

Study of Inverse Association Between Intercellular Adhesion and Malignant Cell Propagation in Gall bladder Carcinoma- Immunohistochemical Study of E-Cadherin and Autocrine Motility Factor Receptor

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Abstract: Though incidence of Gall bladder carcinoma is most common biliary tract cancers, nonspecific presenting symptom, late detection of the disease add to the dismal prognosis We tried to find association between intercellular adhesion and propagation of cancer cell in Adenocarcinoma of Gall bladder. E-cadherin (ECAD) is the strongest intercellular adhesion molecule of epithelial cells and Autocrine Motility Factor (AMFR) is known propagator of cancer cell. In retrospective study, We evaluated simultaneous immunohistochemical expression of these two molecules in 92 cases who were treated surgically by Cholecystectomy with or without lymph node dissection. Normal Gall bladder mucosa reacted strongly with ECAD and weakly with AMFR. With increasing dedifferentiation of the tumour, reversal manifested- more aggressive grades showed varied expression in comparison to its less aggressive type. Gastric adenocarcinoma show weak ECAD (83% vs. 35%) and more AMFR (93.% vs. 35%) expression in comparison to Grade I. Weak ECAD and strong AMFR was also associated with increase in depth of tumour invasion. As ECAD and AMFR is at least partially responsible for varying histologic grade and behavioral pattern of Gall bladder adenocarcinoma, simultaneous evaluation of both parameters is helpful to understand pathway of progression of such cancer cell.

Key words: Gall bladder carcinoma, E- cadherin, Autocrine Motility factor Receptor

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I. Introduction

Gallbladder cancer was first described in 1777. More than 200 years later, late diagnosis and absence of effective treatment for many patients remain typical features of this disease. (1) The highest gallbladder cancer incidence rates worldwide were reported for women in Delhi, India (21.5/100,000), South Karachi, Pakistan (13.8/100,000). (2) In most countries of the European Union (EU), age-standardized mortality rates of Gallbladder carcinomas declined by ~30% among women and by 10% among men in the 1990s, but mortality from Gallbladder carcinomas was still high in central and eastern Europe. Between the early 1980s and mid-1990s, falls in Biliary Tract cancers mortality rates were also observed in the United States and Australia. In contrast, Japan reported a rise of mortality rates for biliary tract cancer. Gallbladder carcinomas is the first cause of death for cancer among women in Chile, and the very high mortality rates did not decline since the 1980s. (3) Female-to-male incidence ratios were generally around 3, but ranged from 1 in Far East Asia to over 5 in Spain and Colombia (2)

The highest frequency of the disease is found among females over the age of 65.7. There is a marked regional and ethnic variation in the incidence of gallbladder cancer. The highest mortality rates have been reported among Chilean Mapuche Indians and Hispanics, among Bolivians, North American Indians, and Mexican Americans. Incidence rates are much lower in Europe and India. (1) Symptoms and signs of Gallbladder carcinomas are not specific and often appear late in the clinical course of the disease. For this reason, diagnosis is generally made when the cancer is already in advanced stages, and prognosis is poor—only about a 32 percent five-year survival rate for lesions confined to the gallbladder mucosa and a 10 percent one-year survival rate for more advanced stages.

Despite advances in diagnosis, the disease is usually detected after invasion of the muscularis mucosae, -very often after involvement of muscularis propria. Furthermore, surgery and chemotherapy have limited value in advanced disease and there is a paucity of molecular markers for targeted therapy. Since cancer of gallbladder

has a relatively poor prognosis a new look at the results of epidemiological and experimental studies is important to establish strategies for early precise detection and prognosis.

At cellular level, progression of malignancy is dependent variably on many cellular properties including intercellular adhesion, motility and proteolysis,(4)for infiltration of malignant cell into surrounding stroma , reduction of intercellular adhesion and increment of cell motility appeared two necessary simultaneous incidents.It is established that E-cadherin(ECAD) is strongest intercellular adhesion molecule in epithelial cell.(5) which is regulated by ECAD and ECAD associated proteins including catenins.(6,7).Many researchers has indicated correlation between of infiltration of malignant cell and diminished ECAD and catenins both in vitro and in vivo in malignant lesion of various organs(6,7) is modulated by property of cell motility-which in turns is affected by various motility factors like Hepatocyte Growth factor, Epidermal growth factors(8-11) and as Silletti et el found that loss of intercellular adhesion up regulate the protein expression and opromoter activity of AMFR(15).

Autocrine Motility Factor (AMF) has been purified from the culture media of various tumor cells as a specific motility modifier.(16,17) The receptor for AMF (AMFR) has been identified as a cell surface glycoprotein (gp78; molecular weight,78,000) on the B16-F1 melanoma cell line with high metastatic ability.(16,17)Autocrine Motility Factor Receptor (AMFR) concentrates on the leading edge of the cell surface, then is phosphorylated and internalized by binding with AMF.(18) Finally, it induces rearrangement of integrin, causing cells to move.(19) In this pathway, G protein might be involved, since cell motility is inhibited by a Bordetella pertussis toxin.(19) Up-regulation of AMFR and its implication in cancer progression in human cancers of various origin, including the large intestine, placenta, esophagus, and stomach,(20-23) has been reported.

Review of literature reveled in epithelial cell, ECAD is strongest intercellular adhesion molecule(5),association between ECAD and AMFR is studied in various epithelial malignancies i.e. carcinomas and simultaneous loss of ECAD and increase in AMFR is found in cultured cell lines of Urinary Bladder carcinomas.(25)This simultaneous alteration of ECAD and AMFR, if they are situated on the common signal, enables us to understand that cancer progression more fluently leads to invasion and metastasis.

Many researchers concentrated to find association between destruction of intercellular adhesion and propagation of carcinomatous cell and studied simultaneous co expression of ECAD and AMFR gene in Colon, Rectal, Breast ,and Gastric adenocarcinoma with aid of immunohistochemistry .We are yet to find similar study in Gallbladder adenocarcinoma. We tried to find if there is any significant association of expressions of these two molecules in different grades and stages gallbladder adenocarcinoma and evaluated immunohistochemical expression of ECAD and AMFR protein to that end in our study.

II. Material And Methods

The study population consisted of 92 patients who were finally treated with Cholecystectomy with or without regional lymph node dissection. In this retrospective study (conducted between 2011to 2016), in Medical College, Kolkata,we found that 43.6% cases Gall bladder adenocarcinoma was detected during histological examination of routine cholecystectomy specimen.

Other patients underwent Final surgery within 60 days (with range of 43 to 184 days)of the initial evaluation. To reduce influence on natural history the disease, we selected only patients who have received no anticancer therapy prior to the surgery.

Clinical data including copy of histopathology requisition slips were collected from tertiary treatment center in Kolkata. Fresh Copy of Hematoxyline and Eosin stained tissue sections of Cholecystectomy with or without regional lymph node dissection specimens were prepared from paraffin blocks. Team of Physicians Surgeon and Pathologist in Medical College, Kolkata went through the clinical data and tissue sections as per previously fixed protocol and parameters.

Hematoxyline and Eosin stained tissue 0.5 micrometer thick sections were studied and Tumor was classified in Well differentiated (Grade 1),Moderately differentiated (grade 2) and Poorly differentiated (grade3) type based on histomorphology. Depth of Tumour invasion was noted following established WHO Guideline. Sections for immunohistochemistry was selected among the paraffin blocks which were taken from invasive margins of tumor, and had tumor in 50% or more of total section area.

Immunohistochemistry was performed on sections obtained from representative block of formalin-fixed paraffin-embedded tissue using the Avidin-biotin complex technique. The sections were deparaffinized in xylene, and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. The slides were subsequently incubated at room temperature with reagents. After washing in a 0.05-mol/L concentration of phosphate-buffered saline (PBS), they were incubated with3% normal rabbit serum for AMFR or 3% normal mouse serum for ECD for 30 minutes to block nonspecific conjugation in the tissues. The specimens were incubated sequentially with the primary anti-AMFR monoclonal antibody, 3F3A19, or antihuman ECD antibody, HECD1 (Takara Shuzo, Kyoto, Japan), at 4°C overnight. After washing with PBS,

they were incubated with biotinylated rabbit anti-rat IgG for AMFR or rabbit anti-mouse IgG (Vectastain ABC Kit, Vector, Burlingame, CA), diluted 1:250 in PBS, for 30 minutes at room temperature and with ABC reagent (Vectastain ABC Kit) for 30 minutes at room temperature. The immune conjugate was visualized with a 0.05-mol/L concentration of tris(hydroxymethyl)-aminomethane (Tris)-hydrochloric acid (pH 7.6) containing 0.02% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride and 0.03% (vol/vol) hydrogen peroxide, and counterstaining was performed with Meyer's hematoxylin.

During immunohistochemical evaluation of ECAD and AMFR tumour cells were designated positive or negative as per predetermined criteria. ECAD is normally expressed at glandular lining epithelium-which made us to fix 90% of ECAD expression as cut-off criteria of positive expression. On the other hand, AMFR is only mildly expressed in normal ductal cells and we fixed its 50% expression as cut-off margin for same purpose. For statistical analysis, differences between the 2 groups were assessed by the Mann-Whitney U test, and correlations between 2 parameters were evaluated by the Spearman rank correlation test.

III. Tables

Table 1:

Expression of Autocrine Motility Factor Inhibitor Receptor (AMFR) and E-Cadherin (ECAD)								
	AMFR				ECAD			
	Strong		Weak		Strong		Weak	
Histopathologic Grade (n=92)								
Grade 1 (n=20)	7	7.6%	13	14.1%	13	14.1%	7	7.6%
Grade 2 (n=46)	30	32.6%	16	17.3%	18	15.5%	28	30.43%
Grade 3 (n=30)	28	30.4%	2	2.17%	5	5.4%	25	27.17%
Depth of Invasion								
T1 (n=8)	2	11.1%	6	75%	7	87.5%	1	12.5%
T2 (n=69)	41	59.4%	28	40.6%	44	63.7%	25	36.2%
T3 (n=15)	11	73.3%	4	26.7%	3	20%	8	53.3%

Table 2:

Relationship Between Autocrine Motility Factor Inhibitor Receptor (AMFR) and E-Cadherin (ECAD) Expression					
	ECD Strong		ECAD Weak		Total
	AMFR Strong	14	15.2%	30	
AMFR Weak	26	28.2%	22	23.9%	48
Total	40		52		92

IV. Results

In non-malignant Gall bladder mucosa, ECAD is strongly expressed at intercellular border. In contrast to ECAD expression, AMFR, in such cases is seen in some foci of proliferating zone. (Image 1,2,)

In Gall bladder cancer cells, AMFR frequently was expressed in the cell surface and cytoplasm and ECAD expression frequently was reduced in a homogenous or heterogeneous fashion. Thus, the alteration in Gall bladder cancers was as follows: 65 cases (70.6%) showed strong expression of AMFR, and 60 cases (65.2%) showed weak ECAD expression. The expressions of AMFR and ECAD molecules were correlated with morphologic variant as well as depth of tumor invasion in Gall bladder Adenocarcinoma [Table 2]. Strong expression of AMFR was observed more frequently in poorly differentiated adenocarcinomas (28/30 [93.3%]) and in (30/46 [65.2%]) moderately differentiated adenocarcinoma than in well differentiated type (7/20 [30%]).

Likewise, the frequency of weak expression of ECAD was higher in poorly differentiated type carcinomas (25/28 [89.3%]) and in moderately differentiated adenocarcinoma (28/46 [60.95%]) than in well differentiated-type carcinomas (7/20 [35%]). The alterations of these molecules were associated with poorly and moderately type differentiated carcinomas, which imply a loss of differentiation (P = .005 and P = .0225 for AMFR and ECAD respectively).

Strong expression of AMFR was observed less frequently in superficial (T1) cancer (2/18 [25%]) than those with deeper infiltration (T2,) (41/69 [59.4%]) and T3 (11/15 [73.3%]). There was a significant positive correlation between the depth of invasion and the expression of AMFR (P = .0393); however, the proportion of ECAD reduction (weak expression) was similar in superficial and deep infiltrating tumors.

When the expression of ECAD and AMFR are compared (Table 2), strong expression of AMFR was more frequent in tumors with weak expression of ECD (30/52 [57.7%]) than in tumors with strong expression of ECD (14/40 [35%]), thereby showing a significant negative correlation (P = .0034). When other morphometric parameters were reevaluated according to the coexpression pattern of these molecules, tumors with strong AMFR and weak ECAD expression showed deep tumor invasion (T2,3) more frequently than tumors with weak AMFR and strong ECAD expression.

V. Discussion

Histologically, Gall bladder cancers are classified into different grades-Grade 1, grade 2 and Grade 3. Grade 3 type of tumours showed diffuse pattern of growth and significant signet ring cell component.

3.2 % of our study population was less than 34 years. Age ranged from 29 to 78 years, with mean age of 59.9 years. Female (n=69) outnumbered male (n=23) in our study. Our findings was in close approximation of study of Randi G. et al. (3)

The E-cadherins (ECAD), or “classical” cadherins of type I, belong to the large family of cadherins, transmembrane or membrane-associated glycoproteins, mediating cell-cell adhesion and playing a pivotal role in epithelial cell behavior and tissue morphogenesis or remodeling (26-32). Transcriptional ECAD reprogramming in epithelial cells leads to decreased adhesion to facilitate migration at the epithelial-to-mesenchymal transition (EMT) interface during cancer progression (33).

As for ECAD, it is the characteristic of diffuse type tumors that the function of ECAD is disturbed, even in the presence of its protein expression,(9) because of ECAD gene mutation or tyrosine phosphorylation of ECAD binding proteins.(7) Accordingly, as mentioned previously, loss of cell- cell adhesion induces transcription of the AMFR gene. In the present study, we found more AMFR overexpression in Grade 3 than in Grade 1 tumors. This probably is a consequence of a functional or expression disorder of ECAD.

As ECAD is normally expressed on cell surface, it was advantageous to set the cutoff line at 90% for ECAD expression.(20,34) However, as AMFR was expressed only slightly in normal epithelium and gradually increased in cancer cells, a 50% cutoff was sufficient for separating AMFR expression into 2 groups.(7)

In the present study, we found overexpression of AMFR in about half of the patients with Gall bladder cancer and association of AMFR with dedifferentiation and deep tumor infiltration. In 1 study that examined the role of AMFR in gastric cancers,(23) the observations were consistent with ours.

The mechanism for regulation of AMFR is yet to be known in detail. The AMFR gene is located on 16q2130. In cultured cell lines, cell- cell contact dramatically down-regulated the protein expression and messenger RNA transcription of the AMFR gene.(15) Researchers performed an AMFR promoter assay and found it was suppressed by high cell density. They could not identify the transcription factor but speculated that c- Myc was a candidate, since the amount of c-Myc was correlated inversely with cell density.(24) There is another report that retinoic acid down-regulates AMFR expression.(35) Since retinoic acid induces differentiation in various types of cells, differentiation might be another factor that regulates AMFR expression.

These phenomena convinced us that ECAD is involved in transcriptional regulation of AMFR. For example, ECAD is the strongest cell-cell adhesion molecule (5) and beta-catenin, an ECAD binding protein, is reported to be associated with c- Myc transcription. (36) Retinoic acid is known to up-regulate ECAD expression.(37) Although the suppression of AMFR transcription by ECAD has not been proven directly, the inverse correlation of ECD and AMFR expression has been reported in bladder carcinomas.(38) and we found the same relationship in human gastric cancers in an earlier study. Since ECAD itself is a strong repressor of cancer invasion and metastasis, the reduction of ECD induces cancer invasion and metastasis, both by the function itself and by the regulatory mechanism for AMFR expression.

VI. Conclusion

Different histologic grades of Gall bladder cancers and their properties could be understood partly by the expression of ECAD and AMFR in the present study and as synergistic effect of these two proteins seem to be crucial step of for progression of carcinoma, we find necessity of evaluation both molecules simultaneously.

IMAGES

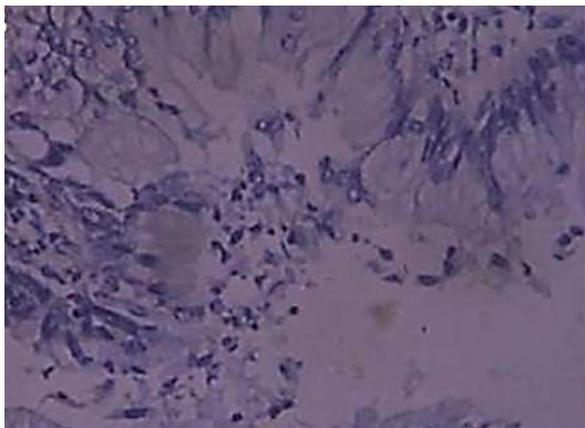


Image 1: Normal AFMR expression in Gall bladder mucosa (x400)

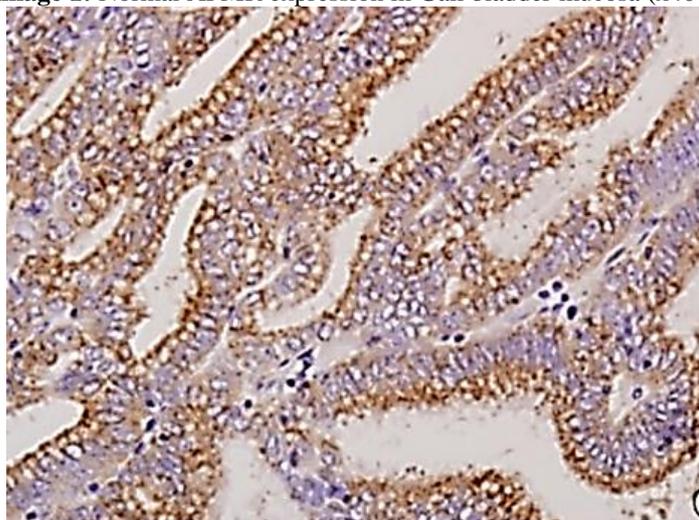


Image 2: Normal ECAD expression in Gall bladder mucosa (x400)

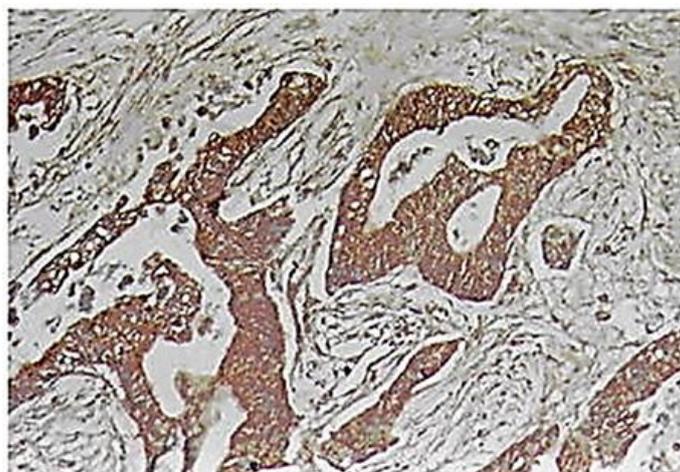


Image 3: ECAD expression in Grade 2 Gall bladder carcinoma (x400)

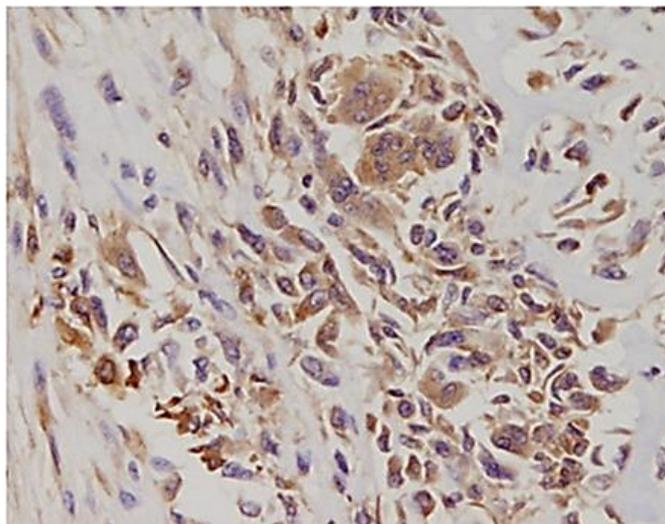


Image 4: AFMR expression in Grade 3 Gall bladder carcinoma (x400)

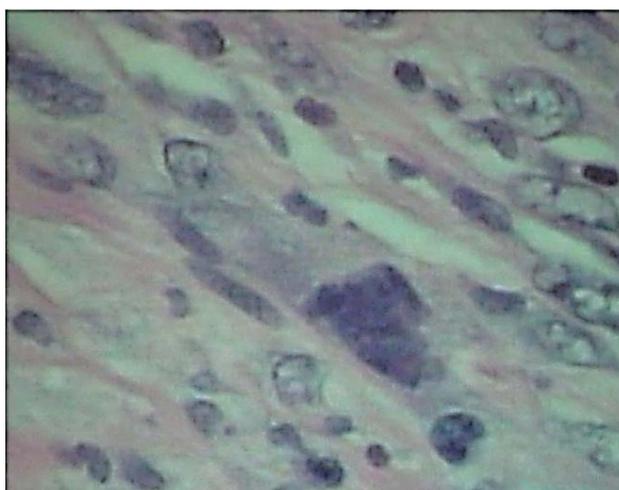


Image 5: ECAD expression in Grade 3 Gall bladder carcinoma (x1000)

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