

Notch Activation Modulates Bevacizumab Activity by CD44 Positive Cancer Stem Cells in Advanced Colon Cancer

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Abstract

Background: Notch pathway is involved in regulating colon cancer stem cells (CSCs), which play an important role in angiogenesis and resistance to conventional therapies. CD44 and CD133 are transmembrane glycoproteins reported as putative markers for isolating CSCs. High expression of Notch Intracellular Cleaved Domain (NICD) has been associated with resistance to anti-vascular endothelial growth factor therapy. Based on these reports, we evaluated NICD, CD44 and CD133 expression in a series of consecutive metastatic colon cancer patients treated with bevacizumab-based chemotherapy within first-line clinical trials.

Methods: Histological samples obtained from 45 primary adenocarcinomas were tested by immunohistochemistry for NICD, CD44 and CD133. A scoring system based on staining intensity and percentage of stained cells was used. Vessels density was measured in hot-spot areas by CD31 antibody.

Results: CD44 levels were higher in high NICD cases than in low NICD cases (63% vs. 16%, respectively, $p=0.014$). High NICD and CD44 levels predicted shorter progression-free ($p<0.001$) and overall survival ($p=0.012$). No significant association was found between CD133 or CD31 and NICD or CD44 expression.

Conclusion: Our data suggest that colon cancer patient with high NICD and CD44 levels have a reduced likelihood of response to bevacizumab-based therapy.

Keywords: Colorectal cancer; bevacizumab; Notch; CD44; Stem cells; Angiogenesis

Introduction

Vascular endothelial growth factor (VEGF)-targeted agents improve the survival of advanced colorectal cancer (CRC) patients, [1-3] but their activity is restricted by mechanisms of both primary and adaptive resistance [4].

Even within an individual patient, tumor contains cell populations with different genetic mutations and at various stages of differentiation, both contributing to cancer cell heterogeneity. In addition, a small fraction of cells is maintained in an undifferentiated stage and defined as “cancer stem cells (CSCs)” with properties including self-renewal, multipotentiality and apoptosis resistance. Recent data from the literature suggest that CRC may originate from this pool of CSCs that may account for tumor heterogeneity [5-10] and resistance to anti-cancer treatments [11,12]. Therefore, understanding the characteristics of CSCs may lead to novel findings in individualized treatment. Moreover, intestinal stem cells are located near the base of crypts [5] and are believed to have a relatively longer life span compared with the normal intestinal epithelial cell; hence, this specific population is potentially exposed to and is able to harbour genetic alterations that are critical mediator of oncogenesis.

CD133 and CD44 are well-established putative cancer stem cell biomarkers expressed in CRC [13-16]. CD133 is a surface

transmembrane glycoprotein whose expression is mainly correlated with tumorigenicity, metastasis, and worse prognosis [17]. CD44, a hyaluronic receptor, promotes cell-adhesion and assembly of cell surface growth factors specifically involved in the maintenance of cell-matrix interactions [14]. CD44 up-regulation increases tumor growth and anti-apoptotic property [16]. It has been demonstrated that colon cancer cell lines expressing both high levels of CD44 and CD133 had more stem cell-related genes, and displayed increased tumorigenic ability. A subset of SW480 cell lines showed enhanced CD44 expression and other markers of chemoresistance [14-18]. Therefore, both CD133 and CD44 could have a strong potential as targets for drug therapies in CRC. These markers are also correlated with the downstream

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molecular pathways that accomplish oncogenic activities such as Wnt, Notch, and BMP-1 [19].

Notch signalling plays a pivotal role for maintenance of the population of colon tumor-initiating cells, a CSC population that retains high metastatic potential and self-renewal [20,21]. When the Notch receptor binds its ligands, the cytoplasmic portion of Notch undergoes proteolytic cleavage through γ secretase [22]. The free cytosolic portion (Notch intracellular domain (NICD)) translocates to the nucleus where it interacts with a multiprotein complex to induce the transcription of Notch target genes [23]. Preclinical studies [24] and a clinical study from our group [25] showed that NICD overexpression is associated with resistance to anti-vascular endothelial growth factor (VEGF) therapy in colon cancer. Based on these observations, we evaluated the immunohistochemical expression of CD44 and CD133 in a series of consecutive metastatic colon cancer patients tested for NICD and treated with anti-VEGF monoclonal antibody (mAb) bevacizumab within first-line clinical trials. Moreover, to determine whether intratumor microvessel density (MVD) could represent a confounding variable of resistance to bevacizumab, the expression of the endothelial cell marker CD31 was assessed.

Materials and Methods

Patients

The study population consisted of consecutive patients with histologically confirmed mCRC enrolled into four prospective clinical trials assessing first-line bevacizumab-based regimens at four Institutions between 2005 and 2011 [26-29]. No patients with mucinous histology (>50% extracellular mucin) were included in the study. The objectives of the study were to evaluate NICD, CD44 and CD133 expression in advanced CRC patients treated with bevacizumab and to correlate these markers with objective tumor response to bevacizumab, progression-free survival (PFS) and overall survival (OS). Inclusion criteria were: available adequate archival tumor samples and measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST; version 1.1) [30].

According to treatment protocol, patients were treated with bevacizumab in combination with either infusional fluorouracil, leucovorin and irinotecan (FOLFIRI) or infusional fluorouracil, leucovorin and oxaliplatin (FOLFOX4) or infusional fluorouracil, leucovorin, oxaliplatin and irinotecan (FOLFOXIRI) or capecitabine. Patients received bevacizumab at the dose of 5 mg/kg on day 1 given every 2 weeks or 7.5 mg/kg on day 1 given every 3 weeks until disease progression, unacceptable toxic effects or withdrawal of consent. This study was conducted according to a protocol approved by the institutional review board/independent ethics committee and informed consent was obtained from patients for the use of tissue samples and the analysis of clinical information.

The response to treatment was evaluated according to RECIST 1.1 [30], taking into account the number of target lesions, sum of the diameters of the target lesions, percentage change since the baseline or tumor nadir (whichever was the least preceding sum diameter), descriptions of nontarget lesions, presence or absence of new lesions.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue blocks from pre-treatment primary lesions, selected on the basis of quality and representativeness of the sample, were cut into 5 μ m thick sections and incubated overnight at 4°C with the primary antibody. The antibodies

used in this study were: NICD polyclonal antibody (Val1744) (Cell Signaling Technology, Danvers, MA, USA), CD44 monoclonal antibody (mAb) (clone DF1485; Dako, Glostrup, Denmark), CD133 mAb (clone AC133; Miltenyi Biotec, Bergisch Gladbach, Germany) and CD31 mAb (clone JC70; Ventana-Roche, Tucson, AZ, USA). Antibody detection was performed using an ADVANCE HRP Link and an ADVANCE HRP Enzyme (Dako Corp., CA, USA). Diaminobenzidine (DAB) was used as chromogen. Samples were counterstained with haematoxylin eosin solution.

NICD and CD44 expression were quantified using a semiquantitative scoring system based on intensity and on percentage of immunostained cells. The immunohistochemical staining was scored as 0 when no expression was found, 1+, 2+ and 3+ when weakly, moderately and strongly positive expression was respectively observed in $\geq 10\%$ of the cells. NICD and CD44 expression was judged positive when a strong staining (3+) was found in >50% and $\geq 60\%$ of cells, respectively. CD133 was evaluated by assessing positive tumor glands compared to all tumor glands. CD133 immunohistochemical staining was scored as 0 when there was no expression at all, 1+, 2+ and 3+ when a weak, moderate and strong expression was respectively detected in $\geq 10\%$ of the whole tumor area. Tumors with 2/ 3+ intensity in $\geq 10\%$ of the whole tumor area were considered as positive.

Vessel density was evaluated after scanning the immunostained section at 200x magnification in order to identify the tumor areas with the highest number of microvessels (hotspots) positively stained by CD31 antibody. Microvessel count was performed from a minimum of 4 to a maximum of 12 fields depending on the cellularity of the tumor at 400x magnification corresponding to an area of 2.3 mm² per field. For each sample, the mean value of vessel count in four fields with the highest MVD was retained as the final value. Any single endothelial cell or cluster that stained for CD31 was counted as a single microvessel, even in the absence of a visible vascular lumen.

Two observers blinded to the clinical and pathological data evaluated all immunohistochemical staining.

Statistical analysis

Statistical significance was set at $p < 0.05$ for each analysis; all analyses were carried out using IBM-SPSS Statistical package, version 22.0 and the widespread open source statistical system 'R', version '3.3.1'. Correlations between interval and ordinal variables were evaluated by Pearson's and Spearman's correlation coefficients, while associations between nominal or dichotomized variables and clinical outcome parameters were estimated by chi-square test and Fisher exact test for 2 \times 2 tables. The Kaplan-Meier method was used to estimate median OS and PFS measured from the date of randomization for all patients. Cox proportional hazard models were fitted to investigate the associations between OS and PFS and covariates. Variables included were: KRAS status, chemotherapy protocol, second-line chemotherapy, NICD expression, CD44 expression, CD133 expression, CD31 expression and MVD. Results were expressed as hazard-ratios (HRs) with 95% confidence intervals (CIs). All tests were two-sided.

Receiver operating characteristic (ROC) curve analysis was carried out to determine a possible cut-off point for CD44 level; for each value, sensitivity, specificity, and total accuracy were obtained as percentages.

Results

Forty-five colon cancer patients with advanced adenocarcinoma, involved into four prospective clinical trials assessing first-line bevacizumab-based regimens, were enrolled in this study. Histological

samples were obtained from 7 biopsies and 38 surgical specimens. Table 1 shows patients' characteristics. NICD immunostaining showed a predominant cytoplasmic localization (Figure 1A). Eight of the 45 (18%) patients showed high levels of NICD expression as defined by a NICD score 3+ in >50% of tumor cells [25]. Among patients with low levels of NICD expression, 2 exhibited a completely negative staining, 9 were scored as 1+ and 26 as 2+.

Characteristics	N (%)
Age (years)	67
Range	36-81
Sex	
Male	28/45 (62)
Female	17/45 (38)
CEA	
<30	16/42 (38)
>30	26/42 (62)
Unknown	3
KRAS	
Mutant	16/33 (48)
Wild type	17/33 (52)
Unknown	12
Number of metastatic sites	
1	29/45 (64)
≥2	16/45 (36)
Subsequent chemotherapy	
Yes	29/42 (69)
Received cetuximab	13/42 (31)
Unknown	3
Positive NICD	8/45 (18)
Positive CD44	11/45 (24)
Positive CD133	7/39 (18)
Not evaluable	6
CD31 (n=35)	
Median MVD/mm ² (range)	20 (4.8-51.3)

Note: Abbreviations: NICD: Notch Intracellular Cleaved Domain; MVD: Microvessel Density

Table 1: Patients' characteristics.

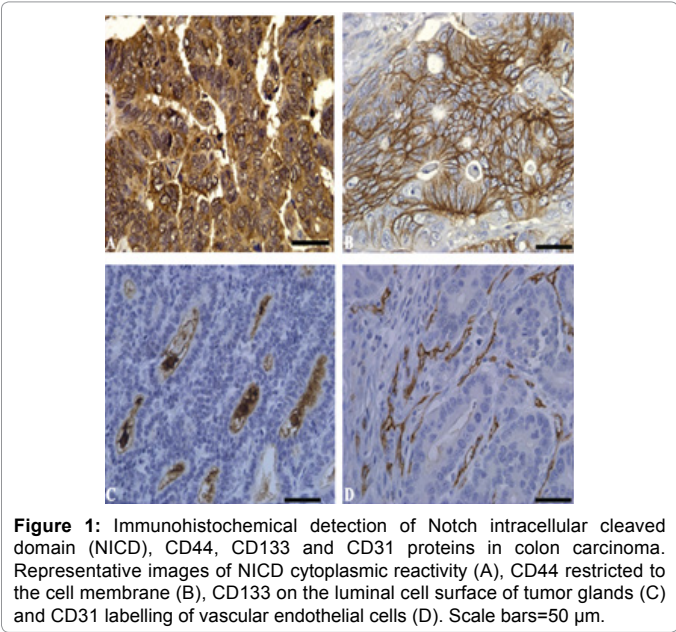


Figure 1: Immunohistochemical detection of Notch intracellular cleaved domain (NICD), CD44, CD133 and CD31 proteins in colon carcinoma. Representative images of NICD cytoplasmic reactivity (A), CD44 restricted to the cell membrane (B), CD133 on the luminal cell surface of tumor glands (C) and CD31 labelling of vascular endothelial cells (D). Scale bars=50 μm.

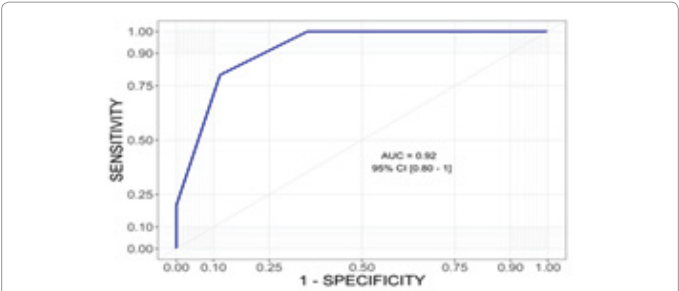


Figure 2: Receiver operating characteristic (ROC) curve for CD44. Analysis relative to logistic model based on CD44 positive staining, with objective tumor response to bevacizumab.

Variables	Low NICD	High NICD
Low CD44	31	3
High CD44	6	5

Fisher's exact test p=0.014

Note: Abbreviation: NICD: Notch intracellular cleaved domain

Table 2: Association between CD44 and NICD expression in colon cancer patients.

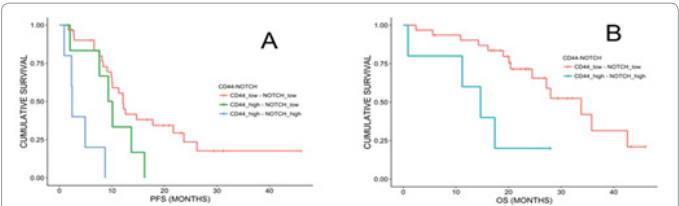


Figure 3: Progression-free survival (A) and overall survival (B) according to Notch intracellular cleaved domain (NICD) and CD44 status.

Immunohistochemical staining for CD44 showed membrane localization of the protein in epithelial tumor cells in the vast majority of cases (Figure 1B). The cut off value for CD44 level, as determined by ROC curve, was $\geq 60\%$ of tumor cells showing 3+ intensity and this threshold was used to divide patients in two groups of 'high' and 'low' CD44 expression. Eleven (24%) of the 45 cases showed a high CD44 expression.

CD133 was evaluable in 39 of 45 cases; 4 cases were not included for technical reasons (suboptimal staining) and two biopsy samples were inadequate. CD133 expression was localized to the glandular-luminal surface of neoplastic glands and on intraglandular cell debris (Figure 1C). Dot-like cytoplasmic staining was occasionally present. Positive CD133 expression was found in 7 of 39 cases (18%) and only 2 cases showed more than 30% CD133 positive cells.

Thirty-five of the 45 samples were evaluable for CD31 expression (among the 10 unassessable cases, 7 were biopsies and 3 had the paraffin block run out). Positive CD31 staining was localized to vascular endothelial cells (Figure 1D). The value of MVD observed in the 35 evaluable samples ranged from 11 to 118 at 40x magnification, with a median value of 20 per mm² (range: 4.8-51.3).

CD44 immunostaining (3+) in $\geq 60\%$ of tumor cells emerged as the best cut-off value to discriminate responders vs nonresponders to bevacizumab (Figure 2). A positive association was found between NICD and CD44 expression (Table 2). Log-rank test showed both a significant shorter PFS ($p<0.001$) and OS ($p=0.012$) for patients having both high NICD and CD44 expression (Figure 3). A median PFS of 12.1 months in low NICD and CD44 expressing tumors was observed

Variables	PFS		OS	
	HR (95% CI)	p-value	HR (95% CI)	p-value
NICD-Low; CD44-Low	Reference value	0.001	Reference value	0.050
NICD-Low; CD44-High	2.163 (0.851 - 5.495)	0.105	1.875 (0.671 - 5.239)	0.231
NICD-High; CD44-Low	1.783 (0.412 - 7.712)	0.439	4.098 (0.878 - 19.13)	0.073
NICD-High; CD44-High	9.194 (2.996 - 28.22)	<0.001	4.157 (1.312 - 13.17)	0.015

Abbreviations: NICD: Notch Intracellular Cleaved Domain; HR: Hazard Ratio; CI: Confidence Interval

Table 3: Cox's regression for progression-free survival (PFS) and overall survival (OS) relative to NICD and CD44 expression.

Variables	Low CD31	High CD31	Low CD133	High CD133
Low CD44	11	10	16	5
High CD44	11	8	22	2
Fisher's exact test, p=0.76		Fisher's exact test, p=0.22		
Low NICD	16	14	28	6
High NICD	6	4	10	1
Fisher's exact test, p=1		Fisher's exact test, p=0.66		

Abbreviation: NICD: Notch intracellular cleaved domain

Table 4: Expression of CD31 and CD133 in relation to CD44 and NICD in colon cancer patients.

compared to 2.4 months in high NICD and CD44 tumors. Likewise, a median OS of 30.4 months was observed in low NICD and CD44 cases compared to 14.7 months in high NICD and CD44 cases.

Cox regression following univariate analysis confirmed that both NICD and CD44 were significantly associated with PFS and OS outcome. Table 3 shows that HR increases significantly from low NICD and CD44 levels to high NICD and CD44 levels.

All major clinical characteristics were comparable among subgroups of patients (data not shown). In particular, no differences were noticed for age, baseline levels of CEA, KRAS status, number of metastatic sites and subsequent chemotherapy.

Cox regression didn't show any significant association between CD133 and CD31 expression and MVD with PFS and OS. Moreover, we didn't find any significant association of CD133 and CD31 expression with CD44 and NICD levels (Table 4).

Discussion

We previously showed that high NICD expression in advanced CRC was negatively associated with response to the VEGF-targeted mAb bevacizumab [25]. Interestingly, in patients treated with chemotherapy alone, survival analysis did not show differences between high and low NICD levels, thus indicating a predictive rather than prognostic value of this molecular marker. Our results were in line with those by Paiva et al. [31], in which low Notch1 levels were able to predict improved OS in a series of 105 metastatic CRC patients treated with chemotherapy plus bevacizumab.

Notch signalling is known to be essential for stem cell maintenance [20]. The deregulated expression of Notch proteins, ligands and targets alters self-renewal and cell fate of stem cells that are normally controlled by both intrinsic and extrinsic pathways resulting in stem cell expansion. For example, Notch signalling is highly active in hematopoietic stem cells (HSC) compared to more differentiated cells and inhibition of Notch signalling promotes differentiation of HSC

[32]. Notch component genes are mainly localized in the base of colonic crypts further indicating that activation of Notch signalling may be involved in the regulation of colonic stem cells [33]. CD44 is critical for proper cell homing to niches microenvironment and for maintaining CSCs in a stem-like state [34,35]. In our series, high levels of NICD expression, evaluated on tissue sections from advanced colon tumors, were significantly associated with high CD44 levels in the majority of cases. Moreover, high NICD and CD44 expression was associated with unfavourable prognosis in patients treated with bevacizumab-based therapy. In pancreatic cancer, subpopulation of quiescent cells [36], enriched of CSCs markers such as CD44 and CD24, can escape therapy and subsequently induce tumor regrowth. Moreover, it has been demonstrated that after chemotherapy CSCs show a relative increase rather than decrease [37], pointing out that a better characterization of CSCs could help in targeting this population in order to inhibit cancer growth and metastasis.

Our results do not seem to support CD133 as a prognostic marker for colon CSCs. CD133 has been reported as a CRC CSC marker based on its high tumorigenicity in immunodeficient mice [6,7]. However, it should be noted that, in accordance with previous literature [38,39], in present study the majority of colon cancers lack the expression of CD133 and that CD133 and CD44 were not detected on the same tumor region. Moreover, it has been reported that knockdown of CD44, but not CD133, strongly prevents tumor initiation *in vivo* [38].

MVD assessment is the most commonly used method to quantify intratumoral angiogenesis in cancer. Vasculogenesis triggered by Notch signalling activation increases blood perfusion and reduce necrosis and hypoxia, leading to a decreased VEGF production and a reduced tumor dependency on VEGF. However, the relationship between Notch and VEGF pathways is not completely defined. Many retrospective studies reported that MVD is inversely related to survival in CRC, but other studies did not reach this conclusion [40]. Our results do not support a prognostic role of MVD in advanced colon cancer patients.

Although this analysis is subject to the limitations of a retrospective study, we utilized data from prospective randomized trials, each of which studied similar patient populations treated with bevacizumab-based chemotherapy. In order to minimize further bias, colon cancers of the mucinous histotype were excluded, given that meta-analyses and previous works suggest that mucinous adenocarcinomas may be associated with worse outcome compared with classical adenocarcinomas [41] as well as with poorer response to chemotherapy [42]. This study design strengthens our findings.

Conclusion

In conclusion, our results suggest that Notch is probably an important regulator for colonic stem cells and support a potential role of stem cells in colon cancer resistance to anti-VEGF therapy. Further studies are needed to clarify the regulatory role of Notch signalling in colon CSCs to provide a rational basis for novel therapeutic approaches to overcome antiangiogenic resistance.

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