Clinicopathological significance of Epo, EpoR, Ki-67 and Bax expression in colorectal cancer

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ABSTRACT

Introduction: Expression of Epo, a glycoprotein secreted by the fetal liver and the adult kidney in response to cellular hypoxia and its receptor have been described in human solid tumors, such as colon and breast cancer.

Purpose: Since activation of Epo-EpoR signaling pathway in erythroid progenitor and precursor cells leads to promotion of proliferation and differentiation or prevention of programmed cell death through Bcl-xl and Bcl-2 it was of interest to investigate expression of Epo, EpoR, apoptosis regulator – Bax and marker of proliferating cells - Ki-67 and assess correlation between them, with regard to clinicopathological variables of colorectal cancer.

Materials and methods: The correlations between expression of Epo, EpoR, Bax and Ki-67 in colorectal cancer were analyzed in regard to patient age, sex, primary localization, histopathological type, grading, staging and lymph node invasion. Statistical analyses were performed by using the Spearman rank correlation test applying a significance level of $p<0,05$.

Results: Correlation between Bax and EpoR is positive and statistically significant at all groups of patients except group pT1+pT2. Positive correlation between Bax and Epo is statistically significant at following groups of patients: all patients, age ≤ 60, age >60, male, female , primary localization in rectum, primary localization in colon, adenocarcinoma, G2, G3. Statistical analysis revealed no significant correlations between expression of neither Ki-67 with Epo nor Ki-67 with EpoR in all groups of patients.

Conclusions: Epo seems to be a pleiotropic cytokine, which can exert its biological effect on several cell types, including neoplastic cells. The effect of Epo-EpoR signaling can differ in various cells and conditions.

Keywords: Colon cancer, erythropoietin, erythropoietin receptor

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INTRODUCTION

Based on rates from 2012 colorectal cancer is the third most common cause of cancer occurrence worldwide with morbidity about 1.4 million people of whom almost 700 000 cases are fatal, making it the fourth leading cause of cancer death [1].

In oncology practice, patients undergoing chemotherapy or radiation therapy due to colorectal cancer have also been treated with recombinant human erythropoietin (rHuEpo) to counteract malignancy-associated anemia, since erythropoietin (Epo) is the pivotal hematopoietic growth factor [2].

Epo is a glycoprotein with molecular weight of 30.4 kDa secreted by the fetal liver and the adult kidney in response to cellular hypoxia [3]. Low tissue oxygen tension results in activation of hypoxia inducible factor-1 (HIF-1), which binds an hypoxia responsive element (HRE) in the 3' flanking region of the Epo gene and mediates the transcriptional response to hypoxia [4]. Epo exerts its biological effects by binding to its specific transmembrane receptor – EpoR. Activation of Epo-EpoR signaling pathway in erythroid progenitor and precursor cells leads to a number of physiological processes, such as promotion of proliferation and differentiation or prevention of programmed cell death through Bcl-xl and Bcl-2 [5,6].

A series of recent studies has indicated that expression of Epo and its receptor – EpoR is not limited to only hematopoietic cells, but has also been found in several nonhematopoietic cell types and tissues [7], which suggested additional, significant functions of Epo in physiological processes other than erythropoiesis. For example, some investigations have emphasized, that Epo is involved in uterine angiogenesis in menstruation cycle or in wound healing process [8,9]. Furthermore, recent study revealed that Epo could contribute to pathological processes, such as neoangiogenesis in proliferative diabetic retinopathy [10].

A challenging problem which arises in this domain is a fact, that expression of EpoR have also been detected in tumor cell lines, such as the myeloma cell line MM- S1 [11] or human melanoma cells [12], which implied that Epo signaling could be involved in several processes in neoplastic tissues. It was reported in the literature that administration of exogenous Epo forced proliferation of MCF7 and BT-549 breast cancer cells up [13]. This effect was also been observed with Caki-2, 786-0 and RAG human renal carcinoma cells [14]. In vitro study on human melanoma cells revealed that Epo is expressed in normoxic condition and its autonomous secretion arising after hypoxia and CoCl2(2) treatment. This study provided also evidences for existence a novel autocrine loop of Epo in melanoma, which might contribute to survival of neoplastic cells in hypoxia [15]. Furthermore, some investigations revealed that expression of Epo and EpoR is responsible for promoting angiogenesis and repressing tumor cells sensitivity to apoptosis [16]. It was reported that in MCF-7 human breast cancer cell line, autonomous secreted Epo, increases expression of Bcl-2 and Bcl-xL, which are known as inhibitors of programmed cell death [17]. In our prior study positive correlation between expression of Epo and Bcl-xL in CRC, was found [18]. In contrast, in our another study on colorectal cancer, some surprising, positive association between presence of Bak, which belongs to the promotors of apoptosis, and Epo, which favors cell survival, was observed [19].

Moreover, expression of two isoforms of EpoR has been described in human solid tumors, such as colon, lung, prostate, ovarian and breast cancer [20]. Also, our earlier in vivo investigation provided evidence for presence of Epo and EpoR in human colorectal cancer [21]. In prostate cancer, expression of Epo and EpoR was uncorrelated with proliferation activity or apoptosis [22]. In contrast, research on human head and neck squamous cell carcinoma reported that administration of recombinant Epo was associated with progression and invasiveness of the tumor [23]. Similarly, a clinical trial on nonanemic patients with metastatic breast cancer was discontinued unexpectedly, because patients, which received rHuEpo were characterized by higher mortality rate compared to placebo group [24].

On the other hand, in series of prior studies on anemic tumor models, rHuEpo beside counteracting anemia, has also been shown as a factor, which increase radio sensitivity and cytotoxicity of chemotherapeutics [25,26].

This data suggests that in malignancy, association between Epo-EpoR signaling pathway and its biological effects on neoplastic cells, such as promotion of proliferation, apoptosis or modulation of response to chemotherapy or radiation therapy, is not yet clear. This remains an open question as treating anemic cancer patients with rHuEpo could have adverse effects, such as promotion of tumor growth and progression.

For present study, it was of interest to investigate expression of Epo, EpoR, marker of proliferating cells - Ki-67 and apoptosis regulator – Bax and assess correlation between them, with regard to clinicopathological variables of colorectal cancer.

MATERIALS AND METHODS

Tissue Samples

The excision specimens were collected from 97 patients undergoing surgery for primary colorectal cancer, prior to chemotherapy or radiation therapy. None of these patients had history of rHuEpo administration.

Research was carried out in accordance with the principles of Declaration of Helsinki. Protocol of the study was approved by the Local Ethical Committee at the Medical University of Bialystok.

Among 97 tumors 56 of them had primary localization in colon and 41 in rectum. Histopathological analysis revealed 83 of adenocarcinoma cases and 14 of mucinous adenocarcinoma cases.

Tumors were classified, according to their extramural depth of invasion, into the following groups: pT1+pT2 for tumors assessed as pT1 or pT2, which counted 8 cases and pT3+pT4 for tumors assessed as pT3 or pT4, which counted 89 cases.

Furthermore, in agreement with guidelines of World Health Organization 69 tumors were classified as moderately differentiated (G2) and 28 tumors were classified as poorly differentiated (G3). Lymph node invasion was positive in 51 samples.

Tissue Staining

The tissue sections were routinely preserved by immersion in 10% buffered formaldehyde, embedded in paraffin and cut on the microtome (Microm H340) into 5 μm thick slices. Prior to staining with hematoxylin and eosin the slices were dewaxed in xylens and rehydrated in graded alcohols. After staining slides were used for routine histopathological analysis.

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using following primary antibodies purchased from Santa Cruz Biotechnology: Epo (rabbit polyclonal, H-162), EpoR (rabbit polyclonal, C-20), Bax (goat polyclonal) and from Dako: Ki-67 (mouse monoclonal, MIB-1).

Prior this procedure, endogenous peroxidase activity was blocked, during incubation with a 2% hydrogen peroxide for 10 minutes.

Subsequently, heat-induced epitope retrieval using microwaves, was applied.

In order to prevent non-specific bonds, incubation with blocking serum, was provided.

Slides with known positive immunostaining cells for Epo, EpoR, Ki-67 and Bax formed positive controls, whereas in negative controls the primary antibodies were omitted.

In order to evaluate immunohistochemical reaction neoplastic cells with positive staining for Epo, EpoR, Ki-67 and Bax were calculated in 10 representative fields of each slide by 2 pathologists.

The evaluation was performed using a light microscope at magnification of 200x.

The staining intensity was presented in percentage and classified as follows: 0, if less than 10% cells were immunoreactive; 1, if positive staining was detected in 10 to 50 % of neoplastic cells; and 2, if more than 50% of cancer cells were positive for tested antigens.

Statistical Analysis

The correlations between expression of Epo, EpoR, Ki-67 and Bax in colorectal cancer were analyzed in regard to patient age, sex, primary localization, histopathological type, grading, staging and lymph node invasion.

Statistical analyses were performed by using the Spearman rank correlation test applying a significance level of $p<0,05$.

RESULTS

Correlation of Ki-67, Epo and EpoR expression in Colorectal Cancers

Statistical analysis revealed no significant correlations between expression of neither Ki-67 with Epo nor Ki-67 with EpoR in all groups of patients.

Correlation of Bax and Epo expression in Colorectal Cancers

As shown in Table 1. the positive correlation between Bax and Epo is statistically significant at following groups of patients:

- all patients ($r=0,569$; $p<0,01$),
- age ≤ 60 ($r=0,665$; $p<0,01$),
- age >60 ($r=0,516$; $p<0,01$),
- male ($r=0,524$; $p<0,01$),
- female ($r=0,620$; $p<0,01$),
- primary localization in rectum ($r=0,422$; $p=0,006$),
- primary localization in colon ($r=0,661$; $p<0,01$),
- adenocarcinoma ($r=0,554$; $p<0,01$),
- G2 ($r=0,526$; $p<0,01$),
- G3 ($r=0,513$; $p=0,005$),
- pT3+pT4 ($r=0,629$; $p<0,01$),
- N(-) ($r=0,587$; $p<0,01$)
- pN(+) ($r=0,562$; $p<0,01$).

According to J. Guilford scale in all this cases correlation was classified as strong except group of patients with primary localization of tumor in rectum ($r=0,422$), where correlation was classified as moderately strong.

Correlation of Bax and EpoR expression in Colorectal Cancers

As shown in Table 2. correlation between Bax and EpoR is positive and statistically significant at all groups of patients except group pT1+pT2 ($r=0,611$; $p=0,108$). According to J. Guilford scale in group of female this correlation was classified as very strong ($r=0,708$).

In following groups of patients correlation was classified as moderately strong: male ($r=0,400$), G3 ($r=0,457$), pN(+) ($r=0,455$). In the case of the others, correlation was classified as strong.

Table 1. Analysis of correlations between Epo, Ki67 and Bax expressions in primary colorectal cancers. Spearman's correlation rank test.

Groups of patients			Epo – Ki67		Epo – Bax	
		n	p	r	p	r
All		97	0.308	0.105	<0.001	0.569
Age	≤ 60	31	0.872	0.030	<0.001	0.665
	> 60	66	0.235	0.148	<0.001	0.516
Sex	male	47	0.972	0.005	<0.001	0.524
	female	50	0.160	0.202	<0.001	0.620
Localization	rectum	41	0.315	0.161	0.006	0.422
	colon	56	0.677	0.057	<0.001	0.661
HP – type	A	83	0.719	0.040	<0.001	0.554
	MA	14	0.177	0.382	0.070	0.497
G	G2	69	0.639	0.057	<0.001	0.526
	G3	28	0.226	0.236	0.005	0.513
T	pT1+pT2	8	0.126	0.588	0.917	-0.044
	pT3+pT4	89	0.483	0.075	<0.001	0.629
N	pN(-)	46	0.492	0.104	<0.001	0.587
	pN(+)	51	0.452	0.108	<0.001	0.562

n – number of cases; **p –** significance level; **r –** correlation coefficient; **A –** adenocarcinoma; **MA –** mucinous adenocarcinoma; **G2 –** moderately differentiated; **G3 –** poorly differentiated; **N(-) –** negative lymph node invasion; **N(+) –** positive lymph node invasion;

Table 2. Analysis of correlations between EpoR, Ki67 and Bax expressions in primary colorectal cancers. Spearman's correlation rank test.

Groups of patients			EpoR – Ki67		EpoR – Bax	
		n	p	r	p	r
All		97	0.465	-0.075	<0.001	0.565
Age	≤ 60	31	0.201	-0.236	<0.001	0.664
	> 60	66	0.964	0.006	<0.001	0.513
Sex	male	47	0.309	-0.152	0.005	0.400
	female	50	0.969	-0.006	<0.001	0.708
Localization	rectum	41	0.307	-0.163	<0.001	0.579
	colon	56	0.895	-0.018	<0.001	0.552
HP – type	A	83	0.288	-0.118	<0.001	0.559
	MA	14	0.967	-0.012	0.048	0.537
G	G2	69	0.145	-0.177	<0.001	0.601
	G3	28	0.566	0.113	0.014	0.457
T	pT1+pT2	8	0.671	0.179	0.108	0.611
	pT3+pT4	89	0.391	-0.092	<0.001	0.557
N	pN(-)	46	0.659	-0.067	<0.001	0.687
	pN(+)	51	0.567	-0.082	<0.001	0.455

n – number of cases; **p –** significance level; **r –** correlation coefficient; **A –** adenocarcinoma; **MA –** mucinous adenocarcinoma ; **G2 –** moderately differentiated; **G3 –** poorly differentiated; **N(-) –** negative lymph node invasion; **N(+) –** positive lymph node invasion;

DISCUSSION

The overall goal of this work was to compare expression of Epo and EpoR with expression of proliferation marker – antigen Ki-67 and apoptosis regulator – Bax.

We did not find evidences for association between expression of Epo or EpoR and increased proliferation ratio, which was assessed by the counting of Ki-67 positive cells of colorectal cancer. A similar conclusion was reached in in vitro investigation on the three tumor cell lines [27]. In contrast, in vitro study on DLD-1 and HT-29 colon cancer cell lines, which was confirmed in in vivo study with nude mice, revealed that stimulation with exogenous Epo promotes cell proliferation in EpoR positive cells trough phosphorylated Akt kinase [28]. The contradictory evidence for increasing proliferation of malignant cell, due to exogenous Epo administration and its interaction with EpoR, could have several possible interpretations. Cells derived from various tumors or tumor models used in in vitro studies may differ in response to EpoR stimulation. Another possibility is that Epo-EpoR signaling may not be functional in some conditions

or counteracting mechanisms, like apoptosis, may be prevalent. Further, we must take into consideration a fact that some investigations assessing expression of EpoR protein in tumors cells were made by using immunohistochemical methods and others using RT-PCR (at mRNA level), what could lead to different results. There is a hypothesis that in some cases detection of EpoR expression in tumor cell lines is limited by lack of specificity of available anti-EpoR antibody, which were used in previous studies [29].

Our results demonstrated also that there is positive correlation between expression Epo as well as EpoR and apoptosis promotor – Bax, in colorectal cancer cells. This surprising finding is consistent with our previous research on colorectal cancer tissue, where we found a positive association of Epo and EpoR with Bak [19]. Oncogenic transformation makes cells resistant to proapoptotic signals, disturbing the balance between cells proliferation and programmed cell death. Deregulation of apoptosis is the leading cause of tumor progression. Coexpression of Epo, which was documented as safeguard factor against apoptosis, which promotes cell survival [6] and apoptosis promotor – Bax, could be explained in several ways. Due to a number of genetic alternations in neoplastic cells, Bax protein despite its increased expression in cells, may be not a functional signaling transducer. As well each one member of death signaling pathway may be defective. On the other hand, the proproliferative influence of Epo with coexisting proper function of Bax protein may increase cellular turnover with prevalence of dividing cells. Bax protein could also be inhibited by prosurvival Bcl-2 protein, which is involved in Epo-mediated signal transduction against apoptosis in erythroid progenitor cells [6].

Although recently both in vitro and in vivo study on DLD-1 and HT-29 colon cancer cell lines, reported that combination treatment with the Bruton's tyrosine kinase inhibitor – LFM-A13 and Epo results in intensification the proapoptotic activity of LFM-A13 [30]. This mechanism is not fully understood, nevertheless implies that Epo action may differ depending on blockage of some cellular signal pathways.

CONCLUSIONS

Epo seems to be a pleiotropic cytokine, which can exert its biological effect on several cell types, including neoplastic cells. The effect of Epo-EpoR signaling can differ in various cells and conditions. It could promote survival, give a signal for proliferation and serve as antiapoptotic factor, but also Epo could serve as a factor which increases effectiveness of the anticancer therapy. This influence can be mediated by augmentation of proapoptotic activity of anti-tumor therapy.

Conflicts of interest
The authors declare that there is no conflicts of interest regarding the publication of this article.

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