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VALUE OF CLAUDIN-4 IMMUNOSTAINING IN DISTINGUISHING ADENOCARCINOMA FROM REACTIVE MESOTHELIUM IN EFFUSION FLUID CYTOLOGY

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ABSTRACT

BACKGROUND- Morphological distinction between metastatic carcinoma cells & reactive mesothelial cells is difficult in many occasions in effusion cytology. Immunohistochemistry plays important role in distinguishing the two. Immunohistochemistry for claudin-4, has shown to distinguish adenocarcinoma from reactive mesothelium effectively.

AIMS- Our aim was to validate and assess the Immunoreactivity profile of claudin-4 in a series of malignant and benign effusions.

MATERIALS AND METHODS:- Total 84 samples were taken, 38 containing reactive mesothelial cells & 46 containing adenocarcinoma cells. Claudin-4 immunohistochemistry was performed on cell-block paraffin sections and scored for staining intensity, staining pattern and percentage of positive tumor cells.

RESULTS- In 46 cases of metastatic adenocarcinomas, 44 cases were positive for Claudin-4 & among the 38 non-malignant effusion cases where the fluid contained reactive mesothelial cells, none of them were positive for Claudin-4. Sensitivity & specificity of claudin-4 in diagnosing adenocarcinoma were 95.6% & 100% respectively. Positive Predictive Value & Negative Predictive Value of claudin-4 in diagnosing adenocarcinoma were 100% & 95% respectively**.**

CONCLUSIONS: Claudin-4 immunohistochemistry effectively distinguishes adenocarcinoma from reactive mesothelium with high sensitivity and specificity.

KEYWORDS : Claudin-4, Effusion cytology, Reactive Mesothelium, Adenocarcinoma.

INTRODUCTION

A variety of benign and malignant disorders can present with serous effusions. Aspiration of serous cavity fluids (pleural, peritoneal) has become a routine procedure for aiding in diagnosis. Diagnostic difficulties can arise as the difference between metastatic carcinoma and atypical reactive mesothelium are subtle. Hence, in serous effusion smears, the morphologic criteria used in cytology have not always ensured diagnostic accuracy and determination of source and cell behavior has always been a matter of diagnostic confusion among investigators all over the world[1]. Hence, in serous effusion smears, the morphologic criteria used in cytology have not always ensured diagnostic accuracy[2].

The presence of antigen selectively on metastatic carcinoma cells, which is absent in reactive mesothelium or vice versa, is the only possible way to identify malignant cells in the body cavity effusions. Therefore, the use of ancillary methods is mandated in all but unequivocal cases and it has become clear that of all the methods available, immunochemical stains are superior in the diagnostic workup of effusion cytology[3].

Claudin-4, a major modulator of tight junctions[4] , identifies neoplasms potentially metastasizing to serosal surfaces, while it is usually not expressed in non-metastatic carcinoma tumors, and claudin-4 is negative in normal mesothelium. This indicates that Claudin-4 is a highly specific and sensitive marker that can be used to discriminate between mesothelial cells and metastatic carcinomas cells in the serous effusions.

The present study was conducted to know whether Claudin-4 should be considered a primary marker to be included in the panels of immunocytochemical markers which can further be applied to differentiate metastatic epithelial cells and mesothelial cells in serous effusions.

MATERIALS & METHODS

The study was done at the Laboratories of the Pathology department of a leading referral teaching Institution of Kolkata(ESI-PGIMSR, Manicktala) with specimens received from the own institution and also referred specimens from other hospitals in the city and state over a period of 2 years (2015-2017). Patients were selected irrespective of their age and sex. Pleural and Peritoneal fluids were taken for study. Only those effusion fluids were taken for study in which reactive mesothelial cells, adenocarcinoma cells or features suspicious of malignancy were found during routine cytological examination. The effusion fluids which were excluded had distortion of cellular morphology on microscopic examination or predominantly inflammatory cells with few scattered mesothelial cells.

Total 84 cases were taken. 38 effusion fluid samples containing reactive mesothelial cells and 46 effusion fluid samples containing adenocarcinoma cells.

All the diagnoses were later confirmed by radiological presentation or histopathology. Centrifuged deposits were stained with MGG and PAP.

The cases morphologically classified as reactive mesothelial Cells were present with following features- cells present singly or more often in berry-like clusters with scalloped contours, no true papillary or acinar structures. Nuclei round with smooth contours. The cells have low nuclear to cytoplasmic ratio. Cytoplasm usually abundant and dense with peripheral fuzziness.

In contrast, cases identified as adenocarcinoma were seen as singly lined or groups of cells with smooth contours and hard anatomical edges. Acinar structures, papillary structures, proliferation spheres and solid three dimensional cell clusters seen frequently. Nuclei of variable size, often with irregular contour, prominent nucleoli and high nucleus to cytoplasmic ratio. Cells with overlapping features were categorized as suspicious of malignancy.[Figure-1]

Formalin-fixed, paraffin-embedded cell blocks were prepared in all cases with using standard cell block preparation method. At least 2 slides comprising serial sections at 3-5 µm were obtained from these cell blocks.

One was stained with Hematoxylin and Eosin (H&E) stain, while the slide was subjected to IHC for Claudin-4. Claudin-4 immunoreactivity was analyzed by evaluating the percentage of cells stained and the intensity of staining for each of the cases . Claudin-4 stained the cell membranes & cytoplasm. [Figure-2]. Scores of 0, 1, 2 and 3 were recorded when 0%, <10%, 10-50% and >50% of the cells stained respectively and intensity scores of 0, 1, 2 and 3 were given when there were none, mild, moderate and strong staining respectively. A sum of the percentage and intensity was taken as the final IHC grade of the marker.

RESULTS

Age range of the patients were 35 – 73 years with a mean age of 55 years. Majority of the patients i.e. 28 out of 84 (33.4%) were in the age group of >60 – 70 years.

Majority of the patients i.e. 54 out of 84 (64.3%) were males with a male female ratio of 1.8 : 1. In the present study the most common presenting symptom was abdominal distension (30.9%) followed by shortness of breath (28.6%) and cough (19.1%). Majority of the fluids(64.3%) taken in the study are pleural fluids, rest (35.7%) are peritoneal fluids. Majority of the fluids (76.2%) were straw coloured and rest (23.8%) were hemorrhagic.

In the study only 21.5% fluids were confidently diagnosed as malignancy in the PAP stained cytological smears, rest are reported as suspicious of malignancy(47.6%) or reactive mesothelial cells.(30.9%). 54.7% cases were finally diagnosed as malignancy and rest 45.3% were non neoplastic based on clinicoradiological/ biochemical/ histopathological correlation.

In the study all the malignancies turned out to be adenocarcinomas metastasizing to serous cavities. 65.3% patients who were diagnosed to have metastatic adenocarcinomas were males and rest 34.7% were females.

Most of the non-malignant effusions turned out to be Tubercular origin. 63.2% those patients who had non-malignant effusions were male, rest were female.

In 46 cases of metastatic adenocarcinomas, 44 cases were positive for Claudin-4 & among the 38 non-malignant effusion cases where the fluid contained reactive mesothelial cells, none of them were positive for Claudin-4.[Table-1], [Table-2].

Sensitivity of claudin-4 in diagnosing adenocarcinoma was 95.6%.Specificity of claudin-4 in diagnosing adenocarcinoma 100%.Positive Predictive Value of claudin-4 in diagnosing adenocarcinoma 100%,Negative Predictive Value of claudin-4 in diagnosing adenocarcinoma 95%[Table 3]. The chi-square statistic is 38.1652. The p-value is < 0.00001. The result is significant at $p < .05$.

DISCUSSION

In our study total 84 cases were evaluated, 46 of them were cases of adenocarcinoma of various primary sites, metastasizing to pleural and peritoneal cavities and 38 of them were benign conditions manifested as serous effusions.

Cytological diagnosis were given then cell blocks were prepared and Immunostaining with Claudin-4 was done. Then cytological report, cell block morphology, Immuno staining results and the final diagnosis by clinicoradiological correlation were considered together to come to the final conclusion of the study.

There has been controversy regarding the optimal amount of fluid to be submitted for examination. In the present study also approximate 50 ml of fluid was found to be adequate for cytological examination. However more fluid can be drained for therapeutic reasons. Physical appreance of pleural fluid varies and can give an idea of underlying etiology. It can be hemorrhagic, straw coloured or purulent. In most of the studies straw-coloured fluid was more commonly encountered. Although straw coloured appearance of pleural effusion is typical of transudates yet it is frequently also seen with exudative effusions. Haemorrhagic effusion are commonly seen in malignancy. In our study majority of the fluids (76.2%) were straw coloured and rest were hemorrhagic.

In our study only 21.5% cases were confidently reported as malignancy but majority of the cases 47.6% were reported as suspicious of malignancy. Those cases which had overlapping features and were inconclusive of malignancy in cytopathology report, were confirmed later by clinicoradiological and Histopathological correlation. In our study 54.7% cases were malignancy and rest 45.3% cases were nonmalignant lesions but in other studies they have taken equal number of malignancy and non-malignant lesions which is attributable to their longer study duration & larger sample size.

In our study out of 46 cases of metastatic adenocarcinoma 44 cases(95.6%) were claudin-4 positive, only 2 case(4.4%) were claudin-4 negative. In 38 cases of reactive mesothelial cells all the cases(100%) were claudin-4 negative. sensitivity, specificity, positive predictive value and negative predictive value of claudin-4 in our study were 95.6%, 100%, 95%, 100% respectively. The only metastatic adenocarcinoma case which was negative for claudin-4 was a case of cholangiocarcinoma metastasizing to pleural cavity.

Study by Lonardi et al(2011)[5] was done to recognize claudin-4 in detection of metastatic tumor cells and the differential with reactive and neoplastic mesothelium. Neoplastic serous effusions obtained from pleura, peritoneum, and pericardium in 345 cases and authors concluded that claudin-4 with high sensitivity (99.1%) and specificity (100%), might be used as an ideal "single-shot" marker for the identification of metastatic carcinoma in serous effusions.

In the study by Afshar-Mogaddam et al(2013) [6] Claudin-4 protein was positive in 40 specimens of metastatic carcinoma, while none of the cases of reactive mesothelium stained with the marker. This was not detected in the mesothelial cells, though. Positive staining for claudin-4 was significantly more frequent in metastatic carcinoma than in the reactive mesothelium ($P > 0.0001$). The sensitivity and specificity of claudin-4 to distinguish reactive mesothelium from metastatic carcinoma were 85% (95% CI, 71.1-93.8%) and 100% (95% CI, 91.1-100%), respectively. The results of this study demonstrated that claudin-4 is less frequently expressed in reactive mesothelium. Thus, this claudin may be helpful in differentiating metastatic carcinoma from reactive mesothelial cells in pleural and peritoneal fluid cytology specimen.

In the study by Vickie Y Jo et al(2014) [7] all cases of mesothelioma were negative for claudin-4 (0 of 64). Eighty-three of 84 cases of adenocarcinoma were positive (99%), 1 case of serous carcinoma was negative. Most adenocarcinomas showed strong and diffuse membranous staining (71 of 84; 84%); 12 cases (14%) showed membranous staining of moderate intensity. The overall sensitivity for adenocarcinoma was 99% (83 of 84).Claudin-4 immunohistochemistry effectively distinguishes adenocarcinoma from malignant mesothelioma with high sensitivity and specificity in the evaluation of malignant effusions.

In the study by Oda et al(2016) [8]the sensitivity and specificity of claudin-4 to distinguish adenocarcinoma from reactive & neoplastic mesothelium were 96.4% and 100% . The study demonstrated a superiority of Claudin-4 over the classical markers Ber-EP4 and MOC-31in the distinction of Metastatic Carcinomas from Reactive Mesotheliums. Thus Claudin-4 may come to be considered one of the best Metastatic Carcinoma markers in effusion cytology.

CONCLUSION

In conclusion, claudin-4 immunostaining effectively distinguishes adenocarcinoma from reactive mesothelium with high sensitivity and specificity in the evaluation of malignant effusions. Furthermore Claudin-4 may be used for Single shot identification of

metastatic adenocarcinomas due to its very high sensitivity and specificity, however studies with larger sample size and prolonged follow up are required to assess the expression of Claudin-4 in different types of metastatic carcinomas and also in reactive & neoplastic mesothelium to evaluate the true value of Claudin-4.

TABLE- 1 Claudin-4 reactivity

Diagnosis	Claudin-4 Positive	Claudin-4 Negative
Adenocarcinomas(46)	44	2
Reactive Mesothelium(38)	0	38

TABLE- 2 Claudin-4 Scoring

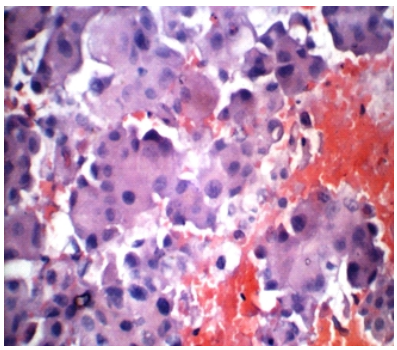
DIAGNOSIS	0	1	2	3	4	5	6
AC(46)	0	0	2	2	2	10	30
RM(38)	38	0	0	0	0	0	0

The best cut-off value for each antibody was staining index score 2.

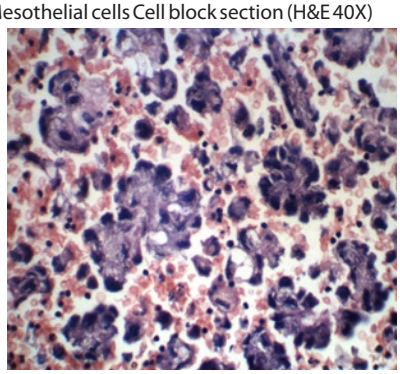
TABLE-3

Claudin-4	Sensitivity	Specificity	PPV	NPV
AC cases-46 True Positive- 44	95.6%	100%	100%	95%

FIGURE 1

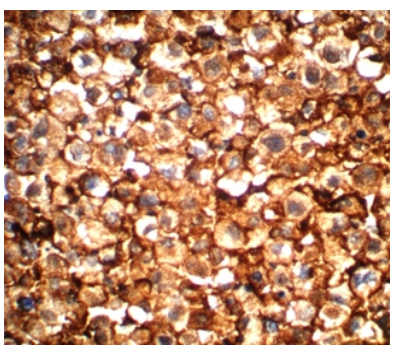


Reactive Mesothelial cells Cell block section (H&E 40X)

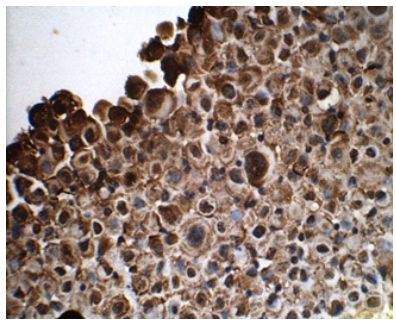


Adenocarcinoma cells Cell block section (H&E 40X)

FIGURE 2



Strong positivity of Claudin-4 in Adenocarcinoma cells (IHC 40X)



Moderate positivity of Claudin-4 in Adenocarcinoma cells (IHC 40X)

DECLARATIONS

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Ethical approval: Not required

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