

BRIEF REPORT

BALANCE BETWEEN THE STEM CELL MARKER CD44 AND CDX2 EXPRESSION IN COLORECTAL CANCER

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ABSTRACT

CDX2 (Caudal-type homeobox transcription factor 2) is a biomarker of differentiated colon enterocytes, whose expression has been associated with a favorable prognosis in colon cancer. The absence of CDX2 has been associated with an aggressive outcome, including an higher risk of relapse. CD44 (Cluster of Differentiation 44) is a transmembrane glycoprotein involved in cell growth, survival, differentiation and migration. It is considered a typical marker of cancer stem cells, with a role in colorectal cancer progression. The aim of this study was to analyze the expression of the stem cell marker CD44 and its relation to CDX2 expression in colorectal cancer.

To this end, 65 consecutive colorectal cancers were immunostained with anti-human CD44 Rabbit monoclonal antibody (clone SP37) and anti-human CDX2 Rabbit monoclonal antibody (clone EPR2764Y). 59 cases were positive for CDX2 and 47 were positive for CD44. Regarding cases positive for CDX2, 49 were positive for CD44. Our findings show the existence of a wide spectrum, ranging from cases CDX2-/CD44- to tumors expressing both markers. Multiple further combinations of the two markers were also found. CD44 immunoreactive tumors showed an high stage at diagnosis, suggesting a possible association of CD44 expression with an aggressive outcome of colorectal cancer.

KEY WORDS

Colorectal cancer; CD44; CDX2; prognostic biomarker; cancer stem cells.

IMPACT STATEMENT

The expression of CD44 in CDX-2 negative tumors could indicate a possible target-therapy targeted to CD44.

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer and the second in terms of worldwide mortality (1).

The regional incidence of CRC varies worldwide. The variability seems related to differences in environmental exposures and eating habits acting on a background of genetic susceptibility. The areas with a higher incidence rate are Europe, New Zealand, Australia and North America. The areas with a lower rate are Africa and South-Central Asia (2). The conventional adenoma-carcinoma pathway is responsible for most colorectal cancers, while 10-20% of CRCs result from serrated lesions (3).

There are many risk factors, genetic and epigenetic, involved in the development of colon cancer. The most potentially preventable risk factors are smoking, high alcohol consumption, unhealthy diet, excess body weight and physical inactivity (4, 5).

The stratification of patients affected by colorectal cancer is a key factor for the identification of patients who require adjuvant chemotherapy after tumor resection. In the absence of simple reliable criteria for the stratification of CRC patients at higher risk of relapse, decision making for adjuvant chemotherapy often represents a dilemma for oncologists (6). To address this problem, many studies explored the possibility of stratifying CRC patients according with the tumor gene expression profiling (7), metastasis-associated gene expression changes (8), the molecular profile of tumor cells (9), the cancer stem cell signature (10, 11) and the correlation with epithelial-mesenchymal transition-related gene expression (5). Given the difficulty of utilizing gene-expression signatures in clinical practice (7), in recent times researchers focused on the immunohistochemical expression of multiple markers with the aim of identifying a signature that could be used to identify the more aggressive forms of CRC. Researchers focused on the identification of immunohistochemical markers possibly associated with an aggressive behaviour of CRC. A project from our group in this field, aimed to identify markers associated with CRC ag-

gressivity, identified Thymosin beta-4 (TB4) (12) at the invasion front of a subset of CRCs (13, 14). In these studies, TB4 was highly expressed in tumor cells undergoing epithelial-to-mesenchymal transition, suggesting a role for this peptide in invasion and metastasis.

Dalerba and coworkers focused on the caudal-type homeobox transcription factor 2 (CDX2), as a biomarker of well differentiated colon enterocyte. The analysis of 466 CRC patients showed that CDX2 expression is associated with an higher disease-free survival as compared with the CDX2-negative patients. Conclusively, this study evidenced that lack of CDX2 expression identifies a group of patients at high risk of relapse, who may benefit from adjuvant chemotherapy, irrespectively of the tumor stage (15). Furthermore, patients with colon cancer without CDX2 expression were more likely to have aggressive features: high grade tumor, mucinous tumors, lymph node involvement and advanced overall pathological staging (16).

Considering that many studies produced evidence on the presence in CRC of self-renewing stem progenitor tumor cells, the so-called cancer stem cells (CSCs), we initiated a search for a biomarker that might better characterize CDX2-negative undifferentiated tumors, focusing on cancer stem cell markers previously described in human colon, including CD44, CD133, CD90, SOX2, SOX9, ALDH1A1 and EpCAM (17).

Our aim was to find, by means of immunohistochemistry, a simple marker of immature colon cancer cell which, joined with CDX2, might be used in clinical practice for identifying the less differentiated and possibly more aggressive forms of CRC.

MATERIALS AND METHODS

We examined 65 cases of colorectal adenocarcinoma diagnosed between 2008 and 2021, ranging in age from 49 up to 85 years, 37 males and 28 females. Ethics Committee approval was obtained

for the study (Protocol number 2020/10912 – code: EMIBIOCCOR) and written informed consent was obtained from all participants for their tissues to be utilized for this work.

Tissue samples were routinely processed for histological observation and stained with hematoxylin-eosin (H.E). For immunohistochemical analysis, 3 µm thick sections were obtained from the paraffin block. All reagents were purchased from *Ventana Medical Systems Inc. 1910 E. Innovation Park Drive Tucson, Arizona 85755 USA*. The sections were automatically dewaxed and rehydrated with EZ Prep 1X (Ref. 950-102) and pre-treated with heat-induced epitope retrieval in Ultra CC1 (Ref. 950-224), following Dealer's instructions. Slides were then incubated at room temperature with anti-human CD44 Rabbit monoclonal antibody – clone SP37 – (Ref. 790-4537) and with anti-human CDX2 Rabbit monoclonal antibody – clone EPR2764Y – (Ref. 760-4380). All immunostaining procedures were performed using the UltraView Universal DAB Detection Kit (Ref. 760-5000) on the BenchMark Ultra (*Ventana Medical Systems Inc. 1910 E. Innovation Park Drive Tucson, Arizona 85755 USA*) instrument, according to the manufacturer's instructions. For CD44 interpretation, we used the following grading score system, based on HER2/neu scheme (**table I** and **figure 1**). For CDX2 evaluation we utilized the scoring system shown in **table II**.

Statistical analysis was performed with the MedCalc Statistical Software Version 14.10.2 (MedCalc Software bvba, Ostend, Belgium; [\[calc.org\]\(http://www.medcalc.org\); 2014\). The association between categorical variables was estimated by the Fisher exact test for categorical binomial variables or by the chi-square test in all other instances.](http://www.med-</p>
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RESULTS

The clinicopathological features of patients here analyzed are reported in **table III**. In our study cohort, the median age was 66 years (range 49-85), 37 patients (57%) were men and 28 (43%) were women. 17 (26.1%) tumors were located in the right colon, 3 in the transverse colon (4.6%), 21 (32.4%) were found in descending colon, 5 (7.7%) in sigma, 2 in sigma-rectum (3.1%), 15 (23.1%) were in the rectum, 1 case affected rectum and right colon (1.5%) and 1 case cecum and transverse colon (1.5%).

CD44 negative or weak membrane staining in less than 10% of tumor cells (score 0) was observed in 18 (27.7%) patients, 15 (23.1%) showed weak membrane staining in at least 10% of tumor cells or moderate in less than 10% of tumor cells (score 1+), 18 (27.7%) moderate membrane staining in at least 10% of tumor cells or intense in less than 10% of tumor cells (score 2+) and 14 (21.5%) intense membrane staining in at least 10% of tumor cells (score 3+) (**table IV**). In this series, CD44 expression was more frequent in cancers of the sigma and rectum (80-100%) versus 70% in the colon.

CD44 EXPRESSION	SCORE
Negative or weak membrane staining in less than 10% of tumor cells	0
Weak membrane staining in at least 10% of tumor cells or moderate membrane staining in less than 10% of tumor cells	1+
Moderate membrane staining in at least 10% of tumor cells or intense membrane staining in less than 10% of tumor cells	2+
Intense membrane staining in at least 10% of tumor cells	3+

Table I. CD44 scoring system

CDX2 EXPRESSION	SCORE
Negative or nuclear staining less than 5% of tumor cells	0
Nuclear staining in 6%-33% of tumor cells	1+
Nuclear staining in 34%-66% of tumor cells	2+
Nuclear staining in more than 66% of tumor cells	3+

Table II. CDX2 scoring system.

		CDX2 +	CD44 +
Age	49-85 years (avg 66 y)	67 years	65 years
Sex			
Male	37 (57%)	33 (89,2%)	27 (73%)
Female	28 (43%)	26 (92.9%)	20 (71.4%)
Site			
Right colon	17 (26,1%)	15 (88.2%)	12 (70.6%)
Transverse colon	3 (4.6%)	3 (100%)	1 (33.3%)
Descending colon	21 (32.4%)	20 (95.2%)	15 (71.4%)
Sigma	5 (7.7%)	5 (100%)	5 (100%)
Sigma-rectum	2 (3.1%)	2 (100%)	1 (50%)
Rectum	15 (23.1%)	12 (80%)	12 (80%)
Rectum and right colon	1 (1.5%)	1 (100%)	1 (100%)
Cecum and transverse colon	1 (1.5%)	1 (100%)	1 (100%)

Table III. The clinicopathological features of 65 patients with CRC.

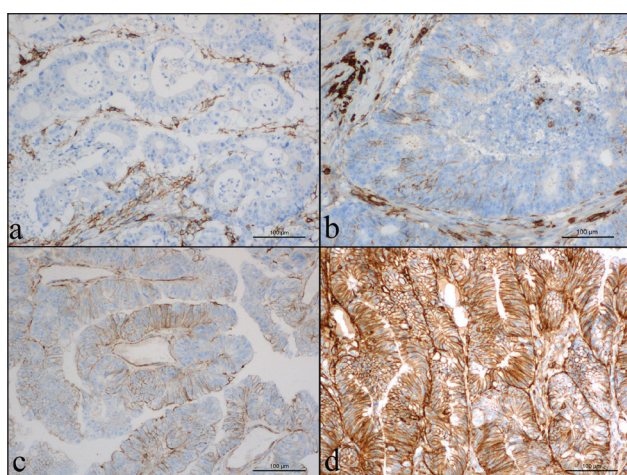


Figure 1. CD44 scoring system: **a.** Score 0: negative or weak membrane staining in less than 10% of tumor cells; **b.** Score 1+: weak membrane staining in at least 10% of tumor cells or moderate membrane staining in less than 10% of tumor cells; **c.** Score 2+: moderate membrane staining in at least 10% of tumor cells or intense membrane staining in less than 10% of tumor cells; **d.** Score 3+: intense membrane staining in at least 10% of tumor cells.

CDX2 total loss of expression (score 0) was observed in 6 (9.2%) patients, 3 (4.6%) showed nuclear staining in 6%-33% of tumor cells (score 1+), 4 (6.2%) stained 34%-66% of tumor cells (score 2+) and 52 (80%) nuclear staining in more than 66% of tumor cells (score 3+) (**table IV**). In this series, CDX2 loss of expression was more common in males (M:F ratio = 2:1).

Following our scoring systems for CDX2 and CD44, we reached 16 groups of patients: 2 CD44 0/CDX2 0; 1 CD44 0/CDX2 1+; 2 CD44 0/CDX2 2+; 13 CD44

		CDX 2			
CD44		0	1+	2+	3+
	0	2	1	2	13
	1+	1	0	2	12
	2+	0	2	0	16
	3+	3	0	0	11

Table IV. Immunoreactivity for CD44 and CDX2.

0/CDX2 3+; 1 CD44 1+/CDX2 0; 0 CD44 1+/CDX2 1+; 2 CD44 1+/CDX2 2+; 12 CD44 1+/CDX2 3+; 0 CD44 2+/CDX2 0; 2 CD44 2+/CDX2 1+; 0 CD44 2+/CDX2 2+; 16 CD44 2+/CDX2 3+; 3 CD44 3+/CDX2 0; 0 CD44 3+/CDX2 1+; 0 CD44 3+/CDX2 2+; 11 CD44 3+/CDX2 3+.

The data regarding immunoreactivity for CD44 and CDX2 are summarized in **table IV**.

In short, according with the different degree of reactivity for CDX2 and CD44, the cases of colon cancer analyzed were differentiated into 16 groups. At the extremes of the spectrum we found 4 cases CDX2 negative and CD44 positive and 16 cases CDX2 positive and CD44 negative. All the other cases showed a more complex co-expression of the two markers (**figure 2**).

CD44 and CDX2 expression did not show a significant correlation with any of the mutational analysis carried out. There was no correlation between CD44 expression and BRAF mutations. BRAF mutations were found in 14.3% of CD44-negative patients *versus* 12.2% of CD44-positive patients ($p =$

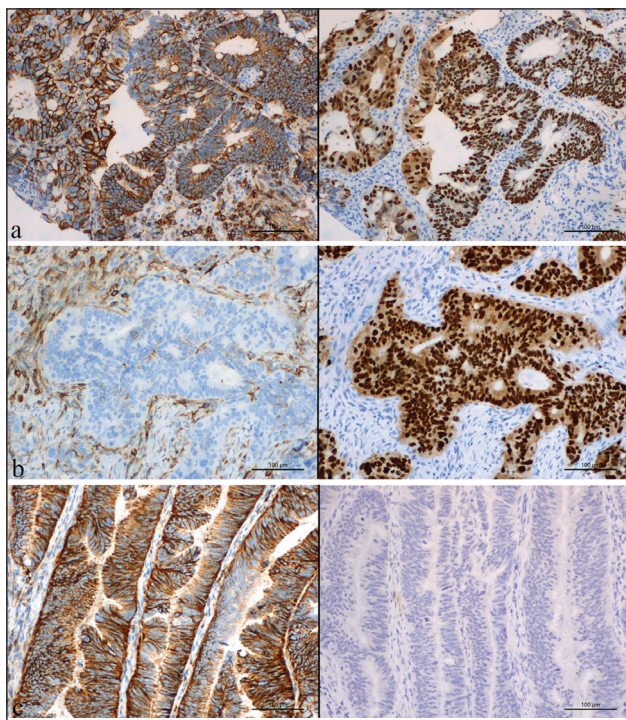


Figure 2. At the extremes of the spectrum: **a.** Case 1: CD44 3+ (on the left) and CDX2 3+ (on the right); **b.** Case 2: CD44 0 (on the left) and CDX2 3+ (on the right); **c.** Case 3: CD44 3+ (on the left) and CDX2 0 (on the right).

0.84). Specifically, relating to the CD44 score: BRAF was mutated in 14.3 % of CD44 negative, in no CD44 1+, in 12.5% of CD44 2 + versus 25% of CD44 3+ patients ($p = 0.3$).

There was a non-statistically significant higher occurrence of mutated BRAF in CDX2- negative patients than in CDX2 positive patients (33.3% versus 12%, $p = 0.3$). Relating to the CDX2 score: BRAF was mutated in 33.3% of CDX2 negative patients, 50% of CDX2 1+, in no CDX2 2+ and 11.4 % of CDX2 3+ ($p = 0.2$).

There was no correlation between CDX2 expression and RAS mutations. RAS mutated was found in 66.7% of CDX2-negative versus 56.9% of CDX2-positive patients ($p = 0.74$). Specifically, relating to the CDX2 score: RAS was mutated in 66.7% of CDX2 negative, in 50% of CDX2 1 +, in no CDX2 2+, and in 56.8% of CDX2 3+ ($p = 0.7$).

There was no correlation between CD44 expression and RAS mutation. RAS was mutated in 53.3% of CD44-negative patients and in 61% of CD44-positive patients ($p = 0.6$). Specifically, relating to CD44 score: RAS was mutated in 53.3% of CD44-negative, in 69.2% of CD44 1+, in 56.2% of CD44 2+, and in 58.3% of CD4 3+ patients ($p = 0.9$).

Furthermore, there was a non-statistically significant higher percentage of patients with a high degree of differentiation in CD44 positive patients. 90% of patients with CD44 positive had a high degree of differentiation (G2-G3) compared to 77.8% of patients with CD44 negative ($p = 0.3$). Mainly all CD44 3 + had a grading of 2-3.

DISCUSSION

Colon cancer is a major problem for the oncologists also because it affects middle aged as well as younger patients. Therefore it is important to study and search for new markers that can allow to stratify patients, to understand which characteristics give the tumor greater aggressivity or less response to therapy. Starting from the article on the New England Journal of Medicine (15) and from our observation of a patient with colon cancer with complete loss of CDX2 expression, we began to study CD44 in a cohort of CRC patients.

CD44 is a multifunctional transmembrane glycoprotein encoded by a single gene on chromosome locus 11p13 expressed ubiquitously throughout the body (18). It is involved in cellular processes such as survival, adhesion, cell division and migration (17). There are multiple CD44 isoforms based on presence of alternative exons at specific site in the extracellular domain (3). Several studies have shown that the expression of different CD44 isoforms seem to play a key role in tumor progression (19). Moreover, CD44 has been shown to transform a non-metastatic cell line into a more metastatic line (19). The different effects of CD44 on cellular processes depend on its binding to different ligands, such as hyaluronic acid (HA), collagens, osteopontin (OPN) and matrix metalloproteinases (MMPs) (17). CD44 has been studied in several organs: it is considered a cancer stem cell marker in colon cancer but CD44 is expressed in other organs, such as breast, lung, prostate and bladder (3). There is evidence showing that high expression of the CD44 variant 2 in CRC patients is associated with a poorer prognosis than other CD44 variants (20).

In our study, at first, we did not find a simple relationship between CDX2 negativity and CD44 positivity. In fact we found that CDX2 and CD44 may be combined in many ways, ranging from the expression of both CDX2 and CD44 up to the absence of both markers. We divided the patients into 16 group. The most represented group is

CD44 2+/CDX2 3+. We found no correlation between expression of CDX2/CD44 and the site of lesions, age or sex.

Further studies are required to clarify the binding between CD44 expression and clinicopathological features of colon cancer.

CONCLUSIONS

Given the complexity, more than expected, regarding the relationship between CDX2 and CD44 expression in CRC, on the basis of our preliminary results, CD44 cannot be simply identified as a marker of undifferentiated CRC. Our initial hypothesis that CD44 expression might be restricted to CDX2-negative tumors has not been confirmed in this study. Relationships between CD44 and CDX2 expression in CRC tumor cells are much more complex than hypothesized. The spectrum is broad, ranging from a modest amount of cases CDX2+ and CD44- (16 out of 65.25%) up to few cases characterized by CD44 reactivity and absence of CDX2 expression (4 out of 65.6%). In the middle, we found the majority of cases analyzed including 39 patients with CDX2 3+ and positivity for CD44, with no significant difference between CD44 1+ (12 cases), CD44 2+ (16 cases) and CD44 3+ (11 cases). The meaning and the clinical significance of the expression of CD44 in CRC has to be clarified, especially with regard to the co-expression with CDX-2.

Our work should be considered as a contribution to assessing the role of CD44 with regard to the ability to metastasis, local infiltration and response to chemotherapy. Consequently, the expression of CD44 in CDX-2 negative tumors could indicate a possible target-therapy targeted to CD44.

ETHICS

Fundings

There were no institutional or private fundings for this article.

Conflict of interests

Prof. Mario Scartozzi has had a role as consultant, advisory board and speakers' bureau for the following companies: Amgen, Sanofi, MSD, CISAI, Merck, Bayer. The remaining authors have declared no conflict of interests.

Availability of data and materials

All the data supporting the findings of this study are available within the article and can be shared upon request to the corresponding author.

Authors' contribution

All authors contributed to manuscript writing and approving the final version.

Ethical approval

Ethics Committee approval was obtained for the study (Protocol number 2020/10912 – code: EMIBIOCCOR).

Consent to participate

Written informed consent was obtained from all participants.

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