Original Research Article

Study of association between expression of E-cadherin and Autocrine Motility Factor in different grades of Colorectal adenocarcinoma

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Abstract

Though incidence of Colorectal carcinoma is relatively less in consideration of world wide data, Indian scenario differs in higher incidence of Grade 3 carcinoma with signet ring cell component. We tried to find association between intercellular adhesion and propagation of cancer cell in Adenocarcinoma of Colorectal region. E-cadherin (ECAD) is the strongest intercellular adhesion molecule of epithelial cells and Autocrine Motility Factor (AMFR) is known propagator of cancer cell. We studied simultaneous immunohistochemical expression of these two molecules in 92 already diagnosed cases that were treated surgically by Colectomy or Abdomino Pelvic Resection. Normal Colorectal mucosa reacted strongly with ECAD and weakly with AMFR. With increasing dedifferentiation of the tumor, reversal manifested- more aggressive grades showed varied expression in comparison to its less aggressive type. Gastric 3 adenocarcinoma show weak ECAD (83% vs. 35%) and more AMFR (93% vs. 35%) expression in comparison to Grade 1. Weak ECAD and strong AMFR was also associated with increase in depth of tumor invasion. As ECAD and AMFR is at least partially responsible for varying histologic grade and behavioral pattern of colorectal adenocarcinoma, simultaneous evaluation of both parameters is helpful to understand pathway of progression of such cancer cell.

Key words

Colorectal carcinoma, E- cadherin , Autocrine Motility factor Receptor.

Introduction

Colorectal cancer is a formidable health problem worldwide. It is the third most common cancer in men (663000 cases, 10.0% of all cancer cases) and the second most common in women (571000 cases, 9.4% of all cancer cases). Almost 60% of cases are encountered in developed countries. The number of Colorectal cancer-related deaths is estimated to be approximately 608000 worldwide [1].

Incidence rates of Colorectal cancer vary 10-fold in both sexes worldwide, the highest rates being estimated in Australia/New Zealand and Western Europe, the lowest in Africa (except Southern Africa) and South-Central Asia .Within Asia, the incidence rates of Colorectal cancer vary widely and are uniformly low in all south Asian countries and high in all developed Asian countries. The burden of Colorectal cancer has risen rapidly in some economically developed Asian countries like Japan, South Korea and Singapore. Fortunately, the age adjusted incidence rates of colorectal cancer in all the Indian cancer registries are very close to the lowest rates in the world [2].

In India, the annual incidence rates for colon cancer and rectal cancer in men are 4.4 and 4.1 per 100000, respectively. The annual incidence rates for colon cancer in women are 3.9 per 100000. Colon cancer ranks 8^{th} and rectal cancer ranks 9^{th} among men. For women, rectal cancer does not figure in the top 10 cancers, whereas colon cancer ranks 9^{th} [1].

The mean age of detection was 47.2 years. Sixty-five percent were males. Patients were symptomatic for an average period of 4 months prior to presentation. The commonest symptoms were rectal bleeding (57%), pain (44%), and altered bowel habits (26%). Thirteen percent of the patients had signet ring tumors. Colorectal cancer in India differs from that described in the Western countries. We had more young patients, higher proportion of signet ring carcinomas, and more patients presenting with an advanced stage [3].

Despite advances in diagnosis, the disease is usually detected after invasion of the muscularis mucosae, Furthermore, surgery and chemotherapy have limited value in advanced disease and there is a paucity of molecular markers for targeted therapy. Since cancer of the colorectum 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 [8-14] and as Silleti, et al. 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.

With intention to find if simultaneous occurrence of destruction of intercellular adhesion is associated with propagation of carcinomatous cells, we studied behavioral pattern of different grades of colorectal carcinoma and studied ECAD and AMFR gene expression with aid of immunohistochemistry.

Materials and methods

The study population consisted of 92 patients who were finally treated with different types of colectomy/ Abdomino Pelvic Resection (APR) with or without regional lymph node dissection. In this retrospective study (conducted between 2014 to 2016), in Medical College, Kolkata, we selected only those patients who underwent endoscopic evaluation followed by Final surgery. Interval between endoscopy and final surgery in our study varied from 28 to 172 days. Most patients underwent Final surgery within 60 days of the endoscopic 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 Hematoxylene and Eosin stained tissue sections of endoscopic biopsy and different types of colectomy/Abdomino Pelvic Resection (APR) with or without regional lymph node dissection specimens were prepared from paraffin blocks. Team of Surgeon and Pathologist in Medical College, Kolkata went through the clinical data and tissue sections as per previously fixed protocol and parameters.

Cases in which histologic slides from endoscopic biopsy were not available for review were excluded. Hematoxylene 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 were 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 antiratIgG for AMFR or rabbit antimouseIgG (Vectastain ABC Kit, Vector, Burlingame, CA), diluted1:250 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) containing0.02% (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 ECD and AFMR tumor cell were designated positive or negative as per predetermined criteria (**Table - 1**).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.

Results

In non-malignant Colorectal 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 Colorectal 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 Colorectal cancers was 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 Colorectal 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%]).

Image - 1: Normal AFMR expression in colorectal mucosa (400X).

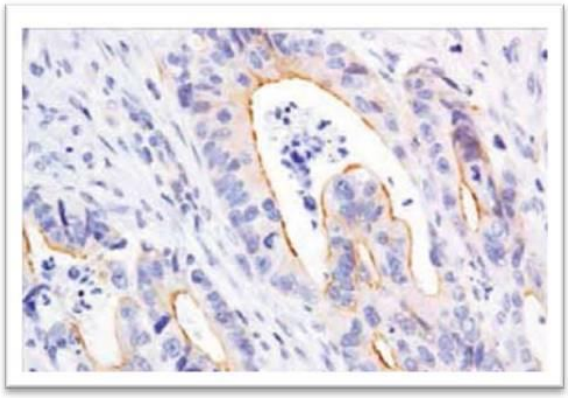
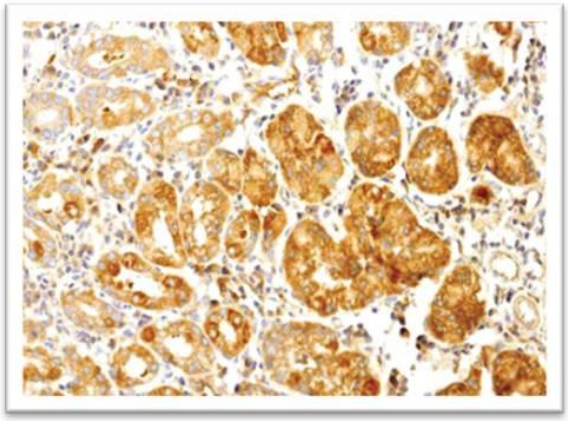


Image - 2: Normal ECAD expression in colorectal mucosa (400X).



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).

Table – 1: Evaluation of Autocrine Motility Factor Inhibitor Receptor(AMFR) and E-Cadherin(ECAD) Expression.

	Strong Expression	**Weak Expression**
Autocrine Motility Factor Inhibitor Receptor (AMFR)	50% or more tumor cell stained	Less than 50% of tumor cell stained
E-Cadherin (ECAD)	90% or more tumor cell stained	Less than 90% of tumor cell stained

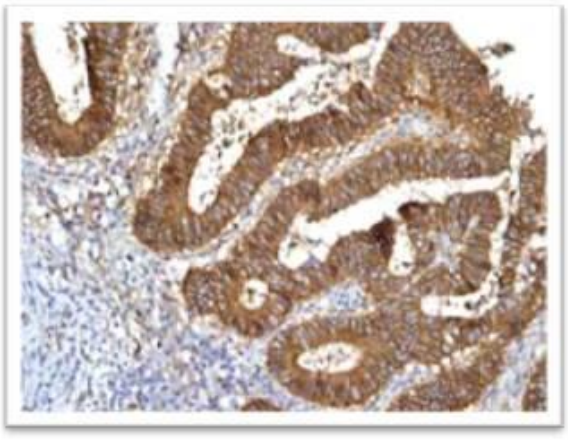
Table – 2: Expression of Autocrin Motility Factor Inhibitor Receptor (AMFR) and E-Cadherin (ECAD).

	AMFR				**ECAD**			
	Strong		**Weak**		**Strong**		**Weak**	
Histopathologic Grade (n =92)								
Grade1 (n=20)	7	7.6%	13	14.1%	13	14.1%	7	7.6%
Grade2 (n=46)	30	32.6%	16	17.3%	18	15.5%	28	30.43%
Grade3 (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 – 3: Relationship Between Autocrine Motility Factor Inhibitor Receptor (AMFR) and E-Cadherin (ECAD) Expression.

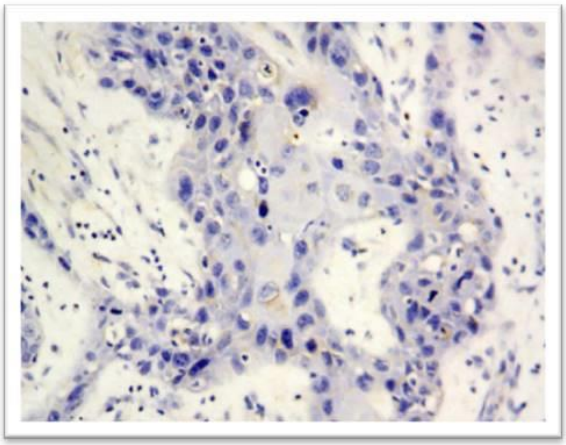
	ECD Strong		**ECAD Weak**		**Total**
AMFR Strong	14	15.2%	30	32.6%	44
AMFR Weak	26	28.2%	22	23.9%	48
Total	40		52		92

Image - 3: ECAD expression in Grade 1 colorectal carcinoma (400X).



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.

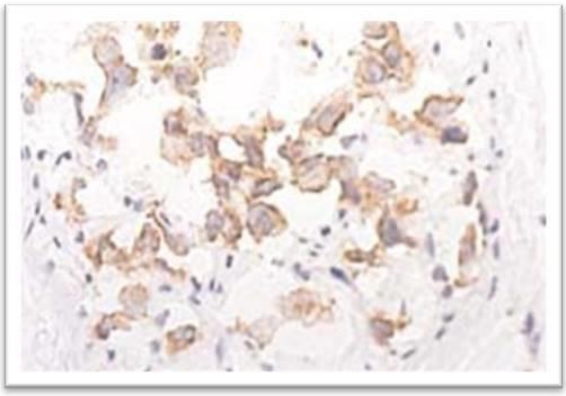
Image - 4: ECAD expression in Grade 3 colorectal carcinoma (400X).



When the expression of ECAD and AMFR are compared (**Table - 3**), 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 (**Images – 3, 4, 5**).

Image - 5: AFMR expression in Grade 3 colorectal carcinoma (400X).



Discussion

Histologically, colorectal cancers are classified into different grades - Grade 1, Grade 2 and Grade 3. Grade 3 types of tumors showed diffuse pattern of growth and significant signet ring cell component. 8.7% of our study population was less than 32 years. Age ranged from 21 to 79 years, with mean age of 39.9 years. Male (n=58) outnumbered female (n=32) in our study. Our findings were in close approximation of study of Prachi S., 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 colorectal cancer and association of AMFR with dedifferentiation and deep tumor infiltration. In one 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.

Conclusion

Different histologic grades of colorectal 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.

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