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# Clinicopathological Significance and Linkage of the Distribution of HIF-1α and GLUT-1 in Human Primary Colorectal Cancer

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**HIF-1α induces GLUT-1 expression, and their presence has been evaluated in colorectal cancer. However, the expressions of GLUT-1 and HIF-1α have not been investigated together with reference to clinicopathological characteristics in human colorectal cancer. The aim of our study was to compare the expression of HIF-1α and GLUT-1 with various clinicopathological features of colorectal cancer. The presence of HIF-1α and GLUT-1 was visualized immunohistochemically in 123 primary tumors. Membranous localization of GLUT-1 was found in multifocally necrotizing cancer samples, while pure cytoplasmic perinuclear, mostly supranuclear GLUT-1 accumulation was characteristic of cancer fields with lack of necrosis. HIF-1α was located in the cytoplasm and occasionally in the nuclei of cancer cells. Immunoreactivity to GLUT-1 was significantly higher in node-positive cancers compared with node-negative ones (p=0.04), confirming our earlier results obtained on a larger number of patients. Non-mucinous adenocarcinomas expressed GLUT-1 and HIF-1α with significantly greater frequency than mucinous adenocarcinomas (p=0.002, p=0.0002, respectively). GLUT-1 and HIF-1α expression did not differ in relation to tumor stage, location, or patients' age or gender. In contrast to that of GLUT-1, expression of HIF-1α correlated with grade (p=0.00003) without difference with regard to pN status. HIF-1α expression correlated with GLUT-1 expression in the whole patient population, as well as in all clinicopathological groups except for the pT1+pT2 group. Although the coexpression of cytoplasmic HIF-1α and GLUT-1 does not directly prove the dependence between HIF-1 as a nuclear transcriptional factor and GLUT-1 as its downstream protein, it is evidence of their simultaneous upregulation. The extranuclear accumulation of HIF-1α and GLUT-1 requires further studies to explain its significance in colorectal cancer.** (Pathology Oncology Research Vol 13, No 1, 15–20)

*Key words:* HIF-1α, GLUT-1, colorectal cancer, cell survival

## *Introduction*

HIF-1 (hypoxia-inducible factor 1) functions as a heterodimeric transcription factor consisting of constantly expressed HIF-1β and hypoxia-induced HIF-1α.[1] HIF-1 upregulates HIF-1-dependent genes like GLUT-1 which encodes a membrane glucose transporter protein.[2] The dependence of GLUT-1 on HIF-1α has been demonstrated on cell lines. Expression of GLUT-1 was decreased in hypoxic culture of hepatocytes which were devoid of HIF-1α, whereas GLUT-1 was upregulated in cells that contained HIF-1α.[3] HIF-1α-negative pancreatic tumors failed to express GLUT-1.[4] HIF-1α-deficient tumor cells, also lacking GLUT-1 expression, were less resistant to hypoglycemia and redox injury and less malignant than tumors with presence of both genes.[5]

HIF-1α and GLUT are predictors of poor prognosis in malignancies.[6,7] The reports on the impact of HIF-1α on prognosis are diverse and sometimes lead to opposing conclusions in colorectal cancer. Combined analysis of HIF-1α and HIF-2α found them of prognostic significance together; however, HIF-1α alone failed to be a predictive factor in colorectal cancer.[8] Furthermore, a poor prognosis and aggressive course of the disease were associated with overexpression of GLUT-1 in colorectal cancer.[9-11]

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HIF-1α regulation of GLUT-1 expression was confirmed by a correlation between HIF-1α and GLUT-1 near sites of necrosis in human breast cancer.[6] In human bladder cancer tissue expressions of HIF-1α and GLUT-1 were also associated with each other, and in separate analysis with unfavorable outcome of patients.[12] They were also evaluated in colorectal cancer but without finding an association with clinicopathological characteristics of the tumors.[13] This is why we decided to determine expression of GLUT-1 and HIF-1α in primary tumors of this neoplasm and their association with clinicopathological features. We also aimed to evaluate correlations between GLUT-1 and HIF-1α in all patients, as well as in groups of different clinicopathological characteristics. To the best of our knowledge, our present report is the first to indicate a strong relationship between GLUT-1 and HIF-1α with regard to various clinical and pathological features of human primary colorectal cancer.

## *Methods*

Our study included tissue samples of surgically removed colorectal cancers from 123 patients. No individuals underwent any radiotherapy or chemotherapy before tumor resection. These human studies were conducted in respect to the ethical standards laid down in the 1975 Declaration of Helsinki and its revision in 1983 and 2000, with the approval of the ethics committee of the Medical University of Bialystok. All subjects gave their informed consent before inclusion in the study. The biopsy material was fixed in 10% buffered formaldehyde solution for 48 hours and then embedded in paraffin blocks at 56°C according to standard procedures. Two independent pathologists produced diagnoses on the basis of 5-ìm-thick hematoxylin and eosin-stained specimens, and determined conventional histopathologic parameters of colorectal cancers, including AJCC/UICC TNM stage, tumor type and grade of differentiation (G). Due to the relatively low number of pT1 tumors, pT1 and pT2 neoplasms were combined into one group that corresponded to stage A of the Dukes' system. pT3 and pT4 tumors comprised a separate group which corresponded to stage B and C of Dukes' classification. This division was justified by the expected dramatically different prognosis of patients with stage A in comparison to those with stage B and C cancers. The tumors were divided in two histopathological groups: non-mucinous adenocarcinomas and mucinous adenocarcinomas; in the latter, the mucin-secreting component made up more than 50% of all microscopically examined cancer fields of each tumor.

Prior to immunohistochemical staining, tissue sections were incubated with blocking serum for one hour. HIF-1α and GLUT-1 proteins were detected with rabbit polyclonal antibodies against HIF-1α (sc-10790, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:150) and GLUT-1 (A3536, Dako, Glostrup, Denmark; 1:250), respectively. Specimens were incubated with the primary antibody overnight at 4°C. Then, reactions for GLUT-1 were developed using the LSAB method with 5 minutes of exposure to DAB (diaminobenzidine), while for HIF-1α detection, the EnVision method was used with 7 minutes of exposure to DAB. Sections were then counterstained with hematoxylin. Negative controls were prepared with the omission of the primary antibodies, while specimens of breast cancer stained for GLUT-1 or HIF-1α served as positive controls.

### *Scoring and statistical analysis*

The results were analyzed with Spearman's rank correlation and Pearson's and Mantel-Haenszel's Chi-square tests. Statistical results with $p<0.05$ were assumed to be significant. We applied a 3-grade scoring system for Spearman's rank correlation as follows: 0 if there were less than 10% immunoreactive cancer cells, 1 if there were 10-50% immunoreactive cancer cells, and 2 if more than 50% of malignant cells were immunoreactive. We used a 2-grade scale for Pearson's and Mantel-Haenszel's Chi-square tests, assigning 0 for less 10% and 1 for more than 10% immunoreactive cells in each tumor. The immunohistochemical stainings for GLUT-1 and HIF-1α were assessed by two pathologists in 10 high-power fields of each tumor with light microscopy, and the mean rate of positive tumor cells was calculated. Besides Pearson's test we performed Mantel-Haenszel's Chi-square test because it is considered more reliable when small numbers of cases are statistically analyzed; however, the results of the two statistical evaluations were very similar, therefore, we present only p values from Pearson's Chi-square test.

## *Results*

HIF-1α and GLUT-1 were visualized in cancer cells by immunohistochemistry. Both HIF-1α and GLUT-1 expressions were generally increased in the vicinity of necrotic foci (*Fig. 1a,c*). Other cells failed to stain for these factors, except for a variable positivity found in dysplastic intestinal epithelium and strong immunoreactivity exclusively to GLUT-1 in red blood cells.

### *Characteristics of HIF-1α immunoreactivity and associations with clinicopathological variables*

The staining for HIF-1α was localized mainly in the cytoplasm of cancer cells in a granular and diffuse pattern. Sometimes the protein accumulated at the nuclear mem-

brane but only occasionally and discretely appeared in the nuclei of colorectal cancer cells. Comparison between G2 and G3 cancers (p=0.00003) resulted in a significantly higher rate of HIF-1α immunoreactivity in moderately differentiated tumors (G2) (*Table 1, Fig. 1a,b*). Non-mucinous adenocarcinomas expressed HIF-1α with a greater frequency than mucinous adenocarcinomas (p=0.0002). There were no significant differences in the expression of HIF-1α according to the depth of invasion (pT), nodal involvement (pN), patients' age, gender and tumor location (*Table 1*).

*Characteristics of GLUT-1 immunoreactivity and associations with clinicopathological variables*

In most of the cases the pattern of immunoreactivity to GLUT-1 was mixed. Namely, it was finely granular in the cytoplasm and formed an intense contour of the cell membrane (approximately 50% of positive specimens). The closer to necrosis the more frequent and intense membranous staining was for GLUT-1. With the increasing distance cancer clusters were characterized by more frequent cytoplasmic localization of GLUT-1. Multifocal necrosis was a common finding in cancer fields, in which exclusively membranous immunoreactive cells outnumbered the cell population with microgranular or diffuse cytoplasmic GLUT-1 staining pattern (about 21% of positive cancer samples) (*Fig. 1c*). Among cases with pure cytoplasmic expression of GLUT-1 (29% of all positive cancers), there were also examples of perinuclear staining (about 12% of all positive cases), which was finely granular or coarse-grained and mostly supranuclear, while rarely an infranuclear pattern of GLUT-1 immunostaining also occurred (*Fig. 1d*).

Significant difference in the expression of GLUT-1 was revealed between node-positive and node-negative primary tumors: a higher rate of GLUT-1 immunoreactive cells was found in cancers that gave metastases to lymph nodes (p=0.04). Non-mucinous adenocarcinomas expressed GLUT-1 in significantly greater numbers than mucinous adenocarcinomas (p=0.002). We did not find any statistically significant difference in GLUT-1 expression between moderately (G2) and poorly differentiated (G3) tumors, pT1+pT2 and pT3+pT4 cancers or according to patients' gender or age, or location of the primary neoplasm (*Table 1*).

***Table 1.*** **Differences in the expression of HIF-1α and GLUT-1 in relation to clinicopathologic parameters of colorectal cancers and patients (Pearson's test)**

| *Groups of patients n=123* | | | *GLUT-1* | | | *HIF-1α* | | |
|---|---|---|---|---|---|---|---|---|
| | | *n* | *negative n (%)* | *positive n (%)* | *p* | *negative n (%)* | *positive n (%)* | *p* |
| *N* | pN (-) | 59 | 26 (44.1) | 33 (55.9) | **0.04195** | 5 (8.5) | 54 (91.5) | 0.09902 |
| | pN (+) | 64 | 17 (26.6) | 47 (73.4) | | 12 (18.8) | 52 (81.3) | |
| *T* | pT1+2 | 11 | 6 (54.5) | 5 (45.5) | 0.15340 | 0 (0.0) | 11 (100) | 0.16395 |
| | pT3+4 | 112 | 37 (33.0) | 75 (67.0) | | 17 (15.2) | 95 (84.8) | |
| *G* | G2 | 88 | 27 (30.7) | 61 (69.3) | 0.11467 | 5 (5.7) | 83 (94.3) | **0.00003** |
| | G3 | 35 | 16 (45.7) | 19 (54.3) | | 12 (34.3) | 23 (65.7) | |
| *HP type* | ad | 107 | 32 (29.9) | 75 (70.1) | **0.00237** | 10 (9.3) | 97 (90.7) | **0.0002** |
| | ad-muc | 16 | 11 (68.8) | 5 (31.3) | | 7 (43.8) | 9 (56.3) | |
| *Sex* | male | 65 | 24 (36.9) | 41 (63.1) | 0.62874 | 10 (15.4) | 55 (84.6) | 0.59481 |
| | female | 58 | 19 (32.8) | 39 (67.2) | | 7 (12.1) | 51 (87.9) | |
| *Age* | ≤60 | 40 | 12 (30) | 28 (70.0) | 0.42328 | 9 (22.5) | 31 (77.5) | 0.05285 |
| | >60 | 83 | 31 (37.3) | 52 (62.7) | | 8 (9.6) | 75 (90.4) | |
| *Location* | rectum | 53 | 16 (30.2) | 37 (69.8) | 0.33430 | 7 (13.2) | 46 (86.8) | 0.86377 |
| | colon | 70 | 27 (38.6) | 43 (61.4) | | 10 (14.3) | 60 (85.7) | |

HP type: histopathologic type, pT: depth of invasion, N: lymph node involvement, G: grading of cell differentiation, ad: non-mucinous adenocarcinoma, ad-muc: mucinous adenocarcinoma

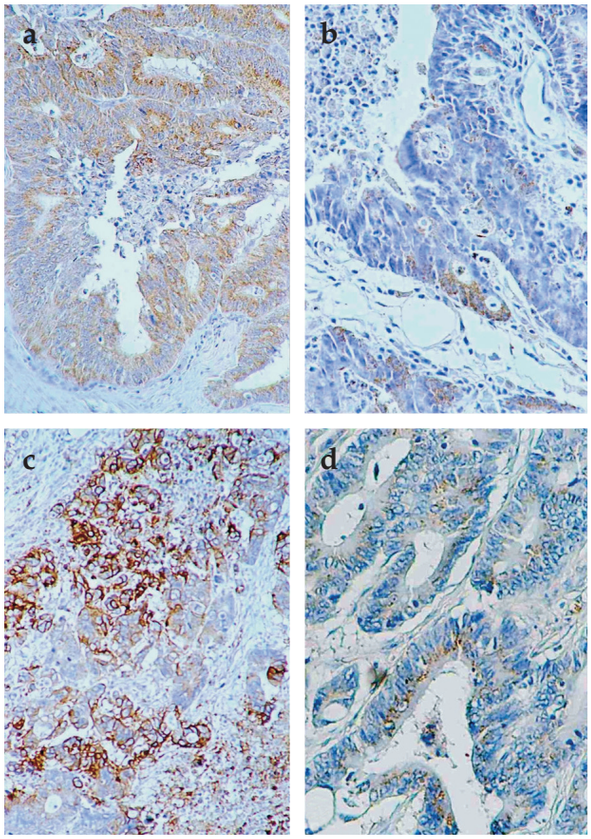


***Figure 1.*** *Immunohistochemistry for HIF-1α and GLUT-1 in human colorectal cancer.* ***(a)*** *Abundant cytoplasmic immunostaining for HIF-1α in gland-like structures of well-differentiated region of colorectal cancer surrounding necrotic foci (x100).* ***(b)*** *Focal and clumped granular, cytoplasmic positivity for HIF-1α dispersed in cells of poorly differentiated colorectal cancer sheet in the vicinity of necrosis (x200).* ***(c)*** *Immunoreactivity to GLUT-1 is intensified predominantly in membranes of colorectal cancer cells in the vicinity of necrosis (x100).* ***(d)*** *Supranuclear accumulation of GLUT-1 in colorectal cancer cells forming gland-like structures (no necrosis present) (x200).*

*Comparison between HIF-1α and GLUT-1 expressions in groups of clinicopathological variables*

The expression of HIF-1α correlated with that of GLUT-1 in the whole patient population, as well as in all groups of various clinicopathological features with the exception of the pT1+pT2 group including relatively small number of cases, which could affect the statistical analysis (*Table 2*).

## *Discussion*

GLUT-1 was shown to be overexpressed in hypoxic cells and to be one of the HIF-1α-induced genes.[2] HIF-1α stimulated survival of cancer cells and expression of GLUT-1, but reversibly dominant-negative HIF-1α accelerated apoptosis and severely decreased expression of GLUT-1 in pancreatic cancer cells.[4] By induction of GLUT-1, HIF-1α improved glucose consumption that increased in hypoxic cancer cells due to glycolysis. The cells tried to adjust to worse circumstances and overexpressed HIF-1α and GLUT-1, which were real proteins of rescue from hypoxic injury. In colorectal cancer cells coexpression of GLUT-1 and HIF-1α was discovered by immunohistochemical analysis of Koukourakis et al[13] who noticed that GLUT-1 positivity was harbored not only by cancer cells but also by the endothelium. Stromal fibroblasts appeared to express HIF-1α and GLUT-1 at low levels while they abundantly expressed monocarboxylate transporter (MCT1) and lactate dehydrogenase 1 (LDH1).

In our studies we observed membranous or cytoplasmic, perinuclear, mostly supranuclear finely granular immunoreactivity to GLUT-1. There were lots of cases with a mixture of these types of protein accumulation. Similarly, two different patterns of GLUT-1 staining (supranuclear and membranous) were also reported earlier in studies of benign colon conditions.[15] Membranous immunoreactivity of GLUT-1, which Fogt et al associated with colon dysplasia,[15] was encountered by us in multifocally necrotizing cancers, in which many cells underwent degenerative changes. GLUT-1 accumulated in the vicinity of necrosis in rectal cancers in other studies.[9] We suggest that the hypoxia and disarrangement of cell structure could associate with membranous location of GLUT-1. The pure supranuclear localization, which was suspected to be caused by specific growth factors,[15] was generally characteristic for cancers with no visible necrosis of examined fields in our studies. In the light of our results we conclude that necrosis strongly affects the pattern of GLUT-1 appearance in cancer cells. The membranous immunoreactivity is a manifestation of functioning GLUT-1 as a transmembrane glucose transporter which is hyperactivated in endangered cells in a hypoxic and necrotizing environment.

The expression GLUT-1 was not significantly higher in tumors with deeper invasion (pT3+pT4) in comparison with more superficial ones (pT1+pT2). The lack of difference could come from the low number of pT1+pT2 cases, which could affect statistical analysis. According to Ito et al, GLUT-1 was coexpressed with factors of neoplastic invasiveness as matrix metalloproteinase-2 (MMP-2) in cell lines of pancreatic cancer. If GLUT-1 was downregulated, levels of this proteinase were also limited, cellular invasiveness was decreased and the metastatic capacity of the tumor was abolished.[16] On the basis of our findings we did not associate upregulation of GLUT-1 with advancement in mural invasion of colorectal cancer. Sakashita et al found that expression of GLUT-1 showed significant differences in well-differentiated colorectal cancers in comparison with moderately and poorly differentiated ones.[17]

***Table 2.*** **Correlation between HIF-1α and GLUT-1 expression in various groups of different clinicopathological features of colorectal cancer (Spearman's correlation test)**

| *Groups of patients* | *Comparison between GLUT-1 and HIF-1α* | | | |
|---|---|---|---|---|
| | | *n (%)* | *p* | *r* |
| *All patients* | | 123 (100.0) | <0.0001 | 0.4492 |
| *N* | *pN (-)* | 59 (48) | <0.0001 | 0.4511 |
| | *pN (+)* | 64 (52) | <0.0001 | 0.5221 |
| *T* | *pT1+2* | 11 (9) | 0.272 | 0.3634 |
| | *pT3+4* | 112 (91) | <0.0001 | 0.4707 |
| *G* | *G2* | 88 (72) | 0.001 | 0.3338 |
| | *G3* | 35 (28) | <0.0001 | 0.6467 |
| *HP type* | *ad* | 107 (87) | <0.0001 | 0.3710 |
| | *ad-muc* | 16 (13) | 0.002 | 0.7088 |
| *Sex* | *male* | 65 (53) | <0.0001 | 0.4351 |
| | *female* | 58 (47) | 0.01 | 0.4381 |
| *Age* | *≤ 60* | 40 (33) | <0.0001 | 0.5825 |
| | *>60* | 83 (67) | 0.001 | 0.3719 |
| *Location* | *rectum* | 53 (43) | 0.015 | 0.3313 |
| | *colon* | 70 (57) | <0.0001 | 0.5229 |

HP type: histopathologic type, pT: depth of invasion, N: lymph node involvement, G: grading of cell differentiation, ad: non-mucinous adenocarcinoma, ad-muc: mucinous adenocarcinoma

Nevertheless, we did not find a significant difference in GLUT-1 expression between G2 and G3 tumors.

In this study we demonstrated that GLUT-1 overexpression was significantly more frequent in cancers with metastases to local lymph nodes, confirming our earlier results obtained from a larger number of patients, in which case the level of significance was greater.[14] Our results are in harmony with the observations of Younes et al who described GLUT-1 as a marker of poor prognosis and lymph node involvement in transitional cell carcinoma of the urinary bladder.[18] Moreover, in our study most node-positive colorectal cancers presented membranous GLUT-1 immunoreactivity (data not shown), which was suggested to predict greater metastasizing potential.[15] Thus, our results showed that increased immunoreactivity for GLUT-1 seems to be a marker of nodal involvement. Association of GLUT-1 expression with carcinomatous involvement of lymph nodes corresponds with the results of Cooper et al, too.[9] They showed that immunoreactivity of the tumor to GLUT-1 predicted shortened survival of rectal cancer patients in association with a faster appearance of metastases. GLUT-1 expression proved a marker of invasiveness of breast cancer cell lines,[19] and a relationship between metastasis-free survival and lack of GLUT-1 expression was discovered in cervical cancer.[20] A question arises as to whether monitoring of GLUT-1 expression can serve as a reliable indicator of disease regression in response to therapy.[9]

HIF-1α overexpression was reported to associate with lymph node involvement in human breast cancer.[21] On the other hand, Yoshimura et al[7] reported that expression of HIF-1α did not correlate with pT stage, grading of histological differentiation and lymph node status in colorectal cancer, but it is worth mentioning that HIF-2 did. Likewise, in our study HIF-1α expression failed to have any relation to the advancement of the growth of colorectal cancer at the primary site. However, in the contrast to Yoshimura's findings, we found positive correlation between HIF-1α expression and grade or different histological types.[7]

Localization of HIF-1α immunoreactivity was various in the different evaluations. It was expected to be inside the nucleus, which would reflect the transcriptional activity of this factor;[7] however, findings appeared to differ from expectations. Human pancreatic endocrine tumors (PET) showed nuclear staining at a low level of histological differentiation, but significantly more well-differentiated benign tumors exhibited intense cytoplasmic staining for HIF-1α.[22] We also associated cytoplasmic appearance of HIF-1α with better differentiated neoplasms. Hypoxia was demonstrated to limit maturation of cancer cells.[23] Moreover, human endometrial carcinomas were characterized by nuclear and cytoplasmic expression of HIF-1α.[24] Some explanations for HIF-1α localization were provided by studies on an orphan nuclear receptor Nur77 that was reported to sustain activation of HIF-1α and stabilize it in human and murine cancer cell lines. Coexpression of Nur77 and HIF-1α caused exclusively nuclear localization of the latter.[25] It cannot be excluded that loss of Nur77 function might lead to cytoplasmic storage of HIF-1α, which we observed.

We would suggest that GLUT-1 and HIF-1α might modulate the progression of colorectal cancer variably. On the basis of our results it is difficult to prove a HIF-1α-dependent induction of GLUT-1,[2,4] because HIF-1α was mostly detected in the cytoplasm and it exerts its transcriptional activity in the nucleus. However, evident positive correlation between HIF-1α and GLUT-1 expressions indicates a close association between these proteins in human colorectal cancer. This linkage was apparent in the whole patient group regardless of the various clinicopathological features considered. In addition, cytoplasmic coexpression of HIF-1α and GLUT-1 is evidence for their simultaneous upregulation. Their extranuclear storage calls for further studies to elucidate its significance in colorectal cancer.

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