Original paper

Immunohistochemical expression and association of hypoxia-inducible factor 1α and carbonic anhydrase IX in colorectal cancer

Veronika Cígerová[1], Marian Adamkov[1], Marián Grendár[2], Veronika Mešťanová[1]

[1]Department of Histology and Embryology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
[2]Department of Bioinformatics, Biomedical Center Martin, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

The presented work focuses on hypoxia-inducible factor 1α (HIF-1α) and carbonic anhydrase IX (CA IX) in colorectal cancer (CRC). The HIF-1α protein shows increased expression due to hypoxia, resulting in up-regulation of CA IX, which is involved in the survival of hypoxic cancer cells in the tumour microenvironment, with overexpression in various types of carcinomas.
HIF-1α and CA IX immunohistochemical analysis was performed on 111 CRC samples. The primary goal was to determine the correlation of expression of proteins with clinical-morphological parameters and mutual correlation of the proteins in question.
The HIF-1α expression was detected in 72.1% of CRC samples with exclusive nuclear localisation. The immunoreaction intensity was predominantly strong. Carbonic anhydrase IX protein was expressed in 75.7% of cases. The membrane positivity and strong immunoreaction intensity were mainly noticed. No statistically significant correlation between the expression of studied proteins and clinical-morphological parameters was confirmed. However, the results proved a statistically significant correlation in mutual co-localisation of given proteins.
Despite contradictory scientific data, our findings suggest a mutual correlation between HIF-1α and CA IX in CRC. The presented hypothesis that their overexpression may represent a potential new therapeutic target in colorectal carcinogenesis might unveil novel strategies in disease development.

Key words: hypoxia-inducible factor 1α, carbonic anhydrase IX, colorectal cancer, immunohistochemistry.

Introduction

Colorectal cancer (CRC) is the third most commonly occurring cancer in men and the second in women [1]. Colorectal cancer progression involves changes in normal epithelium of the colorectal mucosa, ranging from benign adenomatous polyps to highly heterogeneous advanced invasive adenocarcinomas [2]. Biological changes in this process include signalling molecules important for hypoxia-driven signalling pathways [3].

Hypoxia-signalling pathway is a common hallmark of solid tumours, such as CRC, resulting in increased expression of hypoxia-inducible factor 1 (HIF-1) within the tumour microenvironment to compensate for insufficient blood supply to the grow-

ing tumour [4]. HIF-1 represents a heterodimeric basic helix-loop-helix transcription factor consisting of hypoxia-inducible factor 1α (HIF-1α) and HIF-1α subunits [5]. The α subunit is commonly overexpressed in many malignant tumours and correlates with poor prognosis [6]. HIF-1α is detectable in many tumours, including carcinoma of the stomach, breast, lung, kidney, prostate, pancreas, brain, and ovaries, melanomas, and CRC [7].

Stabilisation and activation of HIF-1α represent the primary molecular response to oxygen deprivation [8]. In hypoxia, the α subunit is stabilised, translocated into the nucleus, and dimerised with the α subunit to form the HIF-1 transcription factor [9]. Subsequently, HIF-1 binds to the hypoxia-responsive element in target gene promoter regions. HIF-1 is involved in the regulation of the expression of various genes, participating in different processes (pH regulation, angiogenesis, metastasis, apoptosis, etc.) [10]. Proteins encoded by HIF-1-regulated genes are active players in tumour progression by executing the adaptive responses to hypoxia [11].

Hypoxia is involved in the induction of more than 50 genes via HIF-1α. Carbonic anhydrase IX (CA IX) is one of the most inducible genes [12]. This protein increases resistance to therapy, stimulates adaptation of tumour cells to microenvironmental stress, contributes to adhesion and migration, and is vital for metastasis [13], thus indicating that CA IX plays an important role in tumour development and progression [14]. Several findings have shown that its expression is related to poor prognosis [15, 16].

Carbonic anhydrase IX represents an attractive target for cancer therapy due to its over-expression in a wide range of solid tumours and under-expression in normal tissues. Carbonic anhydrase IX is abundant mainly in the gastrointestinal tract, especially in the gallbladder, stomach, and small intestine. On the other hand, this protein is robustly expressed in carcinoma of the lung, breast, cervix, bladder, ovaries, brain, head and neck, oral cavity, and CRC [8, 13, 17].

The presented results describe HIF-1α and CA IX expression in CRC patients and summarise the correlations of given proteins with clinical-morphological features. Due to the conflicting scientific opinions concerning the HIF-1α and CA IX levels in cancer tissues, the authors evaluated their expression pattern in colorectal carcinoma [18–20].

Material and methods

Patients group

Archival blocks of formalin-fixed paraffin-embedded tissue samples from 111 CRC patients were used in the presented retrospective study. The study group comprised both females (39; 35.1%) and males (72; 64.9%), with a mean age of 67.03 years, ranging from 39 to 94 years (Table 1). The samples for immunohistochemical analysis were recruited during the surgery of CRC patients in the hospitals of the catchment area and subsequently transported to the Alpha Medical Patologia, s.r.o. in Martin, Slovakia, for routine primary diagnostics in the period February 2016 – June 2016. At the time of tissue processing and histopathological examination for the presented study, the tissues were not older than 4 months. Classification of CRCs was provided according to World Health Organisation guidelines and pathological staging criteria (pathological assessment of tumor, node, metastasis; International Union Against Cancer – pTNM UICC) (Table 1) [21]. Pathological reports included the clinical-morphological parameters applied in the study. However, the content of individual bioptic reports missed uniformity, resulting in the absence of parameters such as grade, stage, lymph node status, or vascular invasion in particular cases, respectively.

Table I. Clinico-morphological characteristics of colorectal cancer patients

Features	*n*	Percentage
Age, years ($N = 111$)		
Mean	67.03	
Range	39–94	
Gender ($N = 111$)		
Female	39	35.1
Male	72	64.9
Histological grading ($N = 106$)		
G1	34	32.1
G2	60	56.6
G3	7	6.6
G4	5	4.7
Pathological staging ($N = 61$)		
Stage I	12	19.7
Stage II	23	37.7
Stage III	24	39.3
Stage IV	2	3.3
Lesion site ($N = 111$)		
Proximal colon	50	45.0
Distal colon	61	55.0
Lymph node ($N = 65$)		
Positive	24	36.9
Negative	41	63.1
Vascular invasion ($N = 59$)		
Positive	19	32.2
Negative	40	67.8

Immunohistochemical analysis

Paraffin blocks were cut into 4-μm-thick sections and submitted to immunohistochemical examination. HIF-1α and CA IX protein expression was detected on 2 sections from each case.

According to the manufacturer's protocol, the paraffin sections were stained with automated slide stainer Ventana BenchMark® ULTRA. To detect the HIF-1α protein, the automated program for immunohistochemistry included deparaffinisation at 72°C; cell condition (pH 8.0, 95°C, 8 min); incubation with a UV peroxidase inhibitor (4 min); incubation with a 1:200 diluted rabbit monoclonal HIF-1α antibody (Clone EP118, Epitomics, USA) (36°C, 28 min); incubation with a UV HRP multimer (8 min); incubation with a UV DAB chromogen and UV DAB H_2O_2 (8 min); incubation with a UV DAB copper (4 min); and incubation with haematoxylin (8 min); and incubation with a bluing reagent (8 min). To detect the CA IX protein, the automated program for immunohistochemistry included deparaffinisation (72°C); cell condition (pH 8.0, 97°C, 8 min); incubation with a 1:300 diluted mouse monoclonal CA IX antibody (Clone H-11, Zeta corporation, USA) (36°C, 4 min); incubation with a UV red multimer (12 min); incubation with a UV red enhancer (4 min); incubation with a UV fast red A and UV red naphthol (8 min); incubation with a UV fast red B (8 min); incubation with haematoxylin (8 min); and incubation with bluing reagent (8 min) [22].

Subcellular localisation, the intensity of immunoreaction (weak, moderate, strong), and the percentage of positive cells (a percentage ≤ 10% was considered negative) were assessed. The subcellular localisation of CA IX was classified as the membranous (M) or the combined (membrane and cytoplasmic) (MC), and the localisation of HIF-1α was only nuclear (N). These parameters were quantitatively evaluated using computer-assisted morphometric analysis. Digital microphotographs were taken with a camera microscope (Canon EOS 2000D) installed in an Olympus BX43 bright-field microscope. Microphotographs were analysed with QuickPhoto-Micro Version 3.2 software (Promicra, Prague, Czech Republic). Each slide was examined by 2 independent observers (VC, MA).

Statistical analysis

For the statistical analysis, the χ^2 test of the R software (version 3.2.3) was applied [23]. The results with $p < 0.05$ were considered statistically significant.

Results

Evaluation of immunohistochemical staining

Immunohistochemical analysis showed HIF-1α expression in 80/111 CRC cases (72.1%), with exclusive nuclear staining in all positive cases. The intensity of immunoreaction was predominantly strong (71.2%), while moderate intensity was detected only in 0.9% of cases. The percentage of HIF-1α-positive cells was less than 25% in 8/111 cases (7.2%), less than 50% in 24/111 cases (21.6%), and more than 50% in 32/111 cases (28.8%). Observations revealed 62/111 cases (55.9%) showing ≤ 25% of positively stained cells for HIF-1α, 13/111 cases (11.7%) with ≤ 50%, and only 5/111 cases (4.5%) with > 50% of positive cells.

Carbonic anhydrase IX immunohistochemical detection proved positive in 84/111 CRC cases (75.7%) with membrane or combined (MC) immunoreaction. Membrane staining was detected in 68.5% of cases, while combined localisation was observed only in 7.2%. The immunoreaction intensity was assessed as strong in 81/111 cases (73%), while moderate intensity was detected only in 2.7%. The analysis confirmed 28/111 cases (25.2%) showing ≤ 25% of CA IX-positive cells, 24/111 cases (21.6%) with ≤ 50%, and 32/111 cases (28.8%) with > 50% of positively stained cells.

HIF-1α and CA IX expression were observed in epithelial tumour cells. In most cases (64%) positive expression for both HIF-1α and CA IX was observed with coincidence in the same tumour cells (HIF-1α+/CA IX+) (Fig. 1A, B). However, in 12% of the CA IX-positive cases, HIF-1α expression was absent (HIF-1α–/CA IX+) (Fig. 1C, D), and in 8% of the HIF-1α-positive samples, CA IX expression was not detected (HIF-1α+/CA IX–) (Fig. 1E, F).

The statistical analysis confirmed no statistically significant association between clinical-morphological parameters (patient age, gender, grade, stage, lesion site, lymph node status, vascular invasion) and HIF-1α expression (Table 2) as well as CA IX expression (Table 3). Moreover, a positive significant correlation was demonstrated between CA IX and HIF-1α protein expression, intensity, and percentage of positive cells (Table 4).

Discussion

HIF-1α is a key executor of cellular and molecular response to hypoxia and can activate the expression of several genes involved in various essential cellular processes associated with the aetiopathogenesis of heterogeneous diseases. On the other hand, CA IX represents a protein profusely induced by hypoxia as an indicator of chronic hypoxia [24]. Hypoxic conditions are often encountered in solid tumours, including CRC [4].

In the presented study, the authors confirmed HIF-1α expression in 80 cases (72.1%). Constant nuclear staining pattern was typical for HIF-1α expression associated with predominantly strong im-

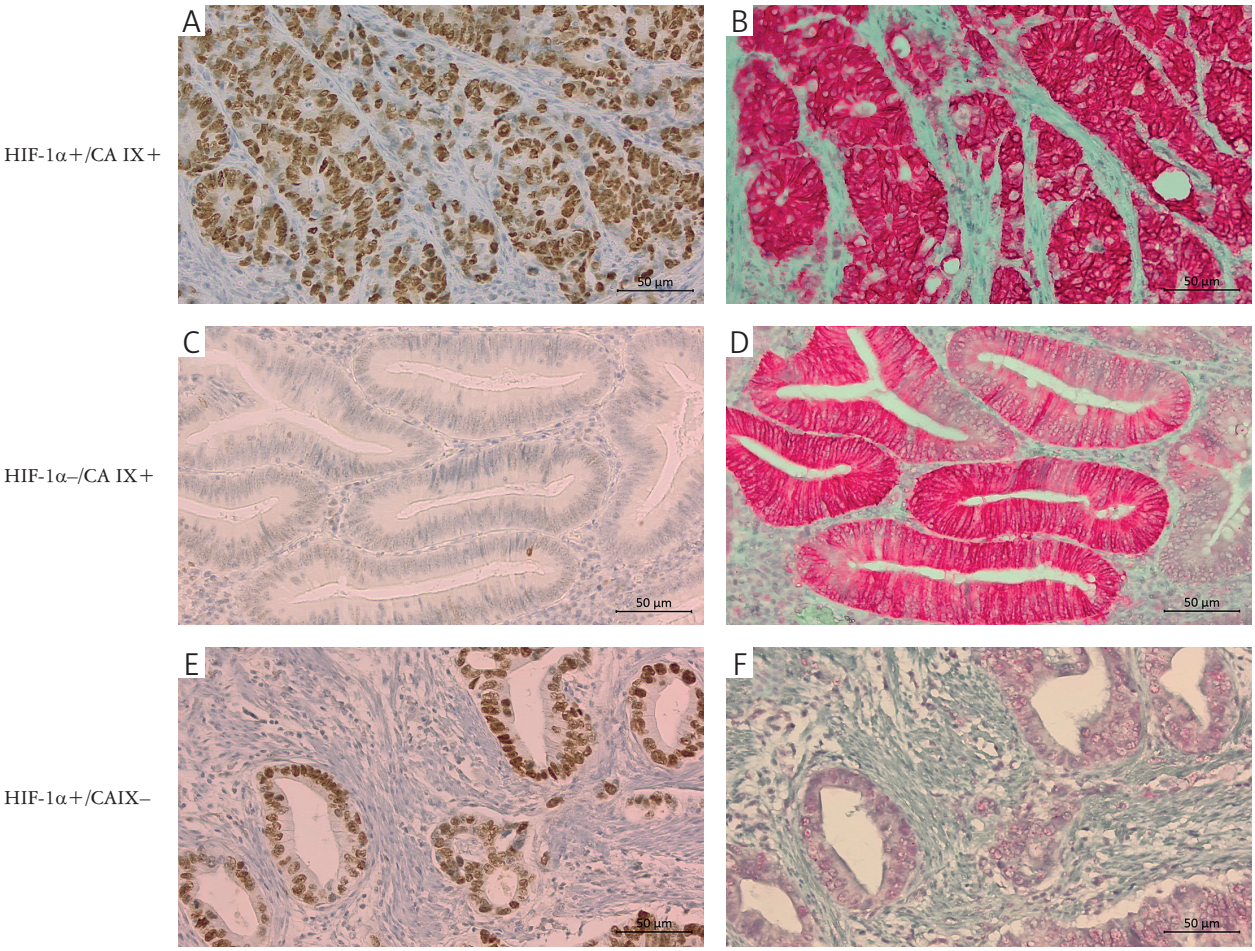

Fig. 1. Mutual relationships between hypoxia-inducible factor 1α and carbonic anhydrase IX expression in colorectal cancer. A, C, E) Immunostained for hypoxia-inducible factor 1α. B, D, F) Immunostained for carbonic anhydrase

munoreaction intensity (71.2%). To compare the observed prevalence of HIF-1α with the other studies, Greijer *et al*. [25] detected HIF-1α in 78% of CRC cases, Saka *et al*. [20] in 94% of cases, similar to Furlan *et al*. [26], who observed the expression of HIF-1α in 77% of CRC cases, which corresponds with the described findings. HIF-1α-positive cells showed moderate or strong nuclear immunoreaction intensity. Based on the abovementioned results, HIF-1α is highly expressed in CRC, with prominent strong immunoreaction intensity, indicating a high accumulation of the questioned protein in colorectal carcinoma.

In parallel with the results of Yoshimura *et al*. [27], statistical analysis confirmed no significant association between HIF-1α expression and clinical-morphological parameters (patient age, gender, histological grading, pathological staging, lesion site, lymph node status, vascular invasion). However, a scientific paper by Fan *et al*. [28] describes a significant correlation between HIF-1α and pathological stage. Moreover, significant associations between HIF-1α and high TNM stage and lymph node involvement were observed by Wu *et al*. [29]. In the case of Saka *et al*. [20], significant correlations were proved only in HIF-1α expression with gender. The discrepancies in published results are obvious, probably a consequence of the limited size of study groups.

Concerning the expression of CA IX, the presented results are consistent with Saarnio *et al*. [30], who mentioned and described 75.7% positivity of observed protein in CRC for the first time. Detailed analysis displayed 29% of weak positive reactions and 47% of moderate to strong reactions. The obtained findings are comparable with the studies of other investigators, who demonstrated high expression of this protein in CRC tissue [19, 31, 32]. Interestingly, the immunoreaction intensity of CA IX in our study group was predominantly strong (73%). Elevated levels of CA IX protein in CRC copy the increasing trend of HIF-1α expression.

The authors of the given study observed membrane and combined (MC) CA IX localisation. This finding supports the fact that CA IX is a transmembrane protein partially protruding into the cytoplasm. Carbonic anhydrase IX membrane localisation was noticed in 68.5% of the cases and combined

was shown only in 7.2% of cases. Identical observations were described by Goethals *et al*. [18], who noticed CA IX membrane localisation and subtle cytoplasmic expression in cases with strong intensity in CRC patients. A similar trend of membrane localisation of CA IX protein in almost 90% of CRC with or without cytoplasmic staining was demonstrated by Saka *et al*. [20].

Table II. Correlation between hypoxia-inducible factor 1α expression and clinical-morphological parameters of colorectal cancer

HIF-1α expression	Negative	Intensity of immunoreaction			Percentage of positive cells		
		Weak intensity	Moderate intensity	Strong intensity	11–25%	26–50%	> 50%
Age (N = 111)							
≤ 50	3	0	0	3	2	0	1
51–60	11	0	0	13	9	4	0
61–70	7	0	1	32	27	5	1
71–80	6	0	0	23	19	3	1
> 80	4	0	0	8	5	1	2
P-value			0.272			0.0955	
Gender (N = 111)							
Female	10	0	1	28	20	6	3
Male	21	0	0	51	42	7	2
P-value			0.375			0.496	
Grade (N = 106)							
G1	12	0	0	22	15	5	2
G2	15	0	1	44	35	8	2
G3	1	0	0	6	5	0	1
G4	1	0	0	4	4	0	0
P-value			0.845			0.657	
Stage (N = 61)							
I	2	0	1	9	7	3	0
II	7	0	0	16	15	1	0
III	5	0	0	19	16	1	2
IV	0	0	0	2	2	0	0
P-value			0.462			0.349	
Lesion site (N = 111)							
Proximal site	13	0	0	37	30	3	4
Distal site	18	0	1	42	32	10	1
P-value			0.594			0.293	
Lymph node (N = 65)							
Positive	5	0	0	19	17	1	1
Negative	13	0	1	27	23	4	1
P-value			0.447			0.585	
Vascular invasion (N = 59)							
Positive	4	0	0	15	15	0	0
Negative	11	0	1	28	22	5	2
P-value			0.661			0.194	

HIF-1α – hypoxia-inducible factor 1α

Regarding the lack of significant correlation between CA IX expression and clinical-morphological parameters (patient age, gender, histological grading, pathological staging, lesion site, lymph node status, vascular invasion), the presented analysis brought similar results to the other studies [16, 20, 33]. However, Cleven *et al*. [19] proved that CA IX expression was significantly associated with lymph node-nega-

Table III. Correlation between carbonic anhydrase IX expression and clinico-morphological parameters of colorectal cancer

CA IX expression	Negative	Intensity of immunoreaction			Percentage of positive cells		
		Weak intensity	Moderate intensity	Strong intensity	11–25%	26–50%	> 50%
Age (N = 111)							
≤ 50	2	0	0	4	0	2	2
51–60	7	0	0	17	8	4	5
61–70	9	0	1	30	8	13	10
71–80	7	0	2	20	10	6	6
> 80	2	0	0	10	2	3	5
P-value			0.847			0.747	
Gender (N = 111)							
Female	11	0	1	27	9	8	11
Male	16	0	2	54	19	20	17
P-value			0.782			0.747	
Grade (N = 106)							
G1	11	0	1	22	11	7	5
G2	13	0	2	45	13	17	17
G3	0	0	0	7	3	2	2
G4	0	0	0	5	1	2	2
P-value			0.419			0.435	
Stage (N = 61)							
I	2	0	0	10	3	3	4
II	5	0	2	16	4	7	7
III	1	0	0	23	9	8	6
IV	0	0	0	2	1	0	1
P-value			0.278			0.684	
Lesion site (N = 111)							
Proximal site	11	0	1	38	10	14	15
Distal site	16	0	1	44	19	13	13
P-value			0.786			0.515	
Lymph node (N = 65)							
Positive	1	0	0	23	10	8	5
Negative	9	0	1	31	7	11	14
P-value			0.073			0.052	
Vascular invasion (N = 59)							
Positive	3	0	0	16	7	5	4
Negative	7	0	0	33	10	11	12
P-value			0.593			0.793	

CA IX – carbonic anhydrase IX, M – membrane, MC – combined membrane and cytoplasmic

Table IV. Correlation between expression of hypoxia-inducible factor 1α and carbonic anhydrase IX proteins

Parameters	HIF-1α	CA IX
Negative cases, *n* (%)	31 (27.9)	27 (24.3)
Positive cases, *n* (%)	80 (72.1)	84 (75.7)
P-value	6.0E–05	
Intensity of immunoreaction, *n* (%)		
Weak intensity	0	0
Moderate intensity	1 (0.9)	3 (2.7)
Strong intensity	79 (71.2)	81 (73.0)
P-value	2.2E–16	
Percentage of positive cells, *n* (%)		
11–25%	62 (55.9)	28 (25.2)
26–50%	13 (11.7)	24 (21.6)
> 50%	5 (4.5)	32 (28.8)
P-value	1.83E–15	

CA IX – carbonic anhydrase IX, HIF-1α – hypoxia-inducible factor 1α

tive CRC. On the contrary, our results manifest only a tendency of correlation between lymph node status and CA IX expression.

Based on the relevant studies [6, 34, 35], HIF-1 significantly correlates with CA IX expression in many types of carcinomas (e.g. breast cancer, nasopharyngeal carcinoma, cervical cancer). However, the studies that examined this correlation in CRC present ambiguous results. On one hand, Greijer *et al*. [25] observed that CA IX was expressed in perinecrotic regions and co-localised with the HIF-1α expression in CRC. On the other hand, Cleven *et al*. [19] as well as Saka *et al*. [20] did not confirm a significant correlation between CA IX and HIF-1α expression in CRC. Their findings suggest HIF-1α-independent induction of CA IX expression, which is probably regulated by other factors and/or signalling pathways within the tumour microenvironment. Limited correlations might be also explained by the inclination of HIF-1α to degradation after extended hypoxia due to the upregulation of prolyl hydroxylase domain proteins. In addition, the half-life of CA IX is long enough (> 24 hrs), and after formation it is still preserved for longer time intervals in the absence of HIF-1α expression [19, 36].

A significant correlation within the studied group of samples was manifested between the HIF-1α and CA IX expression in CRC. The finding follows the CA IX promoter mediation by the HIF-1 pathway and its tight regulation by a HIF-1-dependent HRE lying adjacent to the initiation site [6]. These data demonstrate the primary responsibility of HIF-1 for the induction of CA IX expression in CRC. Moreover, the authors observed HIF-1α expression in the cases, whereas CA IX expression was absent. Kaluz *et al*. [37] presented an explanation for the delayed detection of CA IX protein after HIF-1-induced transcriptional activation. On the other hand, a few samples showed the opposite pattern of expression – CA IX positive while HIF-1α negative expression. According to Kaluz *et al*. [37], labile stability of HIF-1α and extreme stability of CA IX could be responsible for the HIF-1α–/CAIX+ expression in hypoxic cells followed by reoxygenation (loss of HIF-1α expression); however, they preserved CA IX expression.

In general, HIF-1α and CA IX proteins are associated with a poor prognosis in CRC [6, 13, 38]. The key function of CA IX is to maintain the equilibrium between intracellular and extracellular pH, affecting the prognosis of disease [39]. Carbonic anhydrase IX also participates in carcinogenesis and results in a more aggressive phenotype of cancer cells [40]. Because HIF-1α up-regulates the expression of downstream gene *CA IX*, the authors considered these proteins as endogenous hypoxia-related markers and observed that their positive expression is associated with poor prognosis in CRC. Thus, the presence of hypoxia has an adverse effect on the prognosis of the disease, while hypoxic tumours resist chemotherapy and radiotherapy. Hypoxic tumour cell elimination can provide significant therapeutic benefits. The therapeutic approach to such a strategy should be hypoxia-modifying and gene-targeted therapy for CRC patients showing high HIF-1α and CA IX expression.

Conclusions

The presented study demonstrates increased expression of HIF-1α and CA IX in colorectal carcinoma. However, no significant correlations with clinical-morphological parameters were proven. For the first time, the authors bring confirmation of a significant correlation between the mutual positivity of the proteins in question in colorectal carcinoma. Tumour hypoxia is still more recognised as an important therapeutic target. The presented evidence of HIF-1α and CA IX expression in most colorectal carcinomas may shed light on their new roles as potential new therapeutic targets. To elucidate the details of the clinical relationships and roles of HIF-1α and CA IX in colorectal carcinogenesis, further studies are required.

Disclosures

1. The scientific research was conducted ethically, with all study procedures being performed following the requirements of the World Medical Association´s Declaration of Helsinki. The study was approved by

the Independent Ethics Committee of Jessenius Faculty of Medicine, Comenius University registered at the US Office for Human Research Protection, US Department of Health and Human Services (approval number: EK 59/2022, date 7.12.2022). All patients included in the study signed a detailed informed consent form, as well as a consent form for scientific purposes, preoperatively. The presented retrospective study does not require any additional informed consent due to archival material usage only. Data were examined in compliance with the privacy and sensitive data concepts valid in the EU.

2. We thank the laboratory assistants Margaréta Kondeková, Agáta Rešetárová, and Slávka Drahošová for the immunohistochemical preparation of the tissue samples.
3. This study was supported by Comenius University grant (UK/20/2023) and Scientific Grant Agency of the Ministry of Education, Science, Research, and Sport of the Slovak Republic and Slovak Academy of Sciences (VEGA no. 1/0129/16).
4. Conflicts of interest: None.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209-249.
2. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med 2009; 361: 2449-2460.
3. Zou YF, Rong YM, Tan YX, et al. A signature of hypoxia-related factors reveals functional dysregulation and robustly predicts clinical outcomes in stage I/II colorectal cancer patients. Cancer Cell Int 2019; 19: 243.
4. Momin S, Nagaraju GP. The role of hypoxia-inducible factor 1-alpha in colorectal cancer. In: Theranostics approaches to gastric and colon cancer. Springer 2020, 61-68.
5. Masoud GN, Li W. HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharm Sin B 2015; 5: 378-389.
6. Chen Y, Li X, Wu S, et al. Expression of HIF-1α and CAIX in nasopharyngeal carcinoma and their correlation with patients' prognosis. Med Oncol 2014; 31: 304.
7. Courtnay R, Ngo DC, Malik N, et al. Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K. Mol Biol Rep 2015; 42: 841-851.
8. Pastorekova S, Ratcliffe PJ, Pastorek J. Molecular mechanisms of carbonic anhydrase IX-mediated pH regulation under hypoxia. BJU Int 2008; 101: 8-15.
9. Sedlakova O, Svastova E, Takacova M, et al. Carbonic anhydrase IX, a hypoxia-induced catalytic component of the pH regulating machinery in tumors. Front Physiol 2014; 4: 1-14.
10. Wigerup C, Påhlman S, Bexell D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. Pharmacol Ther 2016; 164: 152-169.
11. Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. Nat Rev Cancer 2011; 11: 393-410.
12. Pastorek J, Pastorekova S. Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use. Semin Cancer Biol 2015; 31: 52-64.
13. McDonald PC, Winum J-W, Supuran CT, et al. Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. Oncotarget 2012; 3: 84-97.
14. Lee S-H, Griffiths JR. How and why are cancers acidic? Carbonic anhydrase IX and the homeostatic control of tumour extracellular pH. Cancers 2020; 12: 1616.
15. Korkeila E, Talvinen K, Jaakkola PM, et al. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. Br J Cancer 2009; 100: 874-880.
16. Viikilä P, Kivelä AJ, Mustonen H, et al. Carbonic anhydrase enzymes II, VII, IX and XII in colorectal carcinomas. World J Gastroenterol 2016; 22: 8168-8177.
17. Kalinin S, Malkova A, Sharonova T, et al. Carbonic anhydrase IX inhibitors as candidates for combination therapy of solid tumors. Int J Mol Sci 2021; 22: 13405.
18. Goethals L, Debucquoy A, Perneel C, et al. Hypoxia in human colorectal adenocarcinoma: comparison between extrinsic and potential intrinsic hypoxia markers. Int J Radiat Oncol Biol Phys 2006; 65: 246-254.
19. Cleven AH, van Engeland M, Wouters BG, et al. Stromal expression of hypoxia regulated proteins is an adverse prognostic factor in colorectal carcinomas. Cell Oncol 2007; 29: 229-240.
20. Saka B, Ekinci O, Dursun A, et al. Clinicopathologic and prognostic significance of immunohistochemical expression of HIF-1α, CXCR4 and CA9 in colorectal carcinoma. Pathol Res Pract 2017; 213: 783-792.
21. Bosman FT, Carneiro F, Hruban RH, et al. WHO classification of tumours of the digestive system (4th ed.). Lyon: IARC Press 2010, 417.
22. Kuan SF, Navina S, Cressman KL, et al. Immunohistochemical detection of BRAF V600E mutant protein using the VE1 antibody in colorectal carcinoma is highly concordant with molecular testing but requires rigorous antibody optimization. Human Pathol 2014; 45: 464-472.
23. R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 2021. Available from: https://www.R-project.org/ (accessed: 6.11.2022).
24. Takáčová M, Pastoreková S. Tumour hypoxia – molecular mechanisms and clinical relevance. Klin Onkol 2015; 28: 183-190.
25. Greijer AE, Delis-Van Diemen PM, Fijneman RJ, et al. Presence of HIF-1 and related genes in normal mucosa, adenomas and carcinomas of the colorectum. Virch Arch 2008; 452: 535-544.
26. Furlan D, Sahnane N, Carnevali I, et al. Up-regulation and stabilization of HIF-1alpha in colorectal carcinomas. Surg Oncol 2007; 6: 25-27.
27. Yoshimura H, Dhar DK, Kohno H, et al. Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clin Cancer Res 2004; 10: 8554-8560.
28. Fan LF, Dong WG, Jiang CQ, et al. Role of hypoxia-inducible factor-1α and survivin in colorectal carcinoma progression. Int J Colorectal Dis 2008; 23: 1057-1064.
29. Wu Y, Jin M, Xu H, et al. Clinicopathologic significance of HIF-1α, CXCR4, and VEGF expression in colon cancer. Clin Dev Immunol 2010; 2010: 537531.
30. Saarnio J, Parkkila S, Parkkila AK, et al. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. Am J Pathol 1998; 153: 279-285.
31. Hong YS, Cho HJ, Kim SY, et al. Carbonic anhydrase 9 is a predictive marker of survival benefit from lower dose of bevacizumab in patients with previously treated metastatic colorectal cancer. BMC Cancer 2009; 9: 1-9.
32. Rasheed S, Harris AL, Tekkis PP, et al. Assessment of microvessel density and carbonic anhydrase-9 (CA-9) expression in rectal cancer. Pathol Res Pract 2009; 205: 1-9.
33. Kováčová Z, Hodorová I. Carbonic anhydrase IX and survivin in colorectal adenocarcinoma cells: Slovakian population study. Biology 2021; 10: 872.

34. Vleugel MM, Greijer AE, Shvarts A, et al. Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. J Clin Pathol 2005; 58: 172-177.
35. Iwasaki K, Yabushita H, Ueno T, et al. Role of hypoxia-inducible factor-1α, carbonic anhydrase-IX, glucose transporter-1 and vascular endothelial growth factor associated with lymph node metastasis and recurrence in patients with locally advanced cervical cancer. Oncol Lett 2015; 10: 1970-1978.
36. Sobhanifar S, Aquino-Parsons C, Stanbridge EJ, et al. Reduced expression of hypoxia-inducible factor-1 alpha in perinecrotic regions of solid tumors. Cancer Res 2005; 65: 7259-7266.
37. Kaluz S, Kaluzova M, Liao SY, et al. Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: A one transcription factor (HIF-1) show? Biochim Biophys Acta 2009; 1795: 162-172.
38. Baba Y, Nosho K, Shima K, et al. HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. Am J Pathol 2010; 176: 2292-2301.
39. Neri D, Supuran CT. Interfering with pH regulation in tumours as a therapeutic strategy. Nat Rev Drug Discov 2011; 10: 767-777.
40. Lock FE, McDonald PC, Lou Y, et al. Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. Oncogene 2013; 32: 5210-5219.

Address for correspondence

Veronika Mešťanová
Department of Histology and Embryology
Jessenius Faculty of Medicine in Martin
Comenius University in Bratislava
Martin, Slovakia
e-mail: veronika.mestanova@uniba.sk