHISTOCHEMISTRY TO DETECT MICROMETASTASIS IN LYMPH NODES IN VARIOUS MALIGNANCIES.

KEY WORDS: Micrometastasis, CK, EMA

DR.AKANCHHA RESIDENT OF PATHOLOGY ,INDEX MEDICAL COLLEGE, INDORE

DR.SANJEEV NARANG PROFESSOR , INDEX MEDICAL COLEEGE, INDORE.

DR.RAHUL SINHA ASSISTANT PROFESSOR, INDEX MEDICAL COLLEGE, INDORE.

DR.(MAJ GEN).S.K NEMA PROFESSOR AND HOD , INDEX MEDICAL COLLEGE, INDORE.

ABSTRACT

USE OF IMMUNOHISTOCHEMISTRY TO DETECT MICROMETASTASIS IN LYMPH NODES IN VARIOUS MALIGNANCIES.

Background: The identification of occult metastases in patients with initial stage cancer could have a substantial clinical impact on the prognosis and optimal therapy. We undertook this study to evaluate the efficacy of immunohistochemistry (IHC) and step sectioning in detecting occult lymph node metastases over the routine histological technique.

Methods: The present study was prospective as well as of retrospective analysis of CK7 and EMA as a marker of micrometastasis in lymph nodes in various malignancies conducted at Index Medical college ,Hospital & Research centre, Indore(M.P). We studied 181 lymph nodes in patients of various malignancies which were confirmed negative on histopathological examination. Immunohistochemical staining for CK7 and EMA was done according to CAP protocol.

Results: CK7 & EMA were significantly correlated with increasing tumor size and lymph node size. Age of patient was not found significant with lymph node metastasis. The sensitivity of CK and EMA was 65.4% and 10.5% respectively whereas the specificity of CK and EMA was 34.6% and 47.4% respectively.

Conclusion: Higher tumor size and lymph node size show statistically significant CK and EMA positivity and therefore pronounce a poor prognosis. Lymph node IHC is a useful adjunct to routine histological techniques in detecting micrometastases. Hence, this relatively rapid method can be recommended as a routine procedure in laboratories, especially in high risk group (age of patient ≥50 years, multiple primary tumors, tumor size >5 cm).

INTRODUCTION

The identification of occult metastases in patients with early stage cancer could have a substantial clinical impact on the prognosis and optimal therapy for patients with cancer. At later stages of the disease, it may be useful to determine the presence of and change in the number of residual malignant cells so that the therapies selected can be monitored and adjusted to the changing needs of the patient[1].

We undertook this study to evaluate the efficacy of immunohistochemistry (IHC) and step sectioning in detecting occult lymph node metastases over the routine histological technique and find out the correlation of detected occult lymph node metastases with various morphological parameters

AIM AND OBJECTIVES

AIM:

To study the role of immunohistochemical markers- cytokeratin and EMA antibodies as markers in detecting micrometastases in lymph nodes.

OBJECTIVES:

To detect metastases in lymph nodes reported negative on H & E staining.

To detect the percentage of cases which have immunohis tochemically detectable occult metastasis.

To identify disease recurrence after patients undergo curative resection by detecting micrometastasis.

To determine the usefulness of micrometastasis in recommending adjuvant chemotherapy.

MATERIAL AND METHODS

Source of data

Cancer patients attending the out patient and in patient department in Index hospital attached to Index medical college khudel, Indore during 18 months of study period.

Blocks retrieved from Department of Pathology.

Methods

The specimens are examined for gross details on arrival in the department, routinely processed and 3 to 5 micron thick sections are made from paraffin embedded blocks. These sections are routinely stained with H & E and examined for presence of invasive carcinomas. The lymph nodes reported negative for presence of metastasis are be subjected to immunohistochemical study with primary antibodies against cytokeratin and EMA proteins.

All specimens are formalin fixed and paraffin embedded. Lymph nodes are examined by one cross section through the center of each lymph node. Tumors are classified histologically into differentiated and undifferentiated types according to the World Health Organization tumor classification system. The differentiated type includes well or moderately differentiated tubular adenocarcinoma and papillary adenocarcinoma of Japanese classification, whereas the undifferentiated type includes poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous adenocarcinoma. Patients with tumors that are classified as T2N0M0 are selected for this study.

All available paraffin blocks are obtained, and two consecutive sections measuring 4 mm in thickness were newly cut for H&E staining and immunostaining.Immunohistochemistry was performed using a monoclonal antibody that is reactive with a broad spectrum of human cytokeratins. The tissue sections are deparaffinized, dehydrated and then microwaved for 10 minutes for antigen retrieval and incubated with the antibody at a 1:50 dilution, followed by second antibodies against mouse immunoglobulin.

Immunohistochemistry method:

Adequate tissue fixation in 10% neutral buffered formalin for 6-48 hr was ensured. Paraffin sections (3-4 um) thick sections were selected for IHC. Two sections were taken on Poly-L-Lysine coated slides. Sections were deparaffinised in Xylene followed by hydration in descending Ethanol-grades.

Antigen Retrieval :- By heating at 60 0C followed by 95 0C in pressure cooker in citrate buffer (2.9 gm/dl sodium citrate & 2.1 gm/dl citric acid) and for preparing working solution in 9ml. Citric acid add 450 ml of Distill water. Then cooled with disodium Hydrogen Phosphate buffer. 3% H_2O_2 was added for 10 minutes (to inhibit endogenous Peroxide activity of tissue) and then incubated with Power block for 5 minutes (to reduce Non specific antibody binding). Incubation at 4 oC with primary antibody for 1 hr. Then 3 washes were given with buffer and secondary antibody for 30 minutes. Again 3 washes with buffer. Then 3,3'-Diaminobenzidine (DAB tetrahydrochloride) was applied to section for 10 minutes and counterstained with hematoxylene. Then dehydrated with Ethanol & Xylene and mounted with DPX.

Positive Control :- Lymph nodes positive for metastasis are taken as positive control.

Fig 1. IHC kit

After comparison of immunostaining and H&E staining, monoclonal antibody was selected as the most sensitive for the detection of micrometastases. The immunostained lymph node sections were evaluated without knowledge of clinicopathologic information. Micrometastasis was defined as the presence of tumor cells detected only by cytokeratin specific immunostaining that could not be detected by ordinary H&E staining. Cytokeratin positive tumor cells had diffuse cytoplasmic staining with a more intensely stained peripheral band. There were two patterns of micrometastases:

Micrometastases consisting of a single cell were classified as the single-cell type (Fig. 2).Micrometastases consisting of clusters of two or more tumor cells were classified as the cluster type (Fig. 3).When both single tumor cells and clusters were observed in a lymph node, they were classified as the cluster type.

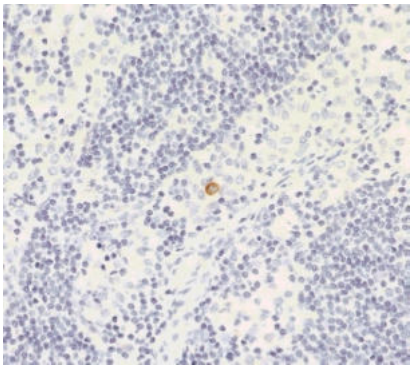


FIGURE 2. Micrometastases of the single-cell type. Cytokeratin positive cells are involved in the lymph nodes (original magnification, 3200).

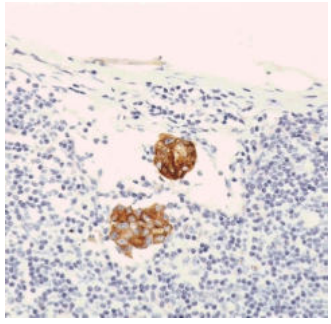


FIGURE 3. Micrometastases of the cluster type. Small clusters consisted of cytokeratin positive cells (original magnification, 3200).

RESULTS

CK is located in the cytoplasm and EMA on the cell membrane . Positive staining cells are brown-yellow, while the negative cells are unstained. Positive staining lymph nodes were confirmed by examination of the structure and morphology of the cells.

CK staining gave brown cytoplasmic reactivity. EMA staining gave brown cytoplasmic reactivity with membrane enhancement. Cells were considered to be occult node metastases if they were (a) Immunoreactive (expressed either CK or EMA antigens) (b)found within the substance of lymph nodes and (c) were morphologically consistent with cancer cells

If IHC-positive cancer cells were detected in the lymph node as a single cell or a small nest of cancer cells <0.2 mm in size, it was defined as ITC. If the size of the cell nest was >0.2 mm but <2 mm, it was defined as MM. However MM and ITC were combined into one group in the subsequent statistical analysis.

Statistical analysis

The data (results) were inferred and they analyzed using the SPSS V21. The mean, median, average and the percentile were calculated accordingly. The statistical correlations between micro-metastasis and the pathological variables were assessed using the chi-square test and the Fisher's exact test, Z tests were used to test the statistical significant differences between the two proportions. The p values <0.05 were considered to be statistically significant and the p value < 0.01 is considered as a highly significant one.

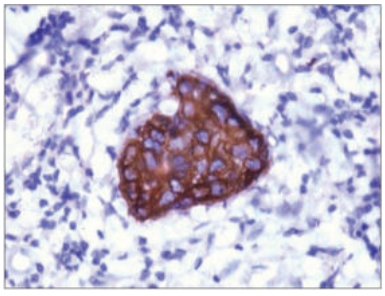


Figure 4. Microphotograph of lymph node showing metastasis by IHC for Ck7

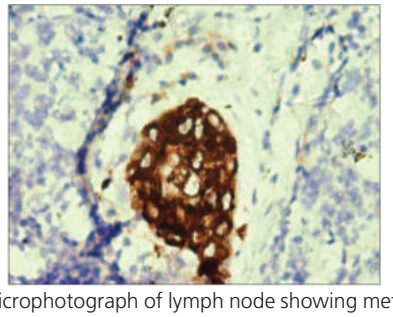


Figure 5. Microphotograph of lymph node showing metastasis by IHC for EMA

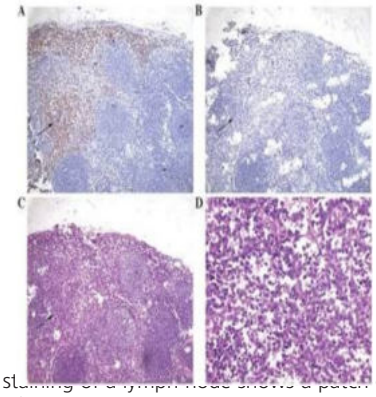


Fig 6. CK staining of a lymph node shows a patch of tumor cells (arrow)(×40)

EMA staining of the corresponding lymph node show no clearly visible tumor cells in the lymph node (arrow)(×40)

HE staining of the corresponding lymph node shows a patch of tumor cells (arrow)(×40)

×100 microscopy of HE slides of the corresponding lymph node proves to be tumor cells.

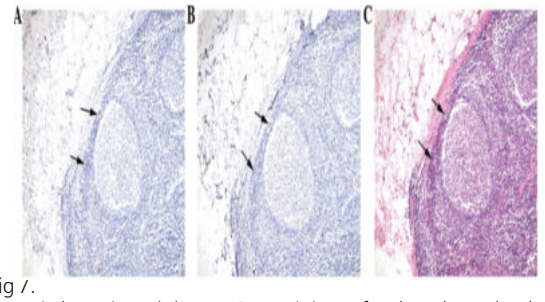


Fig 7.
HE misdetection: (A) pan-CK staining of a lymph node shows several isolated tumor cells(×40).

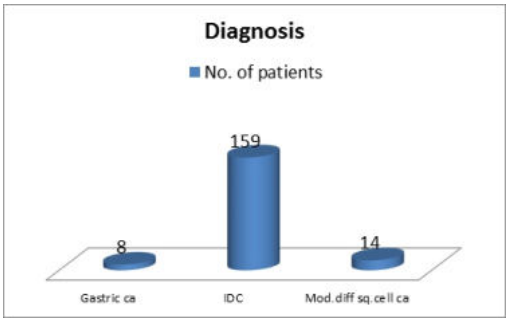
(B) EMA staining of the corresponding lymph node shows several isolated tumor cells(×40)

(C) HE staining of the corresponding lymph node shows no clearly visible tumor cells in the lymph node(×40)

Out of 793 lymph nodes dissected from 77 patients of various malignancies, 612 (77.1%) lymph nodes were positive for metastases. 181 (22.8%) lymph nodes were negative for metastases on H and E examination. These 181 cases were taken up for the study.

One fifty nine cases (87.8%) were diagnosed with infiltrating duct carcinoma not otherwise specified, Fourteen cases (7.7%) as Squamous cell carcinoma and Eight cases (4.4%) as Gastric carcinoma.

GRAPH 1.

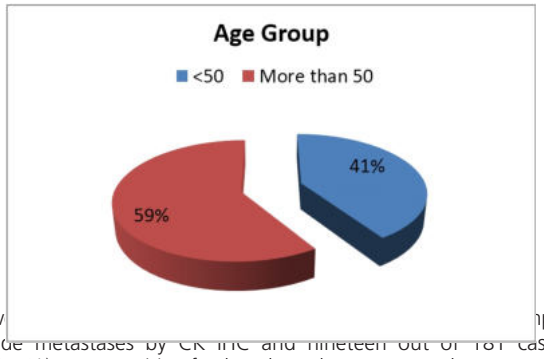


The minimum age of the patients was 30 years and the maximum age was 80 years. The mean age of patients was 53.07 years (standard deviation [SD] – 9.933) and the median age was 54 years.

Table 1:- AGE Calculation

Age group (yrs)	No. of patients
<50	74
≥50	107

Graph 2.



Two hundred and fifty nine lymph node metastases by CK IHC and nineteen out of 181 cases (10.4%) were positive for lymph node metastases by EMA IHC (Table 2).The sensitivity of detection of occult metastases by CK was 65.4% while that of EMA was 10.5%. The specificity of detection was 34.6% for CK and 47.4% for EMA. There was a significant correlation between CK and EMA positive metastases (P = 0.322).

Table 2:- Correlation between lymph node metastases by CK7 and EMA

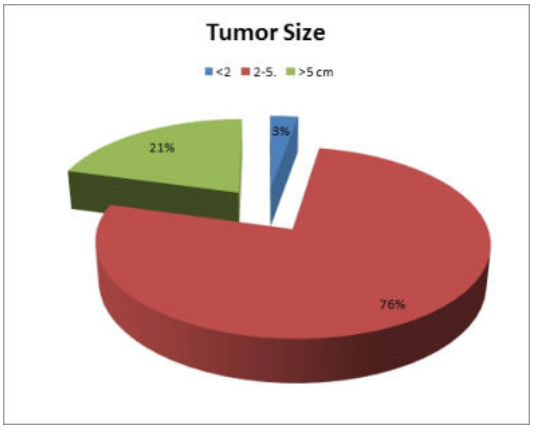
CK	EMA Negative	EMA Positive	Total
Negative	145	10	155
Positive	137	9	26
Total	162	19	181

Most of the cases i.e 137(75.7%) had tumor size 2-5 cm.Thirty seven cases (20.4%) had tumor size > 5cm and five cases (2.8%) had tumor size < 2cm.The mean tumor size was 4.13 cm (SD – 2.36) and median 11.2 cm.

Table 3:- calculation of tumor size

Tumor size(cms)	No. of patients	% of patients
<2	5	2.8
2-5	137	75.7
>5	37	20.4

Graph 3.



One twenty five (68.3%) cases had lymph node size measuring between 1 and 2 cm, forty seven (25.7%) cases < 1 cm and nine (4.9%) cases with > 2 cm.The mean lymph node size was 1.27 cm (SD – 0.55) and median 5.7 cm.

Table 4:- calculation of Lymph node size

Lymp node size	No. of patients	% of patients
	47	**25.7**
1-2	125	68.3
>2 cm	9	4.9

Graph 4.

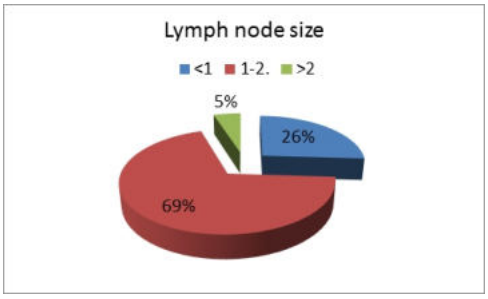


Table 5. Correlation of metastasis detected by CK & EMA with age,tumor size and lymph node size.

Classification	Number and percentage of total cases	Number and percentage of cases with metastases deected by CK (%)	P value for CK	Number and percentage of cases with metastases deected by EMA (%)	P value for EMA
Age(In years)					
<50	74(40.88)	11(14.86)	-0.039(NS)	8(10.81)	-0.073 (NS)
	107(59.12)	15(14.01)		11(10.28)	
Tumor Size (in cm)					
	4(2.21)	1(25)	0.323** (S)	0(0)	0.387** (S)
2-5	140(77.34)	15(10.71)		10(71.42)	
	37(20.44)	10(27.02)		9(24.32)	
Lymp node Size (in cm)					
	37(20.44)	0(0)		2(5.40)	0.232** (S)
1-2	133(73.48)	18(13.53)	.255**(S)	10(9.67)	
	11(6.07)	8(72.72)		7(63.63)	

*. Correlation is significant at the 0.05 level .

DISCUSSION

Lymph node metastasis is an important prognostic factor for patients with malignancy. The clinical significance of nodal metastasis has long been well appreciated. The cure rate drops to nearly half with involvement of regional lymph nodes. Histopathological examination is highly sensitive and specific test for detection of metastasis but earliest stage of metastasis can be difficult to identify by light microscopy. If a single 5um section is required, the 1cm lymph node has to be sectioned 2000 times.

Lindeman et al. demonstrated the absence of cytokeratins in any types of cells of a normal lymph nodes except the metastatic cells from epithelial primary2. He observed the CK7 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.3.Many epithelial inclusions in lymph nodes 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 authenticity of CK as a tumor marker needed it to be used along with other immunohistochemical markers. In our study we used Epithelial membrane antigen as the second marker.

The results of this study confirmed the predicted sources of error in standard pathological assessment of lymph nodes with both immunohistochemical detection and step sectioning resulting in increased detection of metastasis. Serial sectioning of the lymph nodes as a routine procedure is far too time consuming to be practical. This method is labour intensive for both laboratory technician and pathologist.

About 14.36% of the cases were detected to have metastasis by staining with CK and 10.4% by EMA. This increase in detection rate of occult metastasis is seen in concordance with the other studies in the literature.(Table 6)

Table :6

Author	Year	IHC stains used	Increased detection rate %
Wells et al	1984	CK, EMA	15
Trojani et al	1987	CK,EMA,HMFG,HMFG2,AUA1	14
Mascarel et al	1992	CK,EMA,HMFG,HMFG2,AUA1	22.9
Umekita et al	2002	CK AE1/AE3	14.2
Marinho et al	2006	CK AE1/AE3	12.2
Grabau et al	2007	Ck	8

In addition, however occult metastasis were seen more frequently with age of patient more than or equal to 50 years but we did not found a statistically significant correlation between age and lymph node metastases detected by CK and EMA IHC, when patients were grouped as <50 years or >50 years. McGuckin et al. found that immunohistochemically detected micrometastases were associated with a shorter survival by univariate analysis in the under 50 years old group of patients corresponding to 41% (26/64) of the patients in the series[4]. McGuckin et al., de Mascarel et al. and Grabau et al. also found a statistically significant correlation between age and lymph node metastases detected by IHC, when the patients were grouped as <50 years and □50 years[5].

In our study, tumor size correlated significantly with lymph node metastases detected by CK (P = 0.323) and EMA (P = 0.387). Nasser et al. found that occult metastases were more frequent with larger tumor size. McGuckin et al. found lymph node metastases detection rate of 35% in cases with tumor size >2 cm as compared with 20% in cases with tumor size □2 cm and obtained a statistically significant correlation between tumor size and lymph node metastases detected by IHC. In a study by Cote et al., 26% of the cases with tumor size >2 cm were positive for lymph node metastases detected by IHC as compared with 16% of the cases with tumor size □2 cm positive for lymph node metastases by IHC6. Umekita et al. also found a higher incidence of micrometastases detection in cases with tumor size >2 cm7.

There was a statistically significant correlation between lymph node size and lymph node metastases detected by CK and EMA. However, Hainsworth et al. and Grabau et al. found a statistically significant correlation between lymph node number and lymph node metastases detected by IHC[8].

Zhi-Wei Zhou et al in 2005 found no correlation with all these parameters (all p >0.05)[9]. While Noura et al in 2002 found that tumor size alone was significantly larger in the CK-positive group than in the CK-negative group (p = .014)[10].

The detection of micrometastases correlates significantly with the recurrence and survival rate of patients. Immunohistochemical staining is a sensitive and specific method of detecting nodal metastases. It highlights the fallibility of conventional microscopic assessment of lymph nodes.. Although experienced histopathologists, given adequate time and a large number of sections might pick up many of these cases, the important point to emphasize is the ease and confidence with which these micrometastases can be identified by immunostaining. This is of potential clinical significance in the context of chemotherapy trials, in which the treatment is dependent upon the number of involved lymph nodes. Furthermore, metastasis by IHC can be detected by adequately trained scientific staff, which minimizes the workload of specialist pathologists and therefore reduces the cost of implementing such procedures. IHC can be performed in the same time frame as conventional histology and is applicable to most methods of tissue processing including frozen sections.

We have demonstrated the ability to detect lymph node micrometastases by subjecting the entire specimen to complete serial sectioning and IHC for node-negative cancer patients. We conclude that routinely cutting multiple serial sections is not very convenient.

Immunohistochemical detection is not much cumbersome, yet it is expensive or time consuming procedure.

Lymph node IHC is a useful adjunct to routine histological techniques in detecting micrometastases. Hence, this relatively rapid method can be recommended as a routine procedure in laboratories, especially in high risk group (age of patient □50 years, multiple primary tumors, tumor size >5 cm). Immunohistochemical detection after routine histopathological examination is useful for selecting the node negative breast cancer patient subgroup at a high risk for relapse and death. In future, molecular techniques like RT-PCR can be used in combination with IHC to increase the sensitivity and specificity for detection of micrometastases

CONCLUSION

The present study was Prospective as well as of Retrospective analysis of CK7 and EMA as a marker of micrometastasis in lymph nodes in various malignancies conducted at Index Medical college ,Hospital & Research centre, Indore(M.P).

We studied 181 lymph nodes in patients of various malignancies which were confirmed negative on histopathological examination. Immunohistochemical staining for CK7 and EMA was done according to CAP protocol.

CK7 & EMA were significantly correlated with increasing tumor size and lymph node size. Age of patient was not found significant with lymph node metastasis.

The sensitivity of CK and EMA was 65.4% and 10.5% respectively whereas the specificity of CK and EMA was 34.6% and 47.4% respectively.

It is therefore postulated that the higher tumor size and lymph node size show statistically significant CK and EMA positivity and therefore pronounce a poor prognosis in these cases.

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