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Clinicopathologic and prognostic significance of immunohistochemical expression of HIF-1α, CXCR4 and CA9 in colorectal carcinoma☆

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ABSTRACT

Objective: To investigate the immunohistochemical expressions of HIF-1α, CA9 and CXCR4 in resected human CRC specimens in relation to clinicopathologic and prognostic variables.
Methods: A total of 186 patients (mean(SD) age: 56.7(12.6) years, 54.0% were males) with colorectal adenocarcinoma were included in this retrospective study. Resection specimens of the primary tumor were reviewed to confirm the diagnoses and the stage of the disease. Data on age, gender, tumor characteristics (localization, size, macroscopic growth pattern, histologic type, grade, angiolymphatic invasion, TNM stage), applied treatments and clinical outcome (overall survival, local recurrence and distant metastasis) were obtained from the hospital records. Immunohistochemical analysis of tissue specimens was performed to determine HIF-1α, CA9 and CXCR4 expressions.
Results: Overall, 94.0% of cases showed HIF-1α immunoreactivity, 89% showed CXCR4 immunoreactivity, and 15.6% showed CA9 immunoreactivity, while weak expression of immunohistochemical markers was noted in 51.1%, 93.0% and 50.5% of cases, respectively.

HIF-1α expression was higher among males than in females (median (min-max) final score of 6 (0–9) vs. 3 (0–9), $p = 0.013$). CA9 expressed at higher levels in ulcerovegetative and depressed tumors than in polypoid ones [0(0–9) vs. 0(0–6), $p = 0.039$]. CXCR4 expression was significantly higher in tumors <5 cm than ≥5 cm [6(0–9) vs. 3(0–9), $p = 0.028$] and in grade 1–2 than grade 3 tumors [4(0–9) vs. 3(0–9), $p = 0.030$]. No significant difference was noted in survival with respect to strength of HIF-1α, CA9 and CXCR4 immunoreactivity.
Conclusion: In conclusion, our findings revealed weak-to-moderate HIF-1α and CXCR4 immunoreactivity in majority of resection samples, and weak CA9 immunoreactivity in majority of CA9 positive cases. Other than gender (HIF-1α), macroscopic growth pattern (CA9) and tumor size and histologic grade (for CXCR4), none of the clinicopathologic and prognostic factors investigated were associated with expression of immunohistochemical markers and level of immunoreactivity had no impact on survival.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer in males and the second in females [1,2]. CRC remains the second leading causes of cancer-related mortality worldwide, in relation to high rates of recurrence and metastasis and the fact that almost half of the cases are diagnosed at the advanced stage [3–5].

Hypoxia-signaling pathway is a common hallmark of solid tumors, such as CRC, and results in increased expression of hypoxia-inducible factors (HIFs) within the tumor microenvironment to compensate for insufficient blood supply to the growing tumor [6–9]. Hypoxia-inducible factor 1 α (HIF-1 α) is a heterodimeric transcription factor that shows increased expression in response to hypoxia during tumor growth which leads to upregulation of several genes involved in cell proliferation apoptosis, glucose metabolism, pH regulation and angiogenesis [10–12]. Overexpression of HIF-1α plays an important role in regulating cell survival, metabolic changes, angiogenesis and associated with

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tumor growth, metastasis, resistance to chemotherapy and radiotherapy and thus considered to be a poor prognostic factor several types of cancers including CRC [7,13–18].

Low oxygen concentration is accompanied with a decrease in extracellular pH in microenvironment of hypoxic tumor cells [19,20]. Carbonic anhydrase IX (CA9) is a membrane associated zinc metallo-enzyme which plays a key role in tumor acid-base balance by catalyzing the reversible hydration of carbon dioxide to bicarbonate and a proton [21–24]. Thus, CA9 enables hypoxic tumor cells to survive in the acidic microenvironment [25,26] and shown to be over-expressed in various human tumors, including colorectal carcinomas [22,24,27–29].

CA9 is one of the genes upregulated by HIF-1 α and thus expression of CA9 is directly linked to an increase of HIF-1 [23,26,30,31], while correlates to cell survival, proliferation, migration, growth, adhesion, pH value, and cell-signaling pathways [29–31].

'Signaling/homing' is contemporary metastasis theory including release of chemokines (signals) from target organs (home) and their interaction with tumor cells by chemokine receptors leading to metastasis [32]. The C-X-C chemokine receptor type 4 (CXCR4) is one of the key factors in the cross talk between cancer cells and their microenvironment [35,36]. CXCR4 expression was shown to promote angiogenesis and associated with a poor prognosis in several human tumor types, while CXCR4 expression in primary CRC was associated with recurrence and survival and suggested as a risk factor for the development of colorectal liver metastases [33–37].

HIF-1α and CA-9 are potential intrinsic markers of tumor hypoxia and predictor of an adverse disease prognosis [7,22,24,38]. The fact that HIF-1α is also induced by non-hypoxic stimuli [39] raises the concern about the consideration of HIF-1α as a proper marker of hypoxia [40] with likelihood of proteins under the control of HIF-1α such as CA9 to be more relevant markers of tumor hypoxia [41]. HIF-1α is also one of inducers for CXCR4 transcription [42,43], and a correlation reported between HIF-1α and CXCR4 immunohistochemical expressions [44].

Although HIF-1α, CA9 and CXCR4 expression are potential candidates for targeted therapy in CRC patients, most of the previous studies were based on in vitro techniques or focused on tumorigenesis with only a few studies on the probable clinical relevance of these potential markers, particularly among CRC patients. Given the potential prognostic role and possible interaction of HIF-1α, CA9 and CXCR4 in CRC, the present study was designed to investigate the immunohistochemical expressions of HIF-1α, CA9 and CXCR4 in resected human CRC specimens in relation to clinicopathologic and prognostic variables

2. Materials and methods

2.1. Study population

A total of 186 patients (mean(SD) age: 56.7(12.6) years, 54.0% were males) with colorectal adenocarcinoma treated at Gazi University Medical School Hospital between 1993 and 2010, were included in this retrospective study.

The study was conducted in full accordance with local GCP guideline and current legislations, while the permission was obtained from Medical Ethics Committee of Gazi University School of Medicine for the use of patient data for publication purposes.

2.2. Assessments

Resection specimens of the primary tumor were reviewed by the same pathologist to confirm the diagnoses and the stage of the disease according to the American Joint Committee (AJCC-7) on Colon and Rectum Cancer tumor-node-metastasis (TNM) staging system [45]. Data on age, gender, tumor characteristics (localization, size, macroscopic growth pattern, histologic type, grade, angiolymphatic invasion and TNM stage), applied treatments and clinical outcome (overall survival, local recurrence and distant metastasis) were obtained from the hospital records. Immunohistochemical analysis of tissue specimens was performed to determine HIF-1α, CA9 and CXCR4 expressions.

2.3. Immunohistochemistry

Tissue microarray blocks, which contains tumor of 4 cores in 1 mm diameter for each case, were prepared with Veridiam VTA-100 tissue arrayer. Four μm-thick paraffin sections were cut. Sections were mounted on silanized slides and allowed to dry overnight at 56 °C. After deparaffinization and rehydratation, slides were incubated with 3% hydrogen peroxide solution for 10 min. After washing with distilled water, tissue sections were treated for 20 min with buffer in a microwave oven at high temperature. For HIF-1α citrate (pH 6.0), for CA9 ethylenediamine tetraacetic acid (EDTA) (pH 9.0) and for CXCR4 Tris-EDTA (pH 9.0) were used as buffers. The slides were then incubated with the primary antibody for 60 min at room temperature for CA9 and overnight at 4 °C for HIF-1α and CXCR4. The primary antibodies were diluted including HIF-1α (monoclonal mouse antibody, Santa Cruz) 1:50, CXCR4 (polyclonal goat antibody, ABCAM) 1:50 and CA9 (polyclonal rabbit antibody, Santa Cruz, H-120) at 1:100. The slides were stained using the streptavidin-biotin indirect immunoperoxidase technique. Tissue staining was visualized by DAB substrate chromogen solution (Lab-Vision, Neomarkers, USA). Counterstaining was done using Harris hematoxylin. Finally, the slides were dehydrated, and mounted. Positive controls were torsioned testis tissue, clear cell renal cell carcinoma and hepatocellular carcinoma for HIF-1α, CA9 and CXCR4 respectively (Fig. 1).

2.4. Assessment of HIF-1α, CA9 and CXCR4 staining

Immunohistochemical positivity was defined as nuclear staining for HIF-1α, membranous staining for CA9 and cytoplasmic staining for CXCR4. For each marker, extent and intensity was evaluated and scored separately. The extent of staining was scored as: 0, no cells stained; 1, less than 30% of tumor cells stained; 2, 31%–60% of cells stained; and 3, more than 60% of cells stained. Intensity was also scored as 0-none, 1-minimal, 2-moderate, 3-strong by comparing to the positive controls. These two scores were multiplied to obtain 'final score'. According to the final scores, immunoreactivity was regarded as weak (0–3), moderate (4–6) and strong (7–9).

2.5. Statistical analysis

Statistical analysis was made using IBM SPSS Statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0., Armonk, NY: IBM Corp). Chi-square ($\chi 2$) test for the comparison of categorical data and Mann-Whitney *U* test and Kruskal Wallis test for numerical data. Correlation between immunohistochemical markers were analyzed via Spearman's correlation test. Gender, tumor site and macroscopic growth pattern were tested by variance analysis and signed rank tests. Data were expressed as "mean (standard deviation; SD)", median (minimum-maximum) and percent (%) where appropriate. $p < 0.05$ was considered statistically significant.

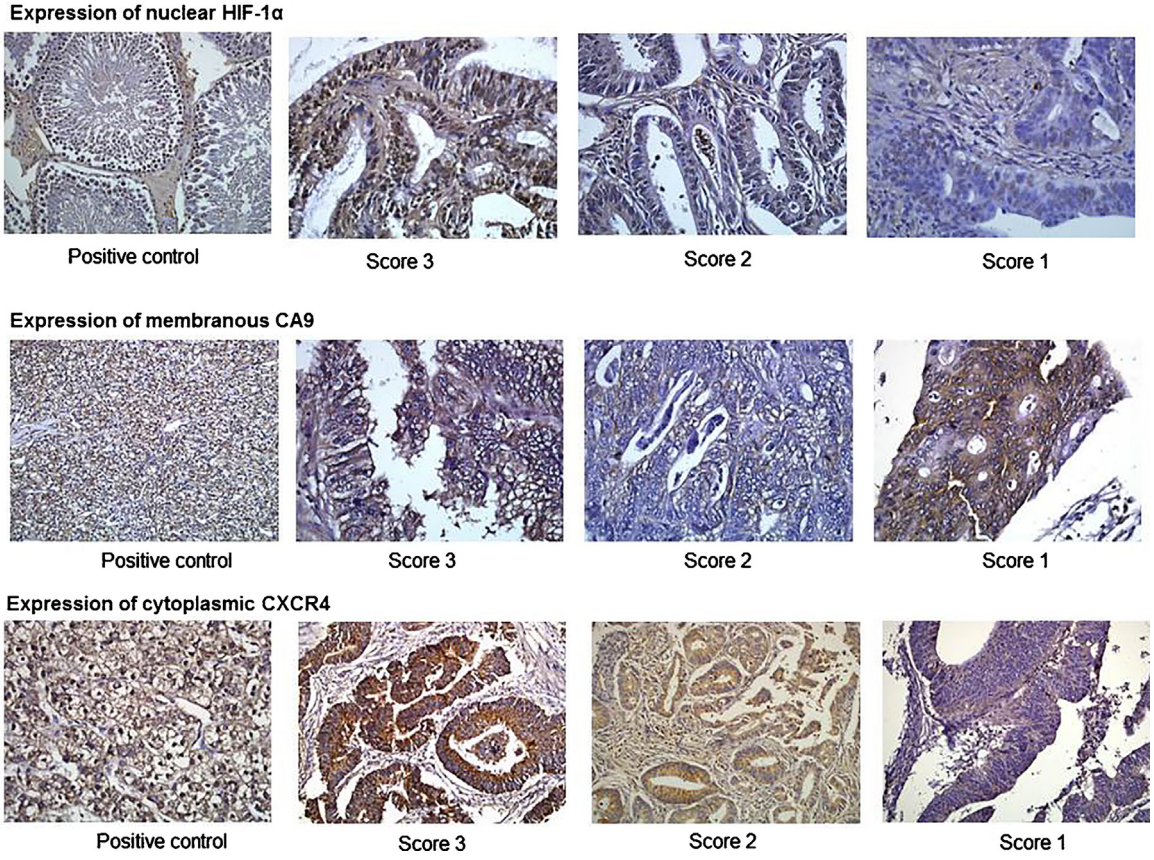

Fig. 1. Immunohistochemistry staining characteristics of colorectal cancer cells with respect to intensity of expression and positive controls of torsioned testis tissue, clear cell renal cell carcinoma and hepatocellular carcinoma for HIF-1α, CA9 and CXCR4, respectively. DABx400.

3. Results

3.1. Patient and tumor characteristics and clinical outcome

Most tumors were located to colon (68.0%), had ulcerovegetative + depressed growth pattern (91.0%) and sized ≥5 cm (53.0%). Conventional adenocarcinoma was the most common type (82.8%), and most tumors were grade1-2 (74.0%), lymph node positive (N1 + N2 in 59.0%), Stage III + IV (67.0%) tumors with angiolymphatic invasion (53.0%), and invasion beyond muscularis (T3 + T4 in 96.0%) (Table 1).

After a median 23.0 months of follow-up, distant metastasis was noted in 27.4% and local recurrence in 7.0% of the patients, while survival rate was 71.0% (Table 1).

All stage III and IV patients received adjuvant chemotherapy. The adjuvant chemotherapy for patients with stage III CRC in our institution was an oxaliplatin-based regimen. In metastatic CRC, patients received irinotecan-based regimen combined with bevacizumab. Three patients had additional neoadjuvant and 22 patients had adjuvant radiotherapy.

3.2. Intensity and patterns of HIF-1α, CA9 and CXCR4 expression

Overall, 94.0% of cases showed HIF-1α immunoreactivity, 89% showed CXCR4 immunoreactivity, and 15.6% showed CA9 immunoreactivity, while weak expression of immunohistochemical markers was noted in 51.1%, 93.0% and 50.5% of cases, respectively (Table 2).

The pattern of HIF-1α expression was mostly nuclear, only a few tumors showed weak cytoplasmic staining in addition to nuclear positivity. CA9 expression was associated with membranous staining in 89.3% of cases with/without accompanying cytoplasmic reaction. CXCR4 was positive only in the cytoplasm (Fig. 1).

3.3. HIF-1α, CA9 and CXCR4 expression with respect to clinicopathologic features

HIF-1α expression was higher (median (min-max) final score of 6 (0–9) vs. 3 (0–9), p = 0.013) among males than in females. No significant difference was noted in final HIF-1α expression scores with respect to clinicopathologic features other than gender (Table 3). No significant difference was noted in strength of HIF-1α immunoreactivity with respect to clinicopathologic parameters (Table 3).

CA9 expressed at higher levels (median (min-max) final score of 0(0–9) vs. 0(0–6), p = 0.039) in ulcerovegetative and depressed tumors than in polypoid ones (median (min-max) final score of 0(0–9) vs. 0(0–6), p = 0.039). No significant difference was noted in final CA9 expression scores with respect to clinicopathologic features other than macroscopic growth pattern (Table 4). No significant difference was noted in strength of CA9 immunoreactivity with respect to clinicopathologic parameters (Table 4).

CXCR4 expression was significantly higher in tumors <5 cm than those ≥5 cm (median (min-max) final score of 6(0–9) vs. 3(0–9), p = 0.028) and also in grade 1–2 than grade 3 tumors (median (min-max) final score of 4(0–9) vs. 3(0–9), p = 0.030). No significant difference was noted in final CXCR4 expression scores with respect to clinicopathological features other than tumor size and histologic grade (Table 5). No significant difference was noted in strength of CXCR4 immunoreactivity with respect to clinicopathologic parameters (Table 5).

Table 1
Clinicopathologic characteristics of CRC.

	n(%)
Age (year), mean(SD)	56.7(12.6)
<50	53(28.0)
≥50	133(72.0)
Gender	
Female	86(46.0)
Male	100(54.0)
Tumor type	
Adenocarcinoma	154(82.0)
Signet ring cell carcinoma	8(4.3)
Mucinous carcinoma	24 (12.9)
Tumor localization	
Rectum and rectosigmoid	59(32.0)
Colon	127(68.0)
Macroscopic growth pattern	
Ulcerovegetative + Depressed	170(91.0)
Polypoid	14(8.0)
Tumor size	
<5 cm	87(47.0)
≥5 cm	99(53.0)
Histologic grade	
Grade 1 and 2	138(74.0)
Grade 3	48(26.0)
Angiolymphatic invasion	
Negative	87(47.0)
Positive	99(53.0)
Invasion depth	
T1 + T2	8(4.0)
T3 + T4	178(96.0)
Lymph node status	
N0	77(41.0)
N1 + N2	109(59.0)
Metastasis status at the time of diagnosis	
M0	135(73.0)
M1	51(27.0)
Stage	
Stage I + II	62(33.0)
Stage III + IV	124(67.0)
Distant metastasis at the time of diagnosis or follow up	
No metastasis	90(48.0)
Liver metastasis[a]	59(32.0)
Non-liver metastasis	37(20.0)
Distant metastasis at follow up	51(27.4)
Local recurrence	
No	173(93.0)
Yes	13(7.0)
Follow up duration (months), median (min-max)	23.0(2–109)
Survival outcome	
Survived	132(71.0)
Died	54(29.0)
[a]	

[a] Isolated or with other distant organ metastasis.

Table 2
Expression of immunohistochemical markers in the overall study population (n = 186).

	Positive n(%)	Strength of immunoreactivity, n(%)		
		weak	moderate	strong
Immunohistochemical markers				
HIF-1α expression	175(94.0)	95(51.1)	73(39.2)	18(9.7)
CA9 expression	165(89.0)	173(93.0)	10(5.4)	3(1.6)
CXCR4 expression	29(15.6)	94(50.5)	60(32.3)	32(17.2)

3.4. HIF-1α, CA9 and CXCR4 expression and survival

Kaplan Meier overall survival curves revealed no significant difference in survival with respect to strength of HIF-1α (Fig. 2A), CA9 (Fig. 2B) and CXCR4 (Fig. 2C) immunoreactivity based on final scores.

3.5. Correlation between HIF-1α, CXCR4, and CA9 expressions

No significant correlation was noted between HIF-1α, CA9 and CXCR4 expression in terms of extent, intensity and final score (Table 6).

4. Discussion

Our findings in a retrospective cohort of CRC patients revealed immunoreactivity for HIF-1α, CA9 and CXCR4 in 94.0%, 15.0% and 89.0% of tumor specimens and weak expression in 51.1%, 93.0% and 50.5% of cases with positive immunoreactivity, respectively. Increased expression of HIF-1α among males, increased expression of CA9 in tumors with ulcerovegetative growth pattern and increased expression of CXCR4 in smaller size and lower histological grade tumors were noted. None of the immunohistochemical markers showed an association with other clinicopathologic factors as well as with prognostic factors, and overall survival rates were also similar with respect to level of HIF-1α, CA9 and CXCR4 immunoreactivity.

HIF-1α overexpression was reported to be evident in nearly 55% of CRC patients [46], associated with poor prognosis resistance to chemotherapy and increased mortality [14,17,47,48] in patients with CRC. HIF-1α overexpression was also shown to be an independent determinant of increased disease recurrence in patients with colorectal liver metastases (CRLM) [49].

A large study of 731 CRC specimens demonstrated that HIF-1α overexpression was independently associated with poor prognosis [14], while data from a meta-analysis of 23 studies comprising 2984 CRC patients also revealed a significant association of HIF overexpression with increased mortality risk including overall survival and disease free survival (DFS) [50].

Although 94.0% of cases showed HIF-1α immunoreactivity in our cohort, weak-to-moderate nuclear expression occurred in majority of cases, despite strong nuclear staining has characteristically been reported for HIF-1α expression in CRC cases [51]. This difference may be related to our method of sampling, since microarray cores were selected from tumor areas away from necrosis in our study.

Notably, no association of the presence of HIF-1α immunoreactivity was shown with clinicopathologic and prognostics factors, and the level of HIF-1α immunoreactivity had no impact on patient survival in our cohort.

Similarly, in a past study from Turkey conducted among patients with metastatic CRC receiving chemotherapy, overexpression of HIF-1α was reported in 55% in tissue samples with no significant impact of HIF-1α expression rate on survival [38]. Authors also noted that HIF-1α expression was similar in the primary tumors of the patients with vs. without metastasis or in patients with single vs. multiple metastases at the time of diagnosis [38].

Preoperative HIF-1α expression assessed via IHC was shown to have a negative predictive and prognostic value in terms of pathologic response and response to chemotherapy in clinical stage II/III rectal cancer patients treated with neoadjuvant chemoradiotherapy [52–54]. However other studies using reverse transcription-polymerase chain reaction to measure HIF-1α expression revealed inconsistent results questioning the role of HIF-1α as a predictive marker in the preoperative treatment

Table 3
HIF-1α expression with respect to clinicopathologic features.

	Immunoreactivity					
	Final score		Strength, n(%)			
	median (min-max)	p value[a]	weak	moderate	strong	p value[b]
Age						
<50	3 (0–9)	0.197	30(56.6)	20(37.7)	3(5.6)	0.423
≥50	4 (0–9)		65(48.8)	53(39.8)	15(11.2)	
Sex						
Female	3 (0–9)	0.013	52(60.4)	28(32.5)	6(6.9)	0.055
Male	6 (0–9)		43(43.0)	45(45.0)	12(12.0)	
Tumor localization						
Rectum and rectosigmoid	4 (0–9)	0.482	29(49.1)	25(42.3)	5(8.4)	0.816
Colon	3 (0–9)		66(51.9)	48(37.8)	13(77.1)	
Macroscopic growth pattern						
Ulcerovegetative + Depressed	3 (0–9)	0.501	88(51.7)	69(40.5)	13(7.6)	0.192
Polypoid	4.5 (1–9)		7(50.0)	4(28.5)	3(21.4)	
Tumor Size						
<5 cm	6 (0–9)	0.081	40(45.9)	40(45.9)	7(8.0)	0.275
≥5 cm	3 (0–9)		55(55.5)	33(33.3)	11(11.1)	
Histologic grade						
Grade 1 and 2	4 (0–9)	0.194	68(49.2)	56(40.5)	14(10.1)	0.704
Grade 3	3 (0–9)		27(56.2)	17(35.4)	4(8.3)	
Angiolymphatic invasion						
Negative	3 (0–9)	0.836	44(50.5)	29(33.3)	12(13.8)	0.070
Positive	3 (0–9)		50(50.5)	44(44.4)	5(5.0)	
Invasion Depth						
T1 + T2	3 (3–6)	0.841	6(75)	2(25)	0(0.0)	0.143
T3 + T4	3.5 (0–9)		89(0.5)	71(39.8)	18(10.1)	
Lymph node status						
N0	3 (0–9)	0.755	39(50.6)	29(37.6)	9(11.6)	0.763
N1 + N2	3 (0–9)		56(51.3)	44 (40.3)	9(8.2)	
Metastasis status at the time of diagnosis						
M0	3 (0–9)	0.228	71(52.5)	54(40)	10(7.4)	0.289
M1	6 (0–6)		24(47.0)	19(37.2)	8(15.7)	
Stage						
Stage I + II	3 (0–9)	0.803	33(53.2)	22(35.4)	7(11.3)	0.715
Stage III + IV	3.5 (0–9)		62(50.0)	51(41.1)	11(8.8)	
Distant metastasis at diagnosis or follow up						
No metastasis	4 (0–9)	0.814	43(47.7)	40(44.4)	7(7.7)	0.573
Liver metastasis[c]	3 (0–9)		30(50.8)	22(37.2)	7(11.9)	
Non-liver metastasis	3 (0–9)		22(59.4)	11(29.7)	4(10.8)	
Local recurrence						
No	3 (0–9)	0.633	88(50.8)	68(39.3)	17(9.8)	0.961
Yes	3 (0–9)		7(53.8)	5(38.4)	1(7.7)	

[a] Mann-Whitey *U* test.
[b] χ^2 test.
[c] isolated or with other distant organ metastasis.

[55,56]. Accordingly, besides different tumor down-staging and tumor regression systems, use of different assessment method has also been suggested to be responsible for the discrepancy between studies in terms of utility of HIF-1α as a predictive and prognostic marker in CRC [54]. Genetic variations in HIF-1α gene has also been suggested to modulate the efficacy of postoperative adjuvant chemotherapy and thus to affect the predictive role of HIF-1α expression of clinical outcomes in CRC patients [54–56].

Increased HIF-1α expression was noted in CRC samples obtained from male than female patients in our cohort. No such gender difference was reported in past studies regarding HIF-1α expression in CRC samples. Nonetheless, gender influence on sensitivity to oxygen deprivation and thus HIF1-dependent cardiac adaptive responses to hypoxia was reported in the literature, with smaller changes in gene expression under hypoxia among females [57].

CA9 is suggested to have a potential role as a diagnostic biomarker, prognostic indicator as well as a tumor therapeutic target in several cancer types [58,59]. Being overexpressed in hypoxic CRC tumor as compared with normoxic condition, CA9 is considered an important biomarker for hypoxic CRC tumor diagnosis [23]. Indeed, a correlation of serum CA9 levels with tumor tissue CA9 levels was reported in clinical CRC patients indicating not only tumor CA9 but also serum CA9 to be considered as a biomarker for hypoxic tumor diagnosis [23].

CA9 expression was shown to occur particularly in areas of high proliferation in colorectal tumors [60], at higher level in late than early stage tumors [23], and with more diffuse staining compared with benign lesions [29].

Broad expression of CA9 was shown in adenoma and T1 colorectal cancer specimens based on immunofluorescent and immunohistochemical staining, flow cytometry, and quantitative real-time-polymerase chain reaction in a past study, indicating the strong likelihood of CA9 to be involved in the carcinogenesis of CRC [61].

Although expression of CA9 is regulated by and directly linked to an increase of HIF-1 [23,30,31], CA9 immunoreactivity was evident only in 15.6% of resection samples with weak immunoreactivity in majority of positive cases in our cohort, despite identification of HIF-1α positivity in 94% of cases. Besides, no correlation was evident between HIF-1α and CA9 immunoreactivity in our cohort.

In a past study on the impact of sampling method of colon carcinoma tissue samples (colon biopsy vs. surgical resection) on the expression levels of potential cancer biomarker genes, significantly increased expression of hypoxia markers GLUT-1 and CA9 was

Table 4
CA9 expression with respect to clinicopathologic features.

	Immunoreactivity					
	Final score		Strength, n(%)			
	median (min-max)	p value[a]	weak	moderate	strong	p value[b]
Age						
<50	0 (0–9)	0.295	46(86.7)	5(9.4)	2(3.7)	0.121
≥50	0 (0–9)		127(95.4)	5(3.7)	1(0.7)	
Sex						
Female	0 (0–6)	0.697	83(96.5)	3(3.4)	0(0.0)	0.081
Male	0 (0–9)		90(90)	7(7.0)	3(3.0)	
Tumor localization						
Rectum and rectosigmoid	0 (0–9)	0.416	54(91.5)	4(6.7)	1(1.7)	0.849
Colon	0 (0–9)		119(94.0)	6(4.7)	2(1.6)	
Macroscopic growth pattern						
Ulcerovegetative + Depressed	0 (0–9)	0.039	159(93.5)	8(4.7)	3(1.7)	0.346
Polypoid	0 (0–6)		12(85.7)	2(14.2)	(0.0)	
Tumor Size						
<5 cm	0 (0–9)	0.436	81(93.1)	5(5.7)	1(1.1)	0.878
≥5 cm	0 (0–9)		92(92.9)	5(5)	2(2.0)	
Histologic grade						
Grade 1 and 2	0 (0–9)	0.844	128(92.7)	8(5.7)	2(1.4)	0.872
Grade 3	0 (0–9)		45(93.7)	2(4.2)	1(2.0)	
Angiolymphatic invasion						
Negative	0 (0–9)	0.880	80(91.9)	3(3.4)	2(2.3)	0.443
Positive	0 (0–9)		91(91.9)	7(7.0)	1(1.0)	
Invasion Depth						
T1 + T2	0 (0-0)	0.217	8(100)	0(0.0)	0(0.0)	0.429
T3 + T4	0 (0–9)		165(92.7)	10(5.6)	3(1.7)	
Lymph node status						
N0	0 (0–9)	0.937	71(92.2)	4(5.2)	2(2.6)	0.700
N1 + N2	0 (0–9)		102(93.8)	6(5.5)	1(0.9)	
Metastasis status at the time of diagnosis						
M0	0 (0–9)	0.170	126(93.3)	7(5.2)	2(1.5)	0.777
M1	0 (0–9)		47(92.2)	3(5.9)	1(1.9)	
Stage						
Stage I + II	0 (0–9)	0.796	58(93.5)	2(3.2)	2(3.2)	0.323
Stage III + IV	0 (0–9)		115(92.7)	8(6.5)	1(0.8)	
Distant metastasis at diagnosis or follow up						
No metastasis	0 (0–6)	0.094	87(96.6)	3(3.3)	0(0.0)	0.20
Liver metastasis[c]	0 (0–9)		52(88.1)	5(8.5)	2(3.4)	
Non-liver metastasis	0 (0–9)		34(91.9)	2(5.4)	1(2.7)	
Local recurrence						
No	0 (0–9)	0.990	161(93.0)	10(5.8)	2(1.2)	0.198
Yes	0 (0–9)		12 (92.3)	0(0.0)	1(7.7)	

[a] Mann-Whitey *U* test.
[b] χ^2 test.
[c] isolated or with other distant organ metastasis.

shown in resection samples [62]. Authors concluded this increase to be as a consequence of induction of hypoxic stress signal by the clamping of part of the colon during surgical resection [62]. This seems notable given that CA9 specific antibodies could not discriminate cells that currently or previously exist in hypoxic conditions since transmembrane CA9 proteins remain stable for a relatively long time after re-oxygenation with a half-life of almost 40 h [23].

In a past study in rectal cancer tumor samples from 166 patients, CA9 staining intensity was shown to differ significantly with respect to treatment categories and response to therapy [29]. Tumors treated with long-course RT without chemotherapy were shown to be CA9 positive with moderate/strong staining intensity, while tumors treated with chemoradiotherapy were reported to be mostly CA9 negative [29]. Only 3 patients in our cohort had neoadjuvant radiotherapy, while none had systemic neoadjuvant chemotherapy. Therefore, low/weak CA9 immunoreactivity in our CRC samples seems not to be associated with chemoradiotherapy related alteration in tumor oxygenation and final immunoreactivity outcome [29,63]. Also, lack of preoperative radiotherapy in majority of our patients excludes the likelihood of radiotherapy related alteration in tumor size, stage and grade, nodal status and thus HIF-1α, CA9 and CXCR4 staining in the resection samples [29].

Nonetheless, higher final scores were noted for CA9 expression in ulcerovegetative-depressed than in polypoid macroscopic growth pattern in our cohort. This seems notable, given the higher rate of submucosal massive invasion and lymph node metastasis and a higher degree dysplasia reported in non-polypoid growth (NPG) than polypoid growth (PG) submucosal CRC [64,65]. CA9 expression was known to correlate to cell survival, proliferation, migration, growth and adhesion [30,31]. Accordingly, higher expression of CA9 in NPG than PG growth tumor specimens in our cohort seems consistent with consideration of NPG to be a more aggressive CRC growth pattern than PG [64].

In a past analysis of tumor sections of CRC patients, a low CA-9 expression in the tumor was correlated with better disease-free survival (DFS) and overall survival [66]. Considering disease outcome in rectal cancer, moderate/strong expression of CA9 and positive HIF-1α expression in resection samples was associated with 47.5-fold risk of disease specific mortality [67], while negative/weak CA9 staining intensity per se was shown to be an independent predictor of longer DFS and disease specific survival (DSS) in rectal cancer [29].

Overall survival rate was 71.0% at a median 23-month follow up with no impact of intensity of HIF-1α, CA9 and CXCR4 expres-

Table 5
CXCR4 expression with respect to clinicopathologic features.

	Immunoreactivity					
	Final score		Strength, n(%)			
	median (min-max)	p value[a]	weak	moderate	strong	p value[b]
Age						
<50	4 (0–9)	0.537	25(47.2)	17(32.0)	11(20.7)	0.701
≥50	3 (0–9)		69(51.8)	43(32.3)	21(15.8)	
Sex						
Female	3 (0–9)	0.188	48(55.8)	23(26.7)	15(17.4)	0.302
Male	4 (0–9)		46(46.0)	37(37.0)	17(17)	
Tumor localization						
Rectum and rectosigmoid	4 (0–9)	0.740	29(49.2)	22(37.3)	8(13.6)	0.502
Colon	3 (0–9)		65(51.2)	38(29.9)	24(18.9)	
Macroscopic growth pattern						
Ulcerovegetative + Depressed	4 (0–9)	0.836	84(49.4)	56(33)	30(17.6)	0.854
Polypoid	3 (0–9)		8(57.2)	4(28.6)	2(14.3)	
Tumor Size						
<5 cm	6 (0–9)	0.028	40(45.9)	26(29.8)	21(24.2)	0.100
≥5 cm	3 (0–9)		54(54.5)	33(33.3)	12(12.1)	
Histologic grade						
Grade 1 and 2	4 (0–9)	0.030	65(47.1)	47(34.0)	26(18.8)	0.270
Grade 3	3 (0–9)		29(60.4)	13(27.1)	6(12.5)	
Angiolymphatic invasion						
Negative	3 (0–9)	0.549	47(54.0)	26(29.8)	12(13.8)	0.409
Positive	4 (0–9)		46(46.7)	33(33.3)	20(20.2)	
Invasion Depth						
T1 + T2	3 (0–9)	0.793	5(62.5)	1(12.5)	2(25.0)	0.741
T3 + T4	3.5 (0–9)		89(50.0)	59(33.2)	30(16.8)	
Lymph node status						
N0	3 (0–9)	0.777	39(50.6)	28(36.4)	10(13.0)	0.636
N1 + N2	3 (0–9)		55(50.5)	32(29.4)	22(20.2)	
Metastasis status at the time of diagnosis						
M0	4 (0–9)	0.386	66(48.8)	51(37.7)	18(13.3)	0.817
M1	3 (0–9)		28(55.0)	9(17.6)	14(27.5)	
Stage						
Stage I + II	4 (0–9)	0.820	30(48.4)	25(40.3)	7(11.3)	0.146
Stage III + IV	3 (0–9)		64(51.6)	35(28.2)	25(20.2)	
Distant metastasis at diagnosis or follow up						
No metastasis	4 (0–9)	0.467	43(47.7)	33(36.6)	14(15.5)	0.317
Liver metastasis[c]	3 (0–9)		31(52.5)	14(23.7)	14(23.7)	
Non-liver metastasis	3 (0–9)		20(54.0)	13(35.1)	4(10.8)	
Local recurrence						
No	4 (0–9)	0.061	85(49.2)	57(32.9)	31(17.9)	0.335
Yes	2 (0–9)		9(69.0)	3(23.1)	1(7.7)	

[a] Mann-Whitey *U* test.
[b] χ^2 test.
[c] isolated or with other distant organ metastasis.

sions on survival in our cohort of CRC patients, while patients with rectum and rectosigmoid tumors composed one third of study population.

Although none of immunohistochemical markers studied was associated with prognostic factors or survival in our cohort and despite positive HIF-1α expression in majority of samples, presence of CA9 immunoreactivity only in 15.6% of resection samples and weak expression in 93.0% of positive samples seems consistent with the favorable survival outcome.

Favorable survival outcome in our cohort seems also in line with the fact that most of our patients had grade 1–2 tumors with no metastasis at the time of diagnosis, and all stage III–IV patients were treated with adjuvant chemotherapy. Nonetheless, given the distant metastasis rate (52.0%, 32.0% to the liver) during 23-month follow up, it should be noted that 5-year survival rate for patients with distant metastatic CRC is 13% [3] and the mortality is caused by liver metastasis in most cases [33].

Besides, albeit strength of immunoreactivity had no impact on survival and both HIF-1α and CXCR4 expressions were similar in patients with vs. without distant metastasis at the time of diagnosis or follow up in our cohort, the association of each immunomarker with poor survival rates and increased metastatic potential has consistently been reported in the past studies [14,18,23,35,36,48,61,66,68].

High expression of CXCR4 in early stage CRC tumor specimens was reported to be associated with an increased risk of loco-regional recurrence and/or liver metastasis, and poor overall survival [68]. Serum CXCR4 levels were also shown to be strongly associated with the number of metastatic sites and liver metastasis in advanced CRC [35], while CXCR4 expression was associated with the outgrowth of colon carcinoma micro-metastases and the development of CRLM [36,37,69]. Hence, CXCR4 expression is considered likely to promote tumor metastases and thus serum CXCR4 levels may be a potential prognostic marker in metastatic/recurrent CRC [33].

Increased risk of recurrence and poor survival in patients with CXCR4 overexpression in primary tumors with CRC is consistent with the role of CXCR4 receptor in intravasation and enhanced lymph node metastases, as strongly related to tumor progression and poor prognosis in advanced CRC [68,70–76].

Increased CXCR4 expression in grade 1–2 than in grade 3 CRC resection samples in our cohort is consistent with a significant correlation between CXCR4 expression and tumor grading in CRC resection specimens reported in a past study [37].

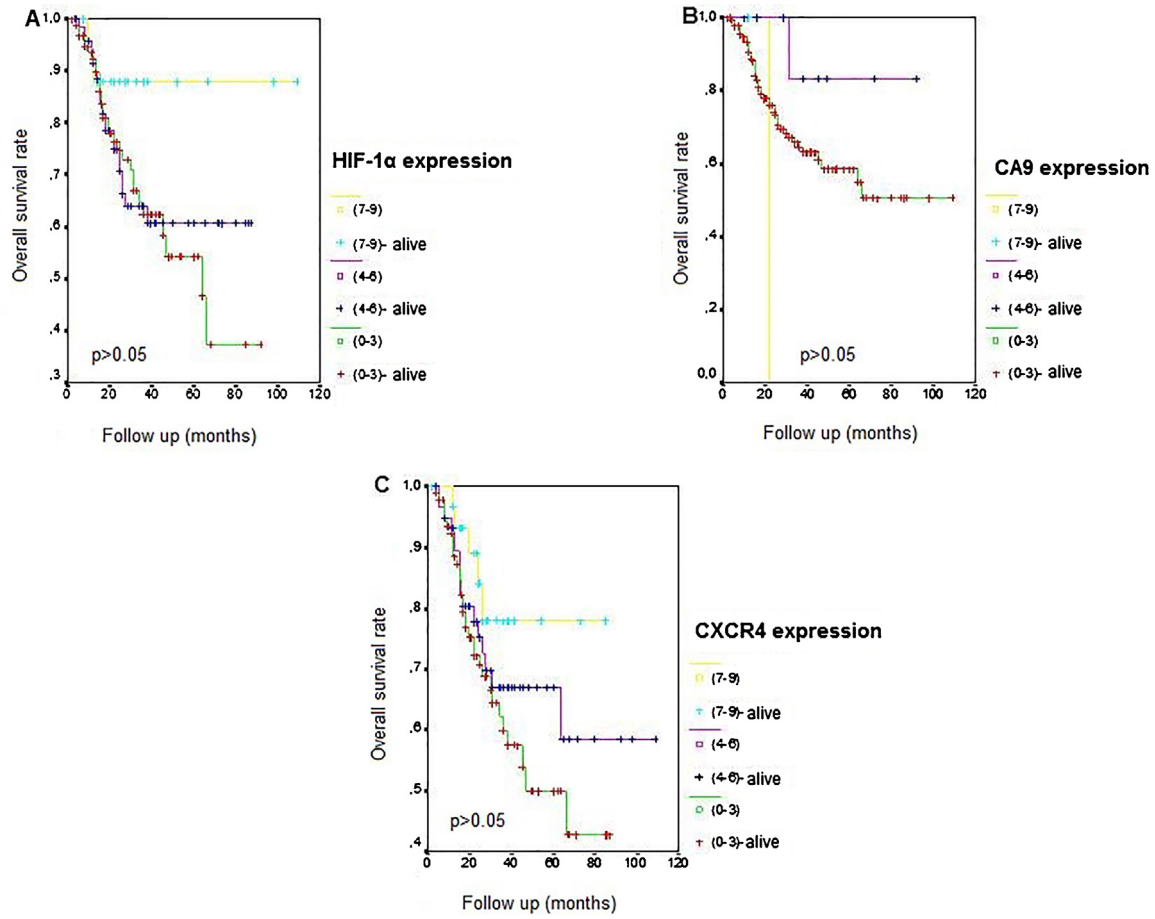

Fig. 2. Kaplan Meier overall survival curves based on A) HIF-1α B) CA9 and C) CXCR4 expression final scores indicating strength of immunoreactivity.

Table 6
Correlation between the extent, intensity and final scores of immunohistochemical markers.

	CA9-CXCR4		CA9-HIF1α		HIF1α-CXCR4	
	r	p	r	p	r	p
Extent	−0.027	0.711	−0.066	0.374	−0.038	0.607
Intensity	−0.030	0.687	0.003	0.968	−0.023	0.755
Final score	−0.040	0.584	−0.019	0.798	−0.020	0.790

r: correlation coefficient. Spearman correlation analysis.

Analysis of tumor tissue samples revealed positive expression rates of CXCR4 to be 61.2% in samples from stage II–III colon cancer patients [77] and to be 38.6% in stage I/II colon cancer patients without chemotherapy [76]. No significant associations between the expression of CXCR4 and clinicopathologic and prognostic factors including gender, age, tumor location, tumor size, TNM staging histological type, lymphovascular invasion in both stage II–III [77] stage I/II [76] colon cancer. Stage II–III colon cancer patients with than without CXCR4 had significantly lower 3-year survival rate (27.3% vs. 76.3%) [77], while no significant difference was noted between low-expression and high-expression groups of CXCR4 in terms of 5-year disease-free survival in stage I/II colon cancer patients [76].

Accordingly, other than gender (HIF-1α), macroscopic growth pattern (CA9) and tumor size and histologic grade (CXCR4), none of the clinicopathologic and prognostic factors studied including tumor localization, histologic type, tumor stage, angiolymphatic invasion, depth of infiltration, lymph node status, local recurrence, metastasis and overall survival was associated with expression of immunohistochemical markers in our cohort. Albeit lack of impact of HIF-1α, CA9 and CXCR4 expression on survival in our cohort is against the past studies, our findings support their findings in terms of no correlation of HIF-1α, CA9 and CXCR4 overexpression with clinical or histopathological and prognostic factors other than survival [29,38,76,77].

Certain limitations to this study should be considered. First, due to retrospective single center design of the present study, establishing the temporality between cause and effect as well as generalizing our findings to overall CRC population seems difficult. Second, HIF-1α, CA9 and CXCR4 expression was based on immunohistochemical analysis not on were evaluated based on protein expression levels, not on genetic levels and results were not confirmed by western blotting analysis. Third, differences in neoadjuvant and adjuvant treatment and prognosis of tumors located to rectum or colon per se is likely to influence clinical outcome. Nevertheless, despite these certain limitations, given the large sample size and long-term follow up, our findings represent a valuable contribution to the literature.

5. Conclusions

In conclusion, our findings revealed weak-to-moderate HIF-1α and CXCR4 immunoreactivity in majority of resection samples, and weak CA9 immunoreactivity in majority of CA9 positive cases. Other than gender (HIF-1α), macroscopic growth pattern (CA9) and tumor size and histologic grade (for CXCR4), none of the clinicopathologic and prognostic factors investigated were associated with expression of immunohistochemical markers and level of immunoreactivity had no impact on survival. Besides interplay

between potential tumor hypoxia markers and cellular signal transduction pathways, prognosis per se depends on a multifactorial process of interacting and correlated parameters. Thus, our findings emphasize the need for further larger scale and longer term studies with standardized and validated assay methodology to clarify the predictive and prognostic role of immunohistochemical markers in CRC in terms of tumorigenesis, clinical outcome and tumor response to therapy.

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