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ORIGINAL ARTICLE

Nestin and CD34 expression in colorectal cancer predicts improved overall survival

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ABSTRACT

Background: Nestin, a class VI intermediate filament protein of the cytoskeleton, and CD34, a transmembrane phosphoglycoprotein, are markers of progenitor cells. This study aimed to evaluate their expression and clinical significance in colorectal cancer.

Methods: A clinically annotated tissue microarray, including 599 patients with colorectal cancer, was analyzed by immunohistochemistry. Furthermore, nestin and CD34 correlations with HIF-1a and a panel of cytokines and chemokines were assessed using quantitative reverse transcription PCR and The Cancer Genome Atlas dataset.

Results: Expression of nestin and CD34 was observed only in the tumor stroma. Patients displaying high expression of nestin and CD34 demonstrated higher rates of T1 and T2 tumors ($p = .020$), lower vascular invasion ($p < .001$) and improved 5-year overall survival (65%; 95% CI = 55–73 vs 45%; 95% CI = 37–53) after adjusting for clinicopathological characteristics (HR: 0.67; 95% CI = 0.46–0.96). A moderate to strong correlation ($r = 0.37$–0.78, $p < .03$) of nestin and CD34 was demonstrated for the following markers; HIF-1α, CD4, CD8, FOXP3, IRF1, GATA3, CCL2, CCL3, CXCL12 and CCL21.

Conclusions: Combined expression of nestin and CD34 expression is associated with better overall survival possibly by modulating a favorable immune response.

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Nestin; CD34; colorectal cancer; tumor microenvironment; hypoxia associated immune response

Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-associated death among adults in Western societies. A total of 1.096.601 new CRC patients with 551.269 deaths were expected in 2018 worldwide [1]. An increase of the global burden of CRC to more than 2.2 million new cases and 1.1 million deaths is estimated by 2030 [2]. In early-stage cancer, the occurrence of disease recurrence is up to 10% in patients with stage I and up to 20% in patients with stage II disease [3]. An estimated 14–34% of patients with non-metastatic disease at the time of diagnosis [4] will develop metastases, with 86% of them within the first three years [5]. Although stage remains the most important prognostic factor for overall survival [6], the necessity to contrive more accurate tools that predict aggressive tumor profiles has emerged.

In recent years, the tumor microenvironment (TME) composition has increasingly been recognized as an essential factor for tumor growth and progression [7]. Therefore, increasing research interest has focused on new prognostic markers and new treatment, potentially impacting tumor stroma's pathophysiology and its interaction with tumor cells.

The cytoskeleton of eukaryotic cells consist of three major filamentous components: actin, microtubules, and intermediate filament proteins [8]. Nestin belongs to class VI of

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Supplemental data for this article can be accessed here.

intermediate filament proteins identified initially in neuroepithelial stem cells [9]. Substantial *in vitro* and *in vivo* information suggests the presence of nestin in cells with stem cell properties [8]. More specifically, tumor cells expressing nestin harbor aggressive clinical features as demonstrated in a variety of malignant tumors of diverse histological origin, including brain (gliomas) [10], breast [11] (triple-negative cancers – regulator of Wnt/β catenin pathway), liver (hepatocellular carcinoma) [12], kidney [13] (cancer stem cell subpopulation in renal cell carcinoma), and prostate [14] (carcinoma initiating stem cells). In some of these tumors, nestin expression correlates with resistance to conventional therapy [8]. Moreover, a possible positive feedback mechanism for tumor neovascularization between nestin-positive cancer cells and endothelial cells of tumor blood vessels has been suggested [8]. Besides, nestin-positive cells in healthy tissues might act as reserve participating in tissue repair processes such as those taking place after focal cerebral ischemia and unilateral ureteral obstruction [8].

CD34 is a transmembrane phosphoglycoprotein, primarily known as a marker of hematopoietic progenitor and stem cells [15]. Accumulating data demonstrates significant CD34 expression in different cell types, such as the multipotent mesenchymal stromal cells, interstitial dendritic cells, and epithelial progenitors [16–19]. CD34 is widely known as a marker of vascular endothelial progenitor cells [20]. Its best-described ligand is L-selectin that mediates adhesion of lymphocytes to CD34 surface proteins in the vascular endothelium [21]. In the gastrointestinal tract, CD34 expression has also been identified in the interstitial cells of Cajal (JCC) [22] in murine and human tissues [23]. Besides, it has been suggested that CD34 enables the trafficking of mast cells and eosinophils into peripheral tissues. Regarding cancer, podocalyxin, a member of the CD34 family, has been associated with malignancy and aggressive tumor profiles in embryonic carcinoma, leukemia, breast, prostate, and pancreatic cancer [16]. In various carcinomas, CD34 has been used to evaluate microvessel density and tumor neovascularization.

Although these data suggest that expression of nestin and CD34 occurs in cells with stem cell properties, so far, nestin and CD34 have been described as markers of neovascularization in CRC [24]. However, their prognostic significance remains to be elucidated. This study aimed to determine the expression patterns of nestin and CD34 in a large cohort of CRC patients and to assess their clinical significance.

Materials and methods

Tissue microarray construction

Five hundred ninety-nine nonconsecutive, clinically annotated surgically removed primary CRC samples collected from the established colorectal tumor tissue bank of the Institute of Pathology of the Basel University Hospital were included in the constructed tissue microarray (TMA). Patients of the present cohort were treated in the period from 1987–1996, and therefore, most of the patients were chemotherapy-naive. Approval by the Regional Ethics Committee (Ethikkommission Nordwest- und Zentralschweiz, ref. nr. EK 120/13) for using the TMA for translational research purposes and written informed patient consent was obtained in advance. The study's conduct follows the principles of the Helsinki declaration, and the report of results proceeds along REMARK guidelines [25].

Standard procedures [26] of the Institute of Pathology of the Basel University Hospital were used to prepare formalin-fixed, paraffin-embedded tissue blocks. Briefly, tissue cylinders with a diameter of 0.6 mm were punched from morphologically representative areas of each donor block and brought into a recipient paraffin block (30x25mm) using a semi-automated tissue arrayer. Only one punch per tumor was taken. HE-stained sections overlayed on the surface of the donor blocks guided sampling from morphologically representative sites in the tumor and containing at least 50% tumor cells previously identified at the microscope from experienced pathologists in the field (L.T.). The latter contributed to the avoidance of heterogeneity within the tissue.

Immunohistochemistry

Immunohistochemistry was performed using standard indirect immunoperoxidase antigen detection. Briefly, slides were deparaffinized and rehydrated in distilled water, while endogenous peroxidase activity was blocked by using 0.5% H2O2. A 20-minute incubation with 10% normal goat serum (DakoCytomation, Carpinteria, CA) was used to block nonspecific binding. The sections were incubated with primary antibodies at room temperature. Primary antibodies were specific for nestin (AbD Serotec OBT1610, stained with Benchmark XT; OptiView DAB IHC Detection Kit, dilution 1:200; incubation time: 16 min) and CD34 (Ventana 790-2927, stained with Benchmark XT: OptiView DAB IHC Detection Kit, dilution: ready to use; incubation time: 12 min). Subsequently, sections were incubated with peroxidase-labeled secondary antibody (DakoCytomation) for 5 min at room temperature. To reveal antigen-antibody reaction, sections were immersed in 3-amino-9-ethylcarbazole plus substrate-chromogen (DakoCytomation) for 30 min and were counterstained with a diluted solution of Gill's hematoxylin (Richard-Allan, Kalamazoo, MI, USA). Heart muscle was used as positive tissue control. Negative control samples were obtained by omitting the primary antibody.

Evaluation of immunohistochemistry

Immunohistochemical staining for nestin and CD34 was assessed by two experienced gastrointestinal pathologists (L.T. and L.M. T.), and interobserver variability was taken into account. Investigators were blinded to clinical and histopathological details. Nestin and CD34 positive cells were counted for each TMA punch (approximately one high power [20x] field). Results were then further evaluated using the semi-quantitative method of histoscores [27–28], obtained by multiplying percentages of positive cells by staining intensities (0 = negative, 1 = weak, 2 = moderate, 3 = strong) on tissue microarray (TMA) tumor punches.

The classification of expression of nestin and CD34 into low or high occurred after determining cutoff scores and threshold values. According to the results of the regression tree analysis, the latter evaluated the sensitivity and false-positive rates for the discrimination of survivors and non-survivors on all tumor samples [27]. Subsequently, cutoff scores of the histoscore values were defined at 198/TMA punch to assess nestin positivity and at 109/TMA punch for considering a specimen as positive for CD34 expression.

Consequently, the associations with clinicopathological features and survival data were analyzed in four groups of nestin/CD34 combined expression (nestin^{high}/CD34^{high}, nestin^{high}/CD34^{low}, nestin^{low}/CD34^{high} and nestin^{low}/CD34^{low}).

Clinicopathological features

In the present dataset, only patients with stage I–III were included in the analysis as this study aimed to assess the impact of the expression of nestin and CD34 in patients with a curative intent treatment. Therefore, patients with unresectable/metastatic disease were a priori excluded from this study. Clinicopathological data were identified from a prospectively collected database and were retrospectively analyzed in a non-stratified and non-matched manner. Annotation included patient's age, gender, tumor diameter, location, pT/pN stage, histologic tumor grading (well differentiated-G1, moderately differentiated-G2, poorly differentiated-G3), histologic subtype, vascular invasion, border configuration, presence of peritumoral lymphocytic (PTL) infiltration at the invasive tumor front and overall survival. Tumor border configuration and PTL were evaluated according to the method introduced by Konoplev and colleagues [29] by using the original hematoxylin and eosin (H&E) slides of the resection specimens corresponding to each tissue microarray punch. MMR status was assessed by immunohistochemical analysis, testing hMLH1, hMSH2, hMSH6 and PMS2 protein. In patients with microsatellite instability Lynch syndrome was ruled out by genetic analysis and counseling.

Outcome of the study

The primary endpoint of the study was overall survival, which was defined as the time from surgery until the occurrence of death.

Quantitative real-time reverse transcription PCR

It was hypothesized that better survival observed in cases with high nestin and CD34 expression might be attributed to mechanisms related to hypoxia or immune response activation. Therefore, the expression of genes encoding *HIF-1α* (hypoxia-inducible factor 1-alpha) and immune markers that have been found to correlate with a better prognosis in CRC and their correlations with nestin and *CD34* gene expression were assessed using qPCR (quantitative real-time reverse transcription PCR) in clinical samples and publicly available databases (TCGA).

qPCR was performed on an independent cohort comprised of 39 patients with CRC stage I–III, according to UICC classification. A summary of the clinicopathological characteristics of the cohort is present in Table A of the Supplementary material. The primers' list for PCR performance is available in Table B of the Supplementary material.

Total RNA was extracted from CRC fresh frozen tissue using the Nucleospin RNA kit (Macherey-Nagel) and reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Quantitative Real-Time PCR (qRT-PCR) was performed in the ABI prism^{TM} 7700 sequence detection system, using TaqMan Universal Master Mix, No AmpErase UNG (from Applied Biosystems) and commercially available primer sequences and quantified using the comparative ΔΔCt 26. All reactions were performed in triplicate. Statistical comparisons were performed using a Mann–Whitney U test (GraphPad Prism 6).

Public database analysis

Correlations of nestin and CD34 with the HIF-1a and the panel of cytokines, chemokines, and immune markers (Figures S1, S2 and S3, Supplementary material) were also assessed using the RNA-sequencing from The Cancer Genome Atlas [30] (colorectal) dataset. Briefly, the Human Protein Atlas database[https://www.proteinatlas.org/ENSG00000132688NES/pathology/tissue/colorectal+cancer] provided colorectal cancer clinical information available for 597 patients. Gene Expression levels (FPKM values) for the genes were downloaded using the TCGAbiolinks R package. After downloading the entire dataset, we excluded the Stage IV CRC and performed the correlation analysis on the remaining 512 samples.

Statistical analysis

Statistical analysis was performed by using the STATA software, version 13.0 (StataCorp, College Station, TX, USA). Descriptive statistics included mean ± standard deviation for continuous variables. Normality of distribution was assessed with histogram and Kolgomorov–Smirnov test. The use of $\chi 2$ test assessed correlations between combined expression of nestin/CD34 and categorical parameters. The assessment of significance was controlled with Chi-Square or Fisher's Exact.

Overall survival curves were estimated with the Kaplan–Meier estimator and were compared with the log-rank test. Patients were followed from surgery and then regularly at the outpatient's clinic or from their family doctor, dependent on oncologic outcomes. For the assessment of overall survival, the last follow-up time after surgery was entered in the analysis. Multivariable Cox regression analysis investigated the effect on survival of clinicopathological features related to the combined expression of nestin and CD34. Hazard ratios (HR) and 95% confidence intervals (CI) were used to determine the prognostic effects on survival time.

Correlations between immune cell markers were evaluated using Spearman correlation assays after statistical

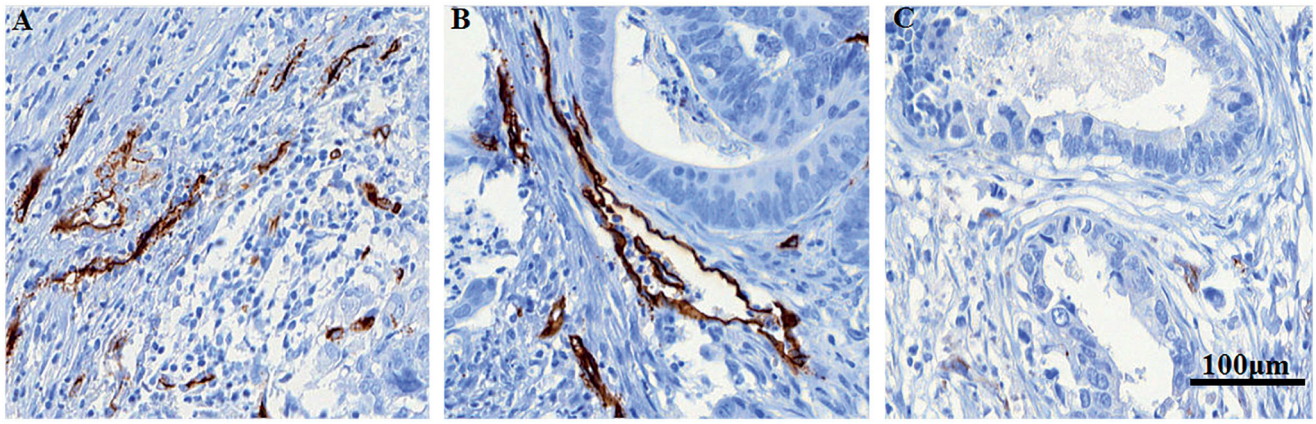

Figure 1. Immunohistochemistry; Representative images of the expression of A.) nestin, B.) CD34 and C.) negative control.

Table 1. Characteristics of CRC patient cohort ($n = 599$)[a].

Characteristics	*N* or Mean, SD	(% or Range)
Age, years mean, (SD)	69.3, (10.9)	36-95
Missing values	0	0
Tumor diameter in mm (mean, SD)	51.3, (20.6)	4-170
Missing values	12	2%
Sex		
Female (%)	331	55.3%
Male (%)	268	44.7%
Missing values	0	0
Anatomic site of the tumor		
Left-sided (%)	398	66.7%
Right-sided (%)	199	33.3%
Rectal cancers (%)	215	36.0%
Rectosigmoid cancers (%)	39	6.5%
Missing values	2	0.3%
T stage		
T1 (%)	29	4.9%
T2 (%)	82	14%
T3 (%)	389	66.3%
T4 (%)	87	14.8%
Missing values	12	2%
N stage		
N0 (%)	322	55.1%
N1 (%)	147	25.1%
N2 (%)	116	19.8%
Missing values	14	2.3%
Tumor grade		
G1 (%)	15	2.6%
G2 (%)	538	91.8%
G3 (%)	33	5.6%
Missing values	13	2.2%
UICC		
Stage IA (%) T1N0	22	3.8%
Stage IB (%) T2N0	61	10.6%
Stage IIA (%) T3N0	207	36%
Stage IIB-C (%) T4N0	27	4.7%
Stage III (%) >N0	258	44.9%
Missing values	24	4%
Tumor border configuration		
Infiltrative (%)	412	70.4%
Pushing (%)	173	29.6%
Missing values	14	2.3%
Vascular invasion		
No (%)	429	73.1%
Yes (%)	158	26.9%
Missing values	12	2%
Microsatellite stability		
high (%)	527	88%
low (%)	72	12%
Missing values	0	0
Overall survival time (median in months)	57	0–151
5-years survival	282	47%

[a]Percentage may not add to 100% due to missing values of same variables. G: histologic tumor grading (well differentiated-G1, moderately differentiated-G2, poorly differentiated-G3).

analysis with the GraphPad Prism 5 software (GraphPad Software). *P* values were further adjusted according to the Benjamini and Hochberg multiple testing correction.

Results

Nestin and CD34 expression in cells within the tumor microenvironment

Nestin and CD34 expression were present in the cytoplasm of endothelial cells of tumor blood vessels and tumor stroma cells'cytoplasm. On the contrary, neither nestin nor CD34 expression was present in CRC cells (Figure 1). After performing qPCR, a strong correlation ($r = 0.68$, $p < .001$, Table S1) was demonstrated between nestin and CD34 gene expression, which was, however not confirmed from the histoscore ($r = 0.34$; $p < .001$).

Correlation of clinicopathological characteristics with the expression of nestin/CD34

Descriptive characteristics of the study population are summarized in Table 1.

Four groups were comparatively analyzed (nestin^{high}/CD34^{high}, nestin^{high}/CD34^{low}, nestin^{low}/CD34^{high} and nestin^{low}/CD34^{low}, Table 2) to assess the correlation eventually occurring between expression of nestin, CD34 and clinicopathological characteristics. No significant correlations were observed between nestin/CD34 expression and age, gender, mucinous histological subtype, tumor location, regional lymph node metastasis, tumor grade, and tumor border configuration. CRC specimens classified as T3 and T4 tumors presented a significantly higher frequency when the tissue specimens displayed a low expression of nestin and CD34 (75.9% vs. 89.2%, $p = .021$). Finally, vascular invasion was significantly lower when the specimens exhibited a high nestin and CD34 expression (18.5% vs. 43.2%, $p < .001$).

Prognostic significance of nestin and CD34 co-expression in the tumor microenvironment of CRC

CRC patients exhibiting a high expression of nestin and CD34 were characterized by a significantly increased 5-year overall survival (Figure 2) as compared with tumors with low

Table 2. Association of nestin and CD34 positive low and high density cell infiltration with clinicopathological features in CRC ($n = 599$).

	Nestin^{high}/CD34^{high}	Nestin^{high}/CD34^{low}	Nestin^{low}/CD34^{high}	Nestin^{low}/CD34^{low}	*p*-Value
Age					
Mean ±(SD)	69.6 ± (11.2)	69.6 ± (11.4)	69.3 ± (11.0)	68.9 ± (10.7)	.858
Tumor diameter (mm)					
mean ±(SD)	48.3 ± (22.5)	49.6 ± (15.3)	51.7 ± (19.9)	52.8 ± (21.0)	.107
Gender					
female	57 (51.3%)	9 (64.3%)	185 (55.9%)	80 (55.9%)	.753
male	54 (48.7%)	5 (35.7%)	146 (44.1%)	63 (44.1%)	
Tumor location					
left-sided	78 (70.9%)	11 (78.6%)	217 (65.8%)	92 (64.3%)	.556
Right-sided	32 (29.1%)	3 (21.4%)	113 (34.2%)	51 (35.7%)	
Mucinous subtype					
Yes	3 (2.7%)	0 (0.0%)	23 (6.9%)	13 (9.1%)	.175
No	108 (97.3%)	14 (100%)	308 (93.1%)	130 (90.9%)	
pT stage					
T1-2	26 (24.1%)	3 (25.0%)	67 (20.4%)	15 (10.8%)	.021
T3-4	82 (75.9%)	9 (75.0%)	261 (79.6%)	124 (89.2%)	
pN stage					
N0	69 (64.5%)	7 (53.8%)	178 (54.9%)	68 (48.2%)	.084
N1-2	38 (35.5%)	6 (46.2%)	146 (45.1%)	73 (51.8%)	
Tumor grade					
G1	5 (4.6%)	1 (8.3%)	5 (1.5%)	4 (2.9%)	.060
G2	99 (91.7%)	10 (83.4%)	308 (93.9%)	121 (87.7%)	
G3	4 (3.7%)	1 (8.3%)	15 (4.6%)	13 (9.4%)	
Vascular invasion					
absent	88 (81.5%)	9 (75.0%)	253 (77.1%)	79 (56.8%)	<.001
present	20 (18.5%)	3 (25.0%)	75 (22.9%)	60 (43.2%)	
Tumor border					
pushing	37 (34.3%)	3 (25.0%)	97 (29.7%)	36 (26.1%)	.573
infiltrative	71 (65.7%)	9 (75.0%)	230 (70.3%)	102 (73.9%)	
PTL infiltration					
absent	83 (76.9%)	10 (83.3%)	246 (75.0%)	115 (82.7%)	.320
present	25 (23.1%)	2 (16.7%)	82 (25.0%)	24 (17.3%)	
Microsatellite stability					
low	9 (8.1%)	2 (14.3%)	36 (10.9%)	25 (17.5%)	.099
high	102 (91.9%)	12 (85.7%)	295 (89.1%)	118 (82.5%	
5-year survival rate (95% CI)	0.65 (0.55–0.73)	0.50 (0.23–0.72)	0.55 (0.50–0.60)	0.45 (0.37–0.53)	.020

Variables are indicated as absolute numbers.

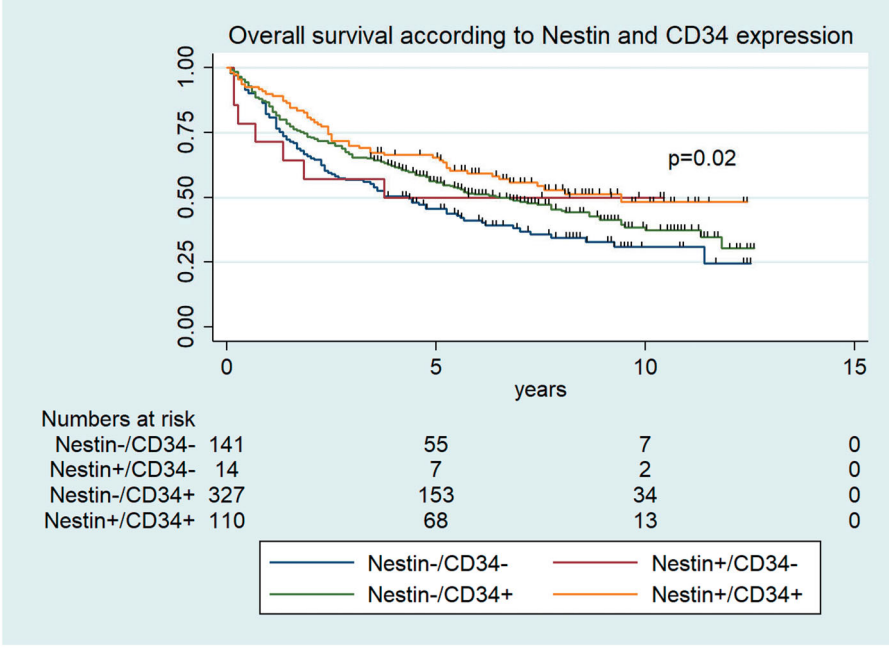

Figure 2. Kaplan Meier curve depicting overall survival in years in the four examined groups according to the levels of nestin and CD34 expression. *P*-value refers to the level of significance of the different overall survival when the four groups of nestin/CD34 expression are compared. The vertical ticks represent censoring.

Table 3. Uni- and multivariable Hazard Cox regression survival analysis according to nestin and CD34 expression patterns.

	Univariate			Multivariable		
	HR	95% CI		HR	95% CI	
Age (years)	1.03	1.02	1.03	1.03	1.02	1.05
Gender (male vs female)	1.38	1.14	1.67	1.64	1.30	2.06
pT (T3 + T4 vs T1 + T2)	3.05	2.20	4.23	2.18	1.45	3.27
pN (N + vs N0)	3.19	2.61	3.90	2.52	1.97	3.21
Grade (high vs low)	5.78	1.86	17.99	2.73	0.66	11.30
Vascular invasion (present vs absent)	2.50	2.05	3.05	1.77	1.38	2.27
Invasive margin (infiltrative vs pushing)	2.03	1.61	2.57	1.25	0.94	1.67
MMR protein status (proficient vs deficient)	1.50	1.10	2.06	1.47	1.00	2.15
$CD34^{low}nestin^{low}$ vs						
$CD34^{low}nestin^{high}$	0.75	0.35	1.61	0.90	0.36	2.24
$CD34^{high}nestin^{low}$	0.76	0.59	0.98	0.88	0.67	1.14
$CD34^{high}nestin^{high}$	0.59	0.42	0.83	0.67	0.46	0.96

Multivariable analyses showing Hazard Ratios and *p*-value for all CRCs ($n = 565$ less than 599, due to missing values) conferred by CD34 and nestin density, age, sex, tumor size, lymph node involvement, tumor grade, vascular invasion, tumor border configuration and microsatellite stability.

expression of both markers (65%; 95% CI = 55–73 and 45%; 95% CI = 37–53, respectively) as illustrated in Table 2.

Multivariable analysis using Cox proportional hazards model confirmed improved survival independent of age, gender, TN stage, tumor grade, vascular invasion, type of invasive margin, and microsatellite instability when a high co-expression of both markers was present ($p = .031$, Table 3).

Nestin, CD34, and HIF-1a gene expression

By qPCR analysis in 39 clinical specimens of stage I–III, a moderate to strong correlation ($r = 0.37$–0.78, $p < .05$, Table S1) with nestin and *CD34* gene expression was observed for the genes encoding *HIF-1α*, *CD4*, *CD8*, *FOXP3*, *IRF1*, *GATA3*, *CCL2*, *CCL3*, *CXCL12* and *CCL21* (Table S1, Supplementary material). Interestingly, *HIF-1α* presented moderate to strong correlations for the same genes ($r = 0.35$–0.69, $p < .05$, Table S1). TCGA analysis comprised of a total of 512 patients with stage I–III CRC revealed a moderate correlation of nestin only with *CXCL12* ($r = 0.39$, $p < .05$, Figure S1, Supplementary material). On the other hand, CD34 gene expression presented moderate to strong correlations ($r = 0.40$–0.74, $p < .0001$) with the expression of HIF1α, *CXCL12*, *FOXP3*, *CD4*, *FCGR3A*, *CCL2*, *CCL21*, *CCL18*, *CCL11* genes (Figures S2 and S3, Supplementary material).

In summary, HIF-1α, nestin, and CD34 gene expression demonstrated a moderate to strong correlation for immune markers that participate in mechanisms associated with improved survival in CRC. These data suggest that hypoxia might be related to a favorable immune response that is probably mediated by nestin and CD34 positive stroma cells.

Discussion

In this large cohort study, we addressed the prognostic significance of the co-expression of nestin and CD34 within CRC stroma. We found that high co-expression of nestin and CD34 is associated with a significantly improved 5-year overall survival irrespectively of age, gender, T and N classification, tumor grade, type of invasive margins, and microsatellite instability.

Previous studies have suggested that cells expressing nestin and CD34 harbor an enhanced progenitor activity [8,15]. In particular, nestin expression has been observed in prostate cancer-initiating cell populations [14], breast cancer stem-like cells [11], glioma [10], lung cancer [31], and hepatocellular carcinoma [12] and has been associated with aggressive clinical profiles. The studies mentioned above from non-CRC patients demonstrated nestin and CD34 expression in cancer cells and not in the tumor microenvironment.

In healthy colorectal tissue, it has been found that CD34 expression occurs in the interstitial cells of Cajal [23]. These cells are of mesenchymal origin, and a subset of them might be functionally related to mast cells [32]. Expression of CD34 has also been observed in fibrocytes recruited to the site of trauma injury, promoting inflammation and wound healing [15]. CD34 positive stromal cells are known to support the maturation and proliferation of adjacent epithelial and mesenchymal stem cells.

Thus, it is tempting to speculate that the presence of both nestin and CD34 positive stromal cells in CRC might be associated with structural remodeling potentially occurring within tumor tissues.

Interestingly, in the present study, the immune markers that presented a moderate to strong correlation with nestin and *CD34* also correlated with *HIF-1α*. In different tissues, nestin-positive cells may act as remodeling regulators, particularly under hypoxic conditions [8,33] and oxidative stress [34]. The family of HIF's induces the expression of several genes, which are implicated in the epithelial to mesenchymal transition as well as other epigenetic alterations such as methylation/demethylation, which promote alterations within cell-cell contact and reduce adhesion to the extra-cellular matrix [35–37]. In addition, cancer cells and cells of the tumor microenvironment, such as fibroblasts, are influenced by hypoxia [38]. Besides, CD34+ hematopoietic progenitors under ischemia-like conditions demonstrate similar metabolic activity to cancer stem cells. However, this feature was not associated with malignant transformation but was an attribute of 'stemness' [34]. As hypoxia is one of the hallmarks in cancer [39] those data suggest a potentially 'protective role' of nestin and CD34 positive cells of the tumor microenvironment *via* a structural remodeling.

Notably, however, improved overall survival associated with the co-expression of nestin and CD34 might also be attributed to immune-mediated effects. Several studies suggest that nestin plays a significant role as a critical modulator in the immune system. Recently it has been observed that in chronic inflammation, nestin-positive stromal cells are involved in re-directing inflammatory cells of different tissues in cooperation with endothelial cells [40]. However, underlying molecular mechanisms are still unclear [41]. Moreover, CD34 is expressed in progenitor cell types and differentiated leukocytes, including mast cells [42], dendritic cell precursors, and eosinophils [43]. Importantly, *CD34* is the ligand for *CD62L* selectin, highly expressed by central memory lymphocytes [44].

In the present study, nestin and CD34 presented a moderate to strong correlation for *CD8*, *FOXP3*, CD4, GATA3, *CCL2,*

CXCL12 and *IRF1*. In CRC, several studies have shown a significant correlation between T-cell infiltration and improved survival [45]. Interestingly, a recent meta-analysis of studies on solid tumors investigating the clinical significance of tumor-infiltrating lymphocytes (TIL) elucidated that the presence of CD3, CD8, and a high CD8/FoxP3 ratio significantly improved survival [46]. CD4+ T cells' role in tumors has been controversial as they can convert from anti-tumor to pro-tumor cells [47–48]. However, recent data suggest that they can exert anticancer actions by enhancing CD8+ T cells' activity or less often by directly recognizing antigens of the cancer cells'surface with subsequent secretion of type 1 cytokines [49] or direct cancer cell destruction [50]. Besides, increasing evidence underlines the crucial role of GATA3 for the development, differentiation, and function of CD4+ T cell and CD8+ T cells [51]. CCL2 and CXCL12 are highly effective chemotactic factors, recruiting monocytes and lymphocytes, respectively [52–53]. Finally, IRF-1 is a nuclear transcription factor with a critical role in inflammation, immunity, cell proliferation, and apoptosis. It is produced in response to IFN-γ (interferon-γ) [54] and has been demonstrated to help suppressing CRC cell growth and metastasis by providing *IRF1/miR-29b* feedback loop [55].

Limitations of our report should be acknowledged. In particular, our data's prognostic significance might be questioned due to the studýs retrospective nature. However, this has allowed us to take advantage of an extensive database with exhaustive clinical annotation. The use of TMA technology also represents a limitation of our work since it might lead to underestimation of intratumor heterogeneity. However, this limitation might be, at least in part, compensated by the large number of cases accessible to analysis in TMA. Another limitation regarding the qPCR gene expression analysis is the relatively low number ($n = 39$) of patients investigated. More experimental '*in-vitro*' and '*in-vivo*' studies are warranted to address these issues. Nevertheless, our findings represent a factual basis for the formulation of sound working hypotheses, capitalizing on reliable clinical data.

The results from our study did not fully match those emerging from TCGA database analysis. Notably, however, TCGA data derive from a highly heterogeneous group of patients with widely different genetic backgrounds, and additionally, TCGA performed the RNA sequencing on the tumor cells mixed with tumor surrounding cells. In contrast, clinical specimens evaluated in our study were processed in a single lab and derived from a mostly homogeneous patient population with standardized technologies.

Disclosure statement

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