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# Significant Co-Expression of Putative Cancer Stem Cell Markers, EPCAM and CD166, Correlate with Tumor Stage and Invasive Behavior in Colorectal Cancer

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## Abstract

The crucial oncogenic role of Cancer Stem Cells (CSCs) in tumor maintenance, progression, drug resistance, and relapse has been clarified in different cancers, particularly in colorectal cancer; on the other hand, the distinguished characterization and isolation of CSC markers remains a debatable topic due to their complex biology. The current study was conducted to evaluate the co-expression pattern and clinical significance of Epithelial Cell Adhesion Molecules (EPCAM) and activated leucocyte cell adhesion (CD166, or ALCAM) in CRC patients. This study was carried out on a total of 458 paraffin-embedded CRC specimens by immunohistochemistry on Tissue Microarray (TMA) slides. Elevated expression of EPCAM and CD166 were observed in 61.5% (246/427) and 40.5% (164/405) of CRC cases. Our analysis showed a significant positive association of EPCAM expression with tumor size (P=0.02), tumor stage (P=0.007), tumor differentiate (P=0.005), vascular (P=0.01), neural (P=0.01), and lymph node (P=0.001) invasion.

There were no significant differences between CD166 expression and clinic pathological parameters. Moreover, combined analysis demonstrated a reciprocal significant correlation between EPCAM and CD166 expression (P=0.02). Interestingly, there was a significant positive correlation between EPCAM/CD166 phenotypes expression and tumor stage (P=0.03), tumor differentiation (P=0.05), neural, and lymph node invasion (P=0.01). The significant correlation of EPCAM and CD166 expression and their association with tumor progression and aggressive behavior is the reason for the suggestion of these two CSC markers as promising targets to promote novel effective targeted-therapy strategies for cancer treatment in the present study.

**Keywords:** Cancer stem cells • EPCAM • CD166 • Colorectal cancer • Tissue microarray

## Introduction

Colorectal Cancer (CRC) is the fourth most common cancer and a leading cause of death, worldwide. With respect to the tumor stage, more than 50 percent of patients are diagnosed with stage III disease, while only 25 percent showed stage I and II. Therefore, recurrence and distant metastasis are the main findings in patients with higher stages. Surgical resection is the most common and first treatments in CRC cases besides chemotherapy and radiotherapy. In this regard, identification and characterization of prognostic cancer biomarkers can pave the way to early treatment and inhibition of tumor progression by targeted-therapy strategies. Increasing evidence has highlighted the role of Cancer Stem Cells (CSCs) in tumor initiation, development, recurrence, metastasis, and drug resistance which are identified by their surface markers. The wide range of CSC markers is recognized in different solid and hematopoietic tumors. Epithelial Cell Adhesion Molecules (EPCAM) and CD166, Leukocyte Cell Adhesion Molecule (LCAM), are two transmembrane glycoproteins which are involved in adhesion interactions between cells, while expressed in malignant cells [1].

The biological role of EPCAM has been proved in most solid tumors, including colorectal cancer. Because of the controversial activity of this marker, different expression patterns and correlation with survival has been reported. EPCAM plays a different role as an oncogenic and/or tumor suppressor gene depending on its microenvironment in different tumor types. Their roles as a hemophilic intercellular adhesion molecule has been reported and justifies its anti-metastatic function and down regulation of EPCAM in metastases of renal clear cell carcinomas and thyroid carcinoma. The above-mentioned points are evidence that show the significant correlation of lower expression of EPCAM with improved patients' survival. In contrast, based on EPCAM activity on cell signaling pathways, its invasive functions in tumor growth and progression have been suggested in the bladder, gallbladder, breast, prostate, lung, pancreas, and renal cell carcinoma. Controversial results have been identified in gastric and colorectal cancer.

CD166 protein is a type-1 glycoprotein from the immunoglobulin superfamily which is known as both putative mesenchymal stem cells marker and maintenance of CSCs characteristic including tumor initiation, proliferation, and invasion has been reported in different cancers such as breast, ovarian, prostate, and CRC. Moreover, the correlation of overexpression of this marker with survival and tumor regression highlighted the CD166 as a potential prognostic marker in esophageal squamous cell carcinoma and CRC patients. However, there have been some controversial results considering the correlation of CD166 expression with clinical significance in CRC specimens.

Regarding the above description and contradictory findings of EPCAM and CD166 expression in the previous studies, this study was conducted to evaluate the co-expression pattern of EPCAM and CD166 and its association with clinic pathological profile in a large series of CRC patients using Tissue Microarray (TMA) based Immunohistochemistry (IHC) analysis.

## Material and Methods

### Sample collection

This study comprised a total of 458 archival paraffin-embedded CRC

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samples and 30 matched adjacent normal tissues collected from Hasheminejad, Rasool Akram, and Firoozgar hospitals between 2009 and 2015 in Tehran, Iran. All histopathological data was recorded from the corresponding hematoxylin and eosin slides including sex, age, tumor size, tumor location, TNM staging classification, tumor differentiation, distance metastases, and the presence of vascular, neural, and lymphnode invasion. None of the CRC patients in this study had received neoadjuvant treatment before surgery [2].

### Tissue Micro Array (TMA) construction

Hematoxylin and Eosin (H and E) stained slides were examined by an expert pathologist to spot the representative area of each tumor tissue, as described previously. In brief, each TMA recipient block contains almost 65 tissue samples with a diameter of 0.6 mm which were constructed in three copies for each specimen; final scoring was evaluated by the mean scoring of three cores. Subsequently, the TMA blocks were cut into 4 m thin serial sections and transferred onto positively charged TMA slides (Superfrost plus, Thermo Scientific, Germany). In TMA-based studies, to overcome the heterogeneity of protein expression, we analyzed three cores of each specimen to elevate the accuracy and validity of the experiment.

### Immunohistochemistry (IHC)

Formalin-fixed, paraffn-embedded sections of the TMA constructed slides were stained using the Biopharmadex kit (Link-Envision; KL5007, Germany), as described previously. After dewaxation at 60°C for 30 minutes followed by rehydration steps, the samples were incubated over-night with anti-EPCAM (1:1000 dilution, ab124825; Abcam, UK) and anti-CD166 (1/500, ab109215; Abcam, UK) at 4°C. Antigen retrieval was done by autoclave for 11 min; anti-EPCAM in citrate buffer (pH=6.0) and anti-CD166 in tris EDTA buffer (pH=8.0). The sections were then treated with the secondary antibody, TM mouse/rabbit Poly Vue HRP/DAB detection kit (standard EnVision-HRP kit (Bio pharmadx), at Room Temperature (RT). This was followed by visualization with 3, 3′-Diaminobenzidine (DAB) substrate as a chromogen for 3 min at RT.

The sections were counterstained with hematoxylin for 15 min, dehydrated, and finally mounted. Human colon adenocarcinoma and liver tissues were selected as the positive controls for anti-EPCAM and anti-CD166, respectively. Replacement of the primary antibodies by preimmune rabbit IgG and Tris Buffer Saline (TBS, pH: 7.4) wash buffer were used as the negative controls [3].

### Scoring system of TMA slides

A semi-quantitative system was used by two pathologists to score each TMA tissue section with no prior knowledge of clinic pathologic parameters of samples. Immunostaining of EPCAM and CD166 was evaluated as described previously. Each marker expression was scored independently and the final scoring assessment was carried out with reinvestigation of the overall distribution of the tumor cells at 10X magnification. Positive cells were then assessed, semi-quantitatively, at higher magnifications (20X or 40X). The intensity of immunostaining was divided into groups 0, 1, 2, and 3 from negative to strong staining. The percentage of positive cells were valued semi-quantitatively, and scored as 0%-100%. The histochemical score (H-score) was obtained by multiplying the intensity (0-3) and percentage scores (0%-100%), and generated scores of 1-100, 100-200, and 200-300. The mean H-score (=196) was chosen as the cutoff point for Anti-EPCAM and (=83) for Anti-CD166. The specimens with H-score $\leq$ 196 and $\leq$ 83 were considered to be low EPCAM and CD166 expressing tissues, and the specimens with H-score >196 and >83 were considered to be high EPCAM and CD166 tissues [4].

### Statistical analysis

Statistical analysis was performed using SPSS software version 22 (SPSS, Chicago, IL, USA). The association between EPCAM and CD166 expression and clinic pathological features was determined by Pearson's Chi-square and Spearman's correlation coefficient test. A P-value <0.05 was considered statistically significant.

## Results

Clinic pathological characteristics of all cases are summarized in Table 1. Patients had a mean age of 60 ± 14.7 years, and males had higher proportion of the distribution of gender with 51.5% (236/458). Based on the tumor size (mean=5 cm); 66% of samples had less than 5 cm in size. Of all patients, 63.5% had moderate/poor differentiation and 36.5% had well differentiated. A total of 71 (16%) specimens had stage I, 172 (38%) stage IIA, 21 (5%) stage IIB, 74 (17%) stage IIIA, 68 (15%) stage IIIB, 17 (4%) stage IIIC, and 21 (5%) had stage IVA [5].

### Expression of EPCAM in colorectal cancer and adjacent normal tissues

Because of technical problems, from all 458 specimens, 415 samples remained for statistics analysis of EPCAM expression. In terms of intensity, membranous expression of EPCAM showed strong (+3) in 150 (36%), moderate (+2) in 170 (41%), weak (+1) in 89 (21.5%), and negative (0) in 6 (1.5%) specimens. Based on H-score scoring; 255 (61.5%) of all the samples had higher and 160 (38.5%) had lower expression of EPCAM. From 30 adjacent normal tissues 6.5%, 16.5%, and 77% of samples demonstrated strong, moderate, and weak intensity staining of EPCAM expression, respectively. Moreover, in terms of H-score; only one sample represented the elevated expression of EPCAM and 29 (96.5%) of normal specimens displayed lower immunoreactivity of EPCAM (Table 1).

### Clinic pathological significance of EPCAM expression

Univariate analysis showed a positive significant association between tumor size, tumor stage, tumor differentiation, vascular, neural, and lymph node invasion and higher expression of EPCAM. The overexpression of EPCAM was demonstrated in 54% of specimens with more than 5 cm tumor size (P=0.02). In terms of tumor stage; 45 (69%), 109\ (69.5%), 11 (55%), 32 (47%), 31 (52%), 6 (40%), and 12 (63.5%) of stage I, IIA, IIB, IIIA, IIIB, IIIC, and IVA displayed higher expression of EPCAM, respectively (P=0.007). Out of 263 moderate/poor differentiated samples 148 (56.5%) and from 148 well differentiated cases, 103 (70%) displayed higher expression of EPCAM (P=0.005). Of 57 samples with positive vascular invasion, 27 (47.5%) had higher expression of EPCAM (P=0.01). From 79 positive neural invasion patients 38 (47.5%, P=0.01), and from 152 positive lymph node invasion, 78 (51.5%) of samples showed higher level of EPCAM expression (P=0.001), Table 1 displayed the correlation of EPCAM expression with all clinic pathological features [6,7].

### Expression of CD166 in colorectal cancer and adjacent normal tissues

Upon IHC staining, CD166 expression mainly localized in membrane and partially in cytoplasmic area of tumor cells. In terms of intensity; from 405 specimens, only 25 (6%) showed strong intensity of staining; moderate, weak, and negative expression of CD166 was found in 112 (27%), 185 (46%), and 83 (21%), respectively. Regarding H-score scoring system; a higher immunoreactivity of CD166 was seen in 164 (40.5%) of samples and a lower CD166 expression was observed in 241 (59.5%) of specimens. Scoring of 30 adjacent normal tissues demonstrated; strong, moderate, weak, and negative intensity staining of CD166 expression in 1 (3.5%), 7 (23.5%), 16 (53%), and 6 (20) specimens, respectively. Furthermore, the higher immunoreactivity of CD66 expression displayed in 8 (27%) normal sample and 22 (73%) showed lower expression of CD166 (Table 2).

Statistics analysis showed that there were no significant association between CD166 expression and clinic pathological features of samples. All data was collected and summarized in Table 1.

### Combined analysis of EPCAM/CD166 expression

Immunohistochemically expression pattern of both EPCAM and CD166 markers suggested a reciprocal significant correlation between two markers (P=0.02). Among 360 combined cases, 77 (21.5%) specimens had $EPCAM^{low}$/$CD166^{low}$ phenotype, 127 (35.5%) samples showed $EPCAM^{high}$/$CD166^{low}$ phenotype, 58 (16%) cases had $EPCAM^{low}$/$CD166^{high}$ phenotype, and 98 (27%)

**Table 1.** Statiscal association of EPCAM and CD166 expression with clinicopathological parameters in colorectal cancer specimens (Pearson $\chi^2$). The bolded values are statistically significant.

| Variables | Total No (%) | EPCAM expression (Mean H-score=196) | | P-value | CD166 expression (Mean H-score=83) | | P-value |
|---|---|---|---|---|---|---|---|
| | | Low | High | | Low | High | |
| **Mean age years** | | | | | | | |
| 60 ≥ | 241 (52.5) | 72 (34) | 140 (66) | 0.03 | 127 (60) | 85 (40) | 0.47 |
| 60< | 218 (47.5) | 88 (43) | 115 (57) | | 114 (59) | 79 (41) | |
| **Gender** | | | | | | | |
| Male | 236 (51.5) | 87 (40) | 130 (60) | 0.26 | 117 (56) | 92 (44) | 0.08 |
| Female | 222 (48.5) | 72 (36.5) | 125 (63.5) | | 123 (63) | 79 (37) | |
| **Tumor size (cm)** | | | | | | | |
| 5 ≥ | 300 (66) | 96 (35) | 175 (65) | 0.02 | 158 (59) | 109 (41) | 0.5 |
| 5< | 154 (34) | 64 (46) | 76 (54) | | 78 (58.5) | 55 (41.5) | |
| **TNM stage** | | | | | | | |
| I | 71 (16) | 20 (31) | 45 (69) | 0.007 | 46 (70) | 20 (30) | 0.2 |
| IIA | 172 (38) | 48 (30.5) | 109 (69.5) | | 81 (52.5) | 73 (47.5) | |
| IIB | 21 (5) | 9 (45) | 11 (55) | | 12 (60) | 8 (40) | |
| IIIA | 76 (17) | 36 (53) | 32 (47) | | 33 (53) | 29 (47) | |
| IIIB | 68 (15) | 29 (48) | 31 (52) | | 39 (68.5) | 18 (31.5) | |
| IIIC | 17 (4) | 9 (60) | 6 (40) | | 9 (69) | 4 (31) | |
| IVA | 21 (5) | 7 (36.5) | 12 (63.5) | | 14 (67) | 7 (33) | |
| **Tumor location** | | | | | | | |
| Cecum | 70 (16.5) | 29 (44) | 37 (56) | 0.09 | 37 (58) | 27 (42) | 0.29 |
| Sigmoid | 140 (34) | 36 (28) | 92 (72) | | 73 (58.5) | 52 (41.5) | |
| Rectum | 114 (27) | 45 (48.5) | 48 (51.5) | | 64 (69) | 29 (31) | |
| Colon ascending | 34 (8) | 11 (34.5) | 21 (65.5) | | 20 (67) | 10 (33) | |
| Colon transvers | 29 (7) | 9 (32) | 19 (68) | | 13 (46.5) | 15 (53.5) | |
| Colon descending | 12 (3) | 5 (45.5) | 6 (54.5) | | 5 (42) | 7 (58) | |
| Recto sigmoid | 18 (4.5) | 8 (44.5) | 10 (55.5) | | 11 (65) | 6 (35) | |
| **Tumor differentiation** | | | | | | | |
| Well | 165 (36.5) | 45 (30) | 103 (70) | 0.005 | 93 (63) | 55 (37) | 0.17 |
| Moderate/poor | 289 (63.5) | 115 (43.5) | 148 (56.5) | | 145 (57.5) | 107(42.5) | |
| **Distant metastasis** | | | | | | | |
| Positive | 25 (6) | 7 (30) | 16 (70) | 0.29 | 17 (68) | 8 (32) | 0.24 |
| Negative | 416 (94) | 146 (38.5) | 232 (61.5) | | 215 (59) | 150 (41) | |
| **Neural invasion** | | | | | | | |
| Positive | 90 (20) | 41 (52.5) | 38 (47.5) | 0.01 | 47 (69) | 22 (31) | 0.15 |
| Negative | 355 (80) | 113 (35) | 212 (65) | | 188 (58) | 138 (42) | |
| **Vascular invasion** | | | | | | | |
| Positive | 69 (15.5) | 30 (52.5) | 27 (47.5) | 0.01 | 37 (65) | 20 (35) | 0.25 |
| Negative | 379 (84.5) | 124 (35.5) | 224 (64.5) | | 201 (59) | 139 (41) | |
| **Lymph node invasion** | | | | | | | |
| Positive | 171 (37.5) | 74 (48.5) | 78 (51.5) | 0.001 | 89 (61.5) | 56 (38.5) | 0.31 |
| Negative | 286 (62.5) | 84 (32) | 177 (68) | | 151 (58) | 108 (42) | |

**Table 2.** Expression of EPCAM and CD166 (intensity and H-score) in colorectal cancer and adjacent normal tissues.

| Scoring system | Carcinoma | | Normal | |
|---|---|---|---|---|
| | EPCAM No (%) | CD166 No (%) | EPCAM No (%) | CD166 No (%) |
| **Intensity of staining** | | | | |
| Strong (+3) | 150 (36) | 25 (6) | 2 (6.5) | 1 (3.5) |
| Moderate (+2) | 170 (41) | 112 (27) | 5 (16.5) | 7 (23.5) |
| Weak (+1) | 89 (21.5) | 185 (46) | 23 (77) | 16 (53) |
| Negative (0) | 6 (1.5) | 83 (21) | 0 (0) | 6 (20) |
| **H-score** | | | | |
| High | 255 (61.5) | 164 (40.5) | 1 (3.5) | 8 (27) |
| Low | 160 (38.5) | 241 (59.5) | 29 (96.5) | 22 (73) |
| Total | 415 | 405 | 30 | 30 |

**Table 3.** Statiscal association of EPCAM/CD166 phenotypes expression with clinicopathological parameters in colorectal cancer specimens (Pearson $\chi^2$). The bolded values are statistically significant.

| Variables | Tota No (%) | EPCAM/CD166 phenotype expression, No (%) | | | | P-value |
|---|---|---|---|---|---|---|
| | | EPCAM Low/CD 166 Low | EPCAM High /CD166 Low | EPCAM Low /CD166 High | EPCAM High /CD166 High | |
| **Mean age years** | | | | | | |
| 60 ≥ | 184 (51) | 32 (17.5) | 71 (38.5) | 29 (16) | 52 (28) | 0.2 |
| 60< | 176 (49) | 45 (25.5) | 56 (32) | 29 (16.5) | 46 (26) | |
| **Gender** | | | | | | |
| Male | 192 (53.5) | 40 (21) | 64 (33.5) | 35 (18) | 53 (27.5) | 0.65 |
| Female | 167 (46.5) | 36 (21.5) | 63 (37.5) | 23 (14) | 45 (27) | |
| **Tumor size (cm)** | | | | | | |
| 5 ≥ | 239 (67) | 47 (20) | 90 (37.5) | 36 (15) | 66 (27.5) | 0.26 |
| 5< | 117 (33) | 30 (25.5) | 33 (28) | 22 (19) | 32 (27.5) | |
| **TNM stage** | | | | | | |
| I | 59 (16.5) | 12 (20) | 28 (47.5) | 5 (8.5) | 14 (24) | 0.03 |
| IIA | 139 (39) | 28 (20) | 42 (30) | 16 (11.5) | 53 (38.5) | |
| IIB | 19 (6) | 6 (31.5) | 5 (26) | 3 (16) | 5 (26) | |
| IIIA | 54 (15) | 8 (15) | 18 (33) | 18 (33) | 10 (19) | |
| IIIB | 49 (14) | 13 (26.5) | 20 (41) | 11 (22.5) | 5 (10) | |
| IIIC | 11 (3) | 5 (45.5) | 2 (18) | 1 (9) | 3 (27.5) | |
| IVA | 19 (5.5) | 5 (26) | 7 (37.5) | 2 (10.5) | 5 (26) | |
| **Tumor location** | | | | | | |
| Cecum | 60 (19) | 18 (30) | 16 (27) | 9 (15) | 17 (28) | 0.05 |
| Sigmoid | 113 (34) | 16 (14) | 46 (41) | 14 (12.5) | 37 (32.5) | |
| Rectom | 73 (22) | 20 (27.5) | 27 (37) | 17 (23.5) | 9 (12) | |
| Colon ascending | 28 (8.5) | 8 (29) | 11 (39) | 1 (3) | 8 (29) | |
| Colon transvers | 27 (8) | 2 (7.5) | 10 (37) | 7 (26) | 8 (29.5) | |
| Colon descending | 11 (3.5) | 2 (19) | 3 (27) | 3 (27) | 3 (27) | |
| Rectosigmoid | 17 (5) | 6 (35) | 5 (29.5) | 1 (6) | 5 (29.5) | |
| **Tumour differentiation** | | | | | | |
| Well | 132 (37) | 25 (19) | 54 (41) | 13 (10) | 40 (30) | 0.05 |
| Moderate/poor | 225 (63) | 52 (23) | 72 (32) | 45 (20) | 56 (25) | |
| **Distant metastasis** | | | | | | |
| Positive | 23 (6.5) | 4 (17.5) | 11 (48) | 3 (13) | 5 (21.5) | 0.65 |
| Negative | 326 (93.5) | 70 (21.5) | 113 (35) | 54 (16.5) | 89 (27) | |
| **Neural Invasion** | | | | | | |
| Positive | 57 (16.5) | 22 (39) | 14 (25) | 10 (17) | 11 (19) | 0.01 |
| Negative | 294 (83.5) | 53 (18) | 110 (37.5) | 47 (16) | 84 (28.5) | |
| **Vascular invasion** | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Positive | 45 (13) | 12 (27) | 14 (31) | 11 (24.5) | 8 (17.5) | 0.18 |
| Negative | 308 (87) | 63 (20.5) | 112 (36.5) | 46 (15) | 87 (28) | |
| **Lymph node invasion** | | | | | | |
| Positive | 126 (35) | 29 (23) | 44 (35) | 29 (23) | 24 (19) | 0.01 |
| Negative | 233 (65) | 47 (20) | 83 (35.5) | 29 (12.5) | 74 (32) | |

samples had EPCAM$^{high}$/CD166$^{high}$ phenotype. The association of EPCAM/CD166 phenotypes expression with clinic pathological characteristics of CRC specimens was examined by one-way ANOVA and Tukey's post hoc analysis tests. The findings observed a significant direct correlation between EPCAM/CD166 phenotypes expression and tumor stage (P=0.03), tumor differentiation (P=0.05), neural, and lymph node invasion (P=0.01). There were no significant correlation between other EPCAM/CD166 phenotypes and clinic pathological variables (Table 3).

## Discussion

Pioneer studies had highlighted the potential function of CSC markers in tumor aggressiveness, drug resistance, and consequently treatment failure in CRC patients after postoperative chemotherapy and/or radiotherapy. Evidence suggests that information regarding EPCAM and CD166 expression and clinical significance are not consistent in different solid tumors. From this point of view, we aimed at evaluating co-expression and the clinical significance of the two putative CR-CSC markers EPCAM and CD166, in a large series of CRC specimens. Our findings showed the higher expression of EPCAM in 61.5% of CRC patients and the direct significant association of EPCAM expression with tumor size (P=0.02), tumor stage (P=0.007), tumor differentiation (P=0.005), and vascular (P=0.01), neural (P=0.01), and lymph node (P=0.001) invasion [8].

Diversity of EPCAM function can cause controversies in expression pattern of this marker in different tumors, especially in CRC cases. Our results are in line with several *in-vivo* and *in-vitro* reports which have revealed the key role of EPCAM in self-renewal, differentiation, migration, and invasion in different solid tumors. Our recent study on clear cell Renal Cell Carcinoma (ccRCC) indicated the higher membranous expression of EPCAM and its direct significant association with nucleolar grade and tumor necrosis. We also found EPCAM to be an independent favorable prognostic marker affecting Progression-Free Survival (PFS) in ccRCC. Previously, Liu, et al. represented the tumor progression, aggressiveness, and chemotherapy resistance in CRC tissues with EPCAM+/CD44+ phenotype [5,9,10].

The immunohistochemically observation in TMA tissues of Went also demonstrated the significant higher expression of EPCAM protein in high-grade CRC tumors. Zhou, et al. noted the high expression of EPCAM in colon cancer and its correlation with lower survival rates in 50 tissues by immunohistochemistry [6]. However, in contrast there are some other studies in the literature which have suggested the negative association of EPCAM expression with tumor grade, invasion, and lymph node metastasis and noted the correlation of a decreased expression of EPCAM with poor survival and cancer recurrence in CRC patients [11].

The diversity of all of these findings can be due to the different biological function of EPCAM CSC marker in different tumor type, particularly CRC, as described previously. EPCAM acts as a double-edged sword protein which has oncogenic and tumor suppressive behavior biologically. Cell formation, adhesive structure and polarity make up the potential traits of EPCAM protein. Although loss of adhesive structure and cell polarity generally happens in tumor cells, higher expression of this protein has been clarified in tumor cells.

In addition, our results revealed that 40.5% of CRC cases had increased levels of CD166 membranous immunoreactivity, and there was no significant correlation with the clinical profile of patients such as clinical stage, distant metastasis, lymph node, neural, and vascular invasion. There is some contentious information regarding the difference between the various localization patterns of CD166 and its relation with demographic features and overall survival of the patient. Because of the different cellular positions of CD166, it is predominantly expressed in cell membrane and partially in cytoplasm. Our findings are consistent with several pieces of evidence suggesting a decreased or no clinical significance of the membranous expression of CD166 in CRC tissues by immunohistochemistry.

A comprehensive study of 1420 CRC samples using TMA constructions observed the lower immunoreactivity of CD166 in high grade tumors, larger tumor size, infiltrating tumor border configuration, and overall less survival cases. Tachezy reported the major membranous localization of CD166 in primary tumors versus secondary and distant metastatic tumors and negative significant clinical differences with tumor differentiation grade.

They introduced CD166 as a good prognostic marker in CRC patients. A study carried out by Weichert noted the cell membrane expression of CD166 in only 31% of CRC tissues and there were no significant association between CD166 expression and clinic pathological features such as grade, stage, and lymph node invasion, however their multivariate analysis showed CD166 as an independent poor prognostic marker in CRC specimens. Another study conducted on 110 CRC samples indicated that 64% of primary tumors had positive membranous expressions of CD166, but they found no significant correlation with clinic pathological parameters. In contrast, other researches represented a direct significant correlation of CD166 expression with tumor regression and worse prognosis effects of this marker in preoperative chemo radiotherapy-treated colorectal adenocarcinoma specimens.

Evidence confirmed the translocation feature of CD166 from the cell membrane to the cytoplasmic localization by a clathrin-dependent pathway. Interestingly, Amanda, et al. evaluated the intracellular and extracellular domain of CD166 by dual stain assay in 105 CRC samples and defined shedding of extracellular expression of CD166 after intracellular localization of this protein [10]. They clarified the correlation of cytoplasmic expression pattern of CD166 with poor prognosis suggesting the surface expression of CD166 in early-stage and cytoplasmic expression in the progressive stage of disease. This may support the contradictory findings of all studies regarding various expressions of CD166 and its clinical significance, described above.

Although the association of CD166 expression with demographic variables of patients was not statistically significant, combined analysis showed the significant association between EPCAM and CD166 expression (P=0.02). Moreover, a significant positive correlation was found between EPCAM/CD166 phenotypes expression and tumor stage (P=0.03), tumor differentiation (P=0.05), neural and lymph node invasion (P=0.01). Thus, co-expression of CD166 with EPCAM (but not alone) was accompanied by a significantly elevated tumor aggressive behavior. Despite a few limitations such as lack of overall survival and follow-up data, our results justify the importance of EPCAM separately and the connection between overexpression of two CSCs markers (EPCAM, CD166) and tumor aggressiveness in CRC tissues. Therefore, management of CRC patients to predict recurrence, relapse, drug resistance, and provide longer survival could come about in the light of using these CSC markers in targeted-therapy strategies.

## Conclusion

Novel molecular therapeutic strategies have shed new light on treatment and found a proper marker on tumorigenic CSCs in the bulk of CRCs and targeting of these cells in order to eradicate them and consequently diminish more of the side effects and damaging processes of non-tumorigenic and normal cells. The results in the current study represented the significant higher expression of EPCAM in tumors with larger size, higher stage, moderate/

poor-differentiation, and positive neural, vascular, and lymph node invasion. No correlation was found between CD166 expression and demographic parameters of patients. A link was also seen between EPCAM and CD166 that represented co-expression of two markers (P=0.02) and a significant direct correlation between EPCAM/CD166 phenotypes expression and tumor stage (P=0.03), tumor differentiation (P=0.05), neural, and lymph node invasion (P=0.01) in CRC tissues. In other words, CD166, dependently, and EPCAM were identified as putative CSC markers with greater tumor progression and aggressiveness in human CRC specimens.

## Ethics Approval and Consent to Participate

The study was approved by the Iran University of Medical Sciences, Human Research Ethics Committee in Iran (Ref No: IR.IUMS.REC1396.9411100005). All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all individual participants, parents or legally authorized representatives of participants at the time of sample collection with routine consent forms.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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