Detection of epithelial specific cell adhesion molecules in colon cancer and the correlation with clinical and pathological characteristics

EpCAM expression in colon cancer

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Aim: *The aim of this study was to evaluate the correlation between EpCAM expression in colon cancer tissue and the clinico-pathological characteristics of the patients.*
Material and Methods: *This is a prospective, longitudinal, observational study on 80 patients undergoing for colon cancer between January - December 2017. EpCAM expression at tumoral level was analyzed in relation with clinical and pathological variables of the patients using anti-EpCAM specific antibody.*
Results: *EpCAM expression was predominant in tumoral tissue compared to normal colonic mucosa and most of the cases (58.7%) showed increased EpCAM expression. Although increased EpCAM expression was observed in advanced stages and in patients with advanced locoregional disease, there was no statistically significant correlation with the clinical and pathological characteristics of the patients.*
Discussion: *The majority of the analyzed samples showed increased EpCAM expression in tumoral tissue suggesting its involvement in the carcinogenesis process. Numerous studies have identified EpCAM overexpression in colon cancer as a negative prognostic factor, being associated with advanced stage of the disease and a poor prognosis of the patient but results are inconsistent. Nevertheless, assessing a possible correlation between EpCAM expression at tumoral level and clinico-pathological characteristics is dependent on the type of antibody used to identify the molecule of interest.*
Conclusions: *EpCAM detection in colon cancer using anti-human CD326/EpCAM clone VU-1D9 does not allow the correlation between its expression and the clinico-pathological characteristics of the patients and it should only be used for EpCAM identification in colon cancer tissues.*

Key Words: Cancer, Colon, EpCAM, Immunohistochemistry

Introduction

Colon cancer is the second most common digestive malignancy among both men and women [1]. The treatment of choice in the majority of colon cancer cases remains the radical surgical resection, accompanied or not, as required by chemotherapy. However, current recommendations for the modern treatment of colon cancer are directed more and more towards targeted molecular therapies, defining the concept of personalized medicine.

EpCAM (epithelial – specific intracellular cell adhesion molecule), also known as CD326, is a type I, homotypic, Ca^{2+} independent transmembrane glycoprotein

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expressed at the basolateral membrane of the cell[2]. It has a molecular weight of 39-42 kDa[2] and presents 3 domains: an extracellular domain (EpEX) of 242 amino acids with epidermal growth factor (EGF)-receptors and a thyroglobulin-like domain, a single transmembrane domain of 23 amino acids, and a short, 26 amino acids intracellular domain (EpICD) [3-5]. EpCAM is particularly involved in intercellular adhesion, but its role in cell signaling, differentiation, proliferation and tumor invasion has also been observed [2-3]. Its expression exclusively in epithelial tissue and epithelial-derived neoplasms allowed its use in differentiating between epithelial and mesenchymal tumors[6]. No EpCAM expression has been observed in lymphomas, sarcomas or mesenchymal tumors [6-7].

In colon cancer, EpCAM was found to be expressed at high levels (between 79-99.7%), condition that has draw attention to it as a possible therapeutic target [4-6,8-9]. While some studies in the literature have reported the increased expression of EpCAM in primary colon tumors as a negative prognostic factor, being associated with: advanced stage of the disease [7,10-12], poor differentiated tumors [10-12], depth of colonic wall involvement and invasion of surrounding organs [10,12], high number of lymph nodes involved [10,13], lymphatic and venous invasion [10-12] but also with an increased risk of metastasis [11] , others have not found the correlation [14]. EpCAM is expressed in liver metastases from colon cancer, while its expression in primary liver tumors was not been reported[15], emphasizing its potential role in colon cancer progression and carcinogenesis [4,6].

This study aims to identify EpCAM expression in colon cancer tissue and to analyze the relationship between its expression at tumor level and the clinical and pathological characteristics of colon cancer patients.

Material and Methods

This is a prospective, longitudinal, observational, analytical, cohort study that included patients diagnosed with colon cancer who underwent radical (curative or palliative) surgery between January and December 2017 in the surgical department of a single tertiary center. All patients signed the informed consent form for inclusion in the study. The study protocol was approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy. Patients under the age of 18, with rectal neoplasms or with a history of preoperative chemo- or radiotherapy or other immunological therapies were excluded from the study.

Tissue Sample Collection Protocol

The surgical specimens resulting from colon resections were used in the study. The samples harvested included colon cancer tissue with adjacent mucosa. The samples were fixed in 4% formaldehyde after routine specimen collection and were processed into paraffin sections and preserved. The resulting tissue fragments were labeled with anti-EpCAM specific antibodies (Anti-Human CD326-EpCAM, clone VU-1D9).

Description of the Immunostaining Technique

The sections of the paraffin blocks were made at 2 μm and stored overnight at 37° C. After dewaxing, antigen retrieval at pH=6 was performed for 30 minutes followed by neutralization of endogenous peroxidase for 5 minutes. The sections were then washed with TBS and incubated with protein block for 5 minutes. A new TBS wash was performed and samples were incubated with the primary EpCAM antibody (clone VU-1D9) dilution 1: 500 pH=6 for 40 minutes at 37° C. After washing with TBS, post-primary incubation was performed for 15 minutes, followed by a new TBS washing and incubation with polymer for 15 minutes. After the DAB solution was applied for 5 minutes, hematoxylin counter-staining was performed.

Calculation of the Total Immunostaining Score (TIS)

EpCAM expression in colon cancer tissue samples was analysed calculating a Total Immunostaining Score (TIS) as the product between the percentage (P) and the intensity (I) of EpCAM-positive cells. The intensity (I) of EpCAM expression can vary between 0 - no expression, 1 - weak, 2 - moderate and 3 - intense. The percentage (P) of cells showing expression of EpCAM can vary between 0 - null, 1 <10%, 2 10-50%, 3 51-80% and 4 >80%. Thus, TIS can take the following values: 0, 1, 2, 3, 4, 6, 8, 9, 12. The results were afterwards grouped into 4 groups: TISG 0 (no expression, TIS 0,), TISG 1 (low expression, TIS 1-4), TISG 2 (moderate expression, TIS 6 or 8), and TISG 3 (high expression, TIS 9 or 12). EpCAM expression in colon cancer tissue samples was analyzed in relation to a normal colon tissue sample (control sample). The immunohistochemical assessment was performed by a single experimented pathologist. An Olympus BX53M microscop was used for tissue sample analysis. The results were recorded using an Excel database.

Data collection

Clinical and pathological data of the colon cancer patients were prospectively collected in a specific databese, different from that of the institution. The following variables were recorded: patient age, CEA carcinoem-

bryonic antigen level (ng/ml), intraoperative aspects (location of the tumor, size in cm, invasion in surrounding organs) as well as histopathological data (histological tumor type, tumor stage, degree of differentiation, colonic wall involvement, lymph node involvement, presence or absence of distant metastases, lymphatic and perivascular invasion). Tissue samples from patients who have died during admission were also recorded and analysed.

Statistical Analysis

Statistical analysis was performed using the MedCalc Statistical Software version 17.9.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017). Data was expressed as median and interquartile range or frequency and percent, whenever appropriate. Differences between groups were verified using the chi-square test. Correlations between variables were verified using Fisher's exact test. A p value <0.05 was considered statistically significant.

Results

The study was conducted on 80 patients that underwent surgery for colon cancer. Gender distribution of patients included in the study was equal, 40 (50%) males and 40 (50%) females. Patients age ranged between 34 and 83 years with a median of 63.5. CEA level (ng/ml) ranged between 3.05 and 9.075 with a median of 5ng/ml. Tumors were predominantly found in the sigmoid colon 38 (47.5%) followed by the ascending colon 34 (42.5%). Less common tumor locations were in descending 6 (7.5%) and transverse 2 (10%) colon. The size of the

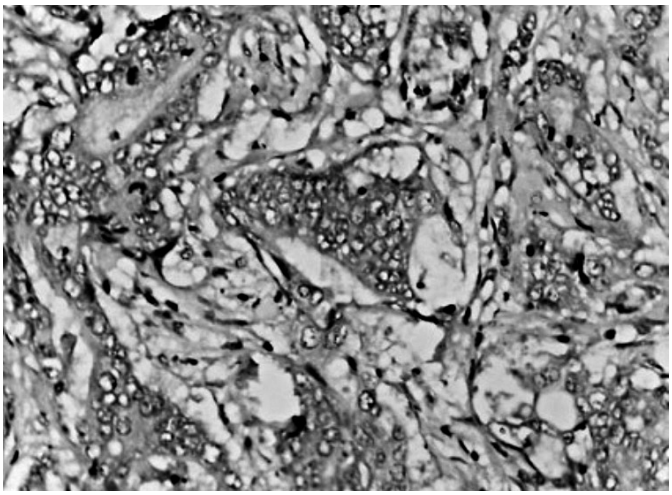

Fig. 2: Weak expression – intensity 1 of EpCAM in tumoral tissue (x400).

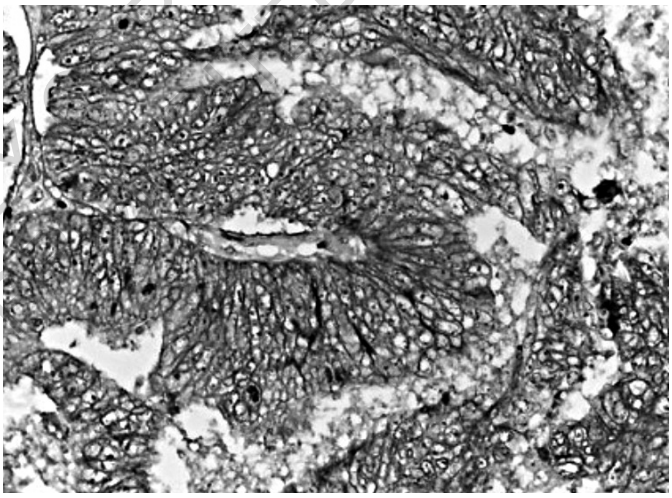

Fig. 3: Moderate expression – intensity 2 of EpCAM in tumoral tissue (x400).

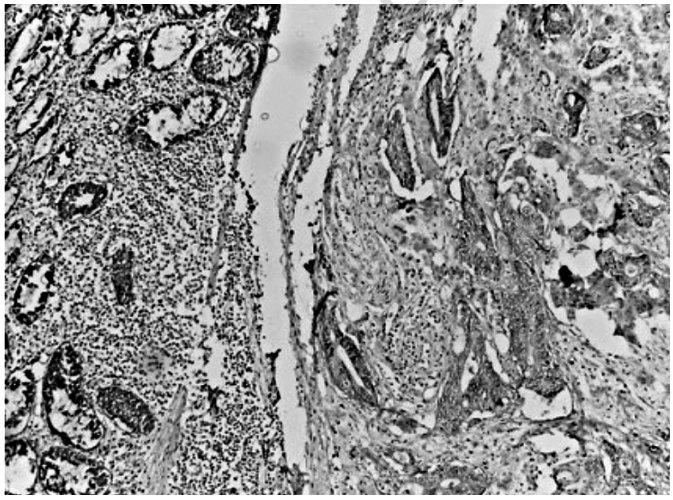

Fig. 1: EpCAM expression in normal colonic mucosa (left side) and in tumoral tissue (right side); x100.

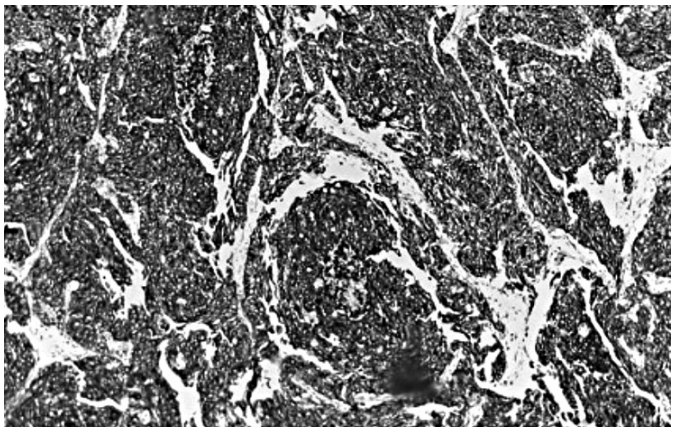

Fig. 4: Strong expsression – intensity 3 of EpCAM in colon tumor (x400).

tumors varied between 1.5 and 9 cm with a median of 4.75cm. Twelve (15%) of the tumors were smaller than 3cm, 30 (37.5%) had 3 to 5cm diameter while the majority had larger dimensions, of over 5cm 38 (47.5%). Twelve (15%) of patients had invasive tumors in adjacent organs (small intestine, bladder, uterus or abdominal wall), while 68 (85%) of patients had tumors limited to the colon. The anatomopathological results revealed that most of the samples were adenocarcinomas 73 (91.3%), while 7 (8.8%) were mucinous carcinomas. Stage II neoplastic disease was the most common 34 (42.5%), followed by stage III 28 (35%), stage IV 14 (17.5%) and stage I 4 (5%) colon cancer. Sixty-four (80%) of the tumors were moderately differentiated (G2), followed by 10 (12.5%) poorly (G3) differentiated tumors. Well differentiated pattern (G1) was observed in a small number of cases 6 (7.5%). Patients presented with distant metastases in 15 (18.75%) of the cases. A number of 4 (5%) patients died during admission.

In the analyzed tissue samples, the expression of EpCAM was highlighted in both normal and tumoral colonic tissue (Fig. 1).

In tumoral tissue, EpCAM expression was observed in three degrees of different intensity (1 – weak intensity – Fig. 2, 2 – moderate intensity – Fig. 3, 3- strong intensity – Fig 4.). In normal colonic mucosa, EpCAM expression intensity remained weak (Fig. 2).

Total Immunostaining Score (TIS) of the samples is presented in Table I.

All in all, EpCAM expression was low (TISG 1) in 20 (25%) of cases, while most cases 47 (58.75%) showed high EpCAM expression (TISG 3). Moderate (TISG 2) expression of EpCAM was seen in 13 (16.25%) cases. No colon cancer tissue sample showed zero (TISG 0) expression.

Further, a Fisher's exact test was applied in order to evaluate the correlation between EpCAM expression in colon cancer samples and its relation with the pathological charecteristics of the patient (stage of the disease, degree of differentiation - G, degree of colonic wall invasion – T, lymph node involvement - N, presence or absence of distant metastatis – M, lymphatic - L and perivascular - V invasion, organ invasion and tumor size). However, the univariate analysis revealed that none of the variables obtain statistical significance (p >0.05) (Table II).

Among locoregionally advanced tumors, 4 (33.3%) showed high EpCAM expression (TISG 3), 5 (41.7%) moderate EpCAM expression (TISG 2) whereas 3 (25%) cases showed low expression (TISG 1). Colonic tumors of deceased patients were characterized by high EpCAM expression (TISG 3). There was one patient out of 4 (25%) with TIS=9, and 3 (75%) with TIS=12. However, univariate analysis did not identify statistical significance between high EpCAM expression at tumor level and death of patients (p>0.05).

Table I - *EpCAM expression and TISG in colon cancer samples.*

TISG	Total Immunostaining Score (TIS)	Patients n (%)
TISG 0, no expression	TIS 0	0 (0)
TISG 1, weak expression	TIS 1	4 (5)
	TIS 2	11 (13.75)
	TIS 3	0 (0)
	TIS 4	5 (6.25)
TISG 2, moderate expression	TIS 6	10 (12.5)
	TIS 8	3 (3.75)
TISG 3, intense expression	TIS 9	9 (11.25)
	TIS 12	38 (47.5)

Table II - *Pathologic characteristics of colon cancer patients and EpCAM e*

Variable	Low EpCAM expression TISG 1 n (%)	Moderate EpCAM expression TISG 2 n (%)	High EpCAM expression TISG 3 n (%)	p
Stage				0.2
I	0 (0)	1 (25)	3 (75)	
II	9 (26.5)	6 (17.6)	19 (55.9)	
III	9 (32.2)	1 (3.6)	18 (64.2)	
IV	2 (14.3)	5 (35.7)	7 (50)	
T				0.5
T1	1 (33.3)	1 (33.3)	1 (33.3)	
T2	1 (20)	1 (20)	3 (60)	
T3	6 (19.35)	4 (12.9)	21 (67.74)	
T4	12 (29.26)	7 (17.07)	22 (53.65)	
N				0.3
N0	11 (25.58)	9 (20.93)	23 (53.48)	
N1	5 (23.8)	2 (9.52)	14 (66.66)	
N2	4 (25)	2 (12.5)	10 (62.5)	
M				0.2
M0	17 (26.2)	8 (12.3)	40 (61.5)	
M1	3 (20)	5 (33.3)	7 (46.7)	
L				0.3
L0	12 (26.7)	9 (20)	24 (53.3)	
L1	8 (22.9)	4 (11.4)	23 (65.7)	
Grading				0.5
G1	1 (16.7)	1 (16.7)	4 (66.6)	
G2	16 (25)	11 (17.2)	37 (57.8)	
G3	3 (30)	1 (10)	6 (60)	
Organ invasion				0.4
Yes	3 (25)	5 (41.7)	4 (33.3)	
No	17 (29.3)	8 (13.8)	33 (56.9)	
Tumor size				0.4
<3cm	2 (16.7)	2 (16.7)	8 (66.6)	
3-5cm	7 (23.3)	4 (13.3)	19 (63.4)	
>5cm	11 (29)	7 (18.4)	20 (52.6)	

Discussions

This study assessed the expression of EpCAM adhesion molecule in colon cancer tissue samples via immunohistochemical analysis using anti-EpCAM specific antibodies (Anti-Human CD326-EpCAM antibody, clone VU-1D9). The relationship between EpCAM expression at the tumoral level and patient's clinical and pathological characteristics was further analyzed.

Immunohistochemical analysis of tissue samples confirmed the expression of EpCAM in the membranous level of both tumoral and normal colonic mucosa, as previously described in the literature [14]. At tumoral level, however, three different degrees of EpCAM expression were observed, while the expression in normal colonic mucosa remained weak. The majority of the analyzed cases (58.75%) showed high EpCAM expression (TISG 3) at tumoral level, which was also found in other papers [9-10], suggesting the involvement of EpCAM adhesion molecule in the process of carcinogenesis. In some studies [10-11], increased EpCAM expression in the colon tumor was associated with advanced loco-regional disease and deep invasion of the colonic wall. In our study, although most of the patients (75%) with advanced loco-regional diseases had increased (TISG 3) and moderate (TISG 2) EpCAM expression in tumor tissue sample, the variable did not reach statistical significance ($p>0.05$). Studies in literature have also highlighted that increased EpCAM expression at tumoral level correlates with advanced stage of the disease[7,10-12], situation that was not confirmed in the present study even if 50% of the stage IV cancer had high EpCAM expression (TISG 3). A low degree of tumor differentiation (G3) [10-12], the number of lymph nodes involved (N)[10,13] but also the peri-lymphatic (L) and peri-neural invasion (V) [10,12] were associated with high EpCAM expression levels in colon cancer tumors. However, in the present study, the aforementioned variables were analyzed in relation with EpCAM expression but no statsitical significance was found ($p>0.05$).

Resuming the analysis of literature studies on the role of EpCAM expression in the prognosis of colon cancer patients highlighted the fact that most studies used MOC31 [16], MAB960 [7], Ber-EP4 [17-18] or Ab 323A3 [19] as antibodies for the detection of EpCAM in the colon tumor when its relation with the clinical and pathological characteristics of patients was also evaluated. The use of anti-EpCAM antibody (clone VU1D9) for the immunohistochemical detection of the cell adhesion molecule in colon cancer has been reported in the literature[9,20] . However, the only study using clone VU1D9 for EpCAM detection in colon cancer that also analyzed EpCAM expression in relation to the clinical and pathological characteristics of the patients was that of Kuhn et al [20]. In the study, it was not possible to establish the link between high EpCAM expression at tumor level and advanced disease, similarly to the present study. Thus, we conclude that the use of clone VU1D9 for the detection of EpCAM in colon cancer does not allow the analysis of its expression in relation to the clinical and pathological characteristics of colon cancer patients. Nevertheless, the use of clone VU1D9 allows detection with good accuracy of EpCAM adhesion molecule in colon cancer tissues.

Following the epithelial-mesenchymal transition process that cancer cells undergo with subsequent detachement from the solid tumor, a down-regulation of EpCAM molecule at the membrane level occurs [21]. Thus, circulating tumor cells (CTC) are characterized by reduced expression of EpCAM, which makes their detection difficult. Recent studies [22], encourage the use of this anti-EpCAM antibody clone VU1D9 as an enrichement method for the accurate detection of circulating tumor cells (CTC) in colon cancer.

Thereby, the anti-EpCAM antibody clone VU1D9, is useful for EpCAM identification in colon cancer tissues and it can be used with promising results in CTC detection, but it should not be used to evaluate the prognostic value of EpCAM expression at tumor level in relation to the clinical and pathological characteristics of the neoplastic patients.

Conclusions

EpCAM expression is highly expressed in colon tumors, which allows its use as a possible therapeutic target. There is no correlation between clinical and pathological characteristics of cancer patients and EpCAM expression when clone VU-1D9 is used for detection.

Riassunto

La diagnosi precoce di uno stadio avanzato della malattia è essenziale per iniziare un trattamento adiuvante adeguato nel carcinoma del colon. L'espressione di EpCAM è stata confermata a livello tumorale da vari studi e anche da un gruppo di studio esterno, ma le contraddizioni esistenti in letteratura riguardo alla capacità di sovraespressione di EpCAM di predire o meno uno stadio avanzato della malattia possono essere dovute ad un'insufficiente identificazione finora della molecola.

L'impossibilità di stabilire il valore predittivo della molecola EpCAM nei pazienti con cancro del colon può essere dovuta a opzioni di rilevamento eterogenee utilizzate dai vari Autori. Sono necessari ulteriori studi per identificare con precisione il ruolo della molecola di EpCAM nello sviluppo del tumore e nella carcinogenesi del cancro del colon.

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