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ORIGINAL PAPER

EPO-R Expression Patterns in Resected Gastric Adenocarcinoma Followed by Adjuvant Chemoradiation Treatment

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Abstract The primary aim was to determine whether Epo-R immunohistochemical expression is related to disease free survival (DFS) in specimens of GC from patients who underwent adjuvant chemoradiation. Specimens of gastric adenocarcinomas obtained from 44 patients who had undergone curative gastrectomy and adjuvant treatment were investigated immunohistochemically expression of Epo-R. Three patterns for Epo-R staining were defined: Pattern A (secretory cells-like staining), Pattern B (parietal-like staining) and Pattern C (chief-like staining). Median DFS was 38 months (CI 95%: 33–43) and 15 months (IC 95%: 3–27) in the pattern *B* and *C*, respectively, but it was not reached in the pattern A ($p = 0.06$). Our findings suggest that there may be a relationship between Epo-R expression and DFS in the patients with GC resected.

Keywords Gastric cancer · Epo-R · adjuvant chemoradiation

Introduction

Erythropoietin (Epo) is a glycoprotein hormone responsible for regulating the erythropoiesis [1]. Epo normally is produced by the kidney and liver in adults. Its gene expression is modulated by tissue hypoxia [2]. Epo acts by binding to Epo-specific receptors (Epo-R), which belong to the citokine receptor type I superfamily. EpoR stimulation in erythroblasts promotes proliferation and differentiation, leads to increased expression of the antiapoptotic proteins Bcl-2 and Bcl-Xl, and inhibits the apoptosis [3]. Epo-R is expressed by a variety of cell types as well, including endothelial cells, neurons [4], mammary epithelial cells [5], and human endometrial cells suggesting a wider biologic role for Epo signalling unrelated to erythropoiesis. Epo also stimulates proliferation and migration of human endothelial cells and promotes angiogenesis. Major signal-transduction pathways activated by Epo include the Jak/signal transducer and activator of transduction (STAT) and Ras/mitogen-activated protein kinase pathways, which are involved in the inhibition of apoptosis and the stimulation of cell proliferation in response to this hormone [6, 7].

Previous studies have shown that cultured human breast and cervical carcinoma cells, as well as other carcinoma cells, express high levels of Epo/Epo-R and both mRNA and protein [6, 8].

These authors have demostrated that exposure of tumour cells transfected with Epo-R to recombinant human Epo (rHuEpo) stimulated tyrosine phosphorylation and subsequent DNA synthesis and proliferation, suggesting that Epo signalling is biologically active in malignant cells [6, 7]. Recently, Mohyeldin has found a correlation between Epo and Epo-R expression and malignant progression in head and neck squamous cancer [9, 10].

Gastric cancer (GC) remains one of the leading causes of cancer related deaths worldwide. Many biological factors

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(p53, Bcl-2, COX-2, c-erb-B2, E-cadherin and Beta-catenin) have been implicated in the genesis and progression of sporadic gastric cancer. However, there are very few data in the medical literature about the role of Epo-R in the carcinogenesis or tumoral progression. This fact is of particular importance, especially in these patients who usually have a high incidence rate of multifactorial anemia [8].

It was the primary aim of the present investigation to determine whether Epo-R immunohistochemical expression is related to disease free survival (DFS) in specimens of GC from patients who underwent adjuvant treatment in accordance with MacDonald scheme [11]. Secondary aims were as follows:

a) To establish the correlation between Epo-R expression and the following clinicopathological variables: stage, differentation grade, and classification systems by Lauren and Bormann.
b) To establish the potential relationship between Epo-R expression and further proteins of interest in GC carcinogenesis and progression, such as p53, COX-2, c-erb-B2, E-cadherin and β-catenin.

Materials and Methods

Patient Population and Tumor Samples

Specimens of primary gastric adenocarcinomas, their paired adjacent normal gastric mucosa and node metastases were obtained from 44 patients who underwent curative gastrectomy and adjuvant treatment based on MacDonalds protocol (5-Fluoroucil and concomitant radiotherapy) [10]. Tissue samples were fixed in formalin and embedded in paraffin according to standard procedures. Sections (4 μm thick) were cut and mounted on glass slides.

Clinical and Pathological Data

We reviewed the clnical records from 44 patients. Clinical data, such as pathological classification according to TNMp (IB, II, IIIA, IIIB, IVA) were recorded. Pathological data taken from the clinical records were differentation grade (G1, G2 y G3) according to WHO classification system; histologic subtype according to Lauren (intestinal and difusse) classification system [12], including the mixed subtype defined by Carneiro [12]; Borman classification system (I: polypoid lesions, II: ulcerated lesions with raised edges, III: ulcerated lesions with stomach wall invasion, IV: infiltrative diffuse lesions, and V: non-categorized lesions). In order to simplify the analysis, tumors were classified into two groups as follows: excrescent (I and II of Borman), and ulcerative (III and IV). We found no cases included in the subtype V [13].

Immunostaining

A rabbit polyclonal antibody against the Epo-R (C20: Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. The anti-Epo-R antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping a carboxy-terminus of Epo-R of human origin. Briefly sections were collected on 3-amino-propyl-triy-thoxysilane-coated slides, the paraffin was extracted by the xylen ethanol scale and in Tris buffer saline (TBS, Ph 7.6) and incubated overnight at 4°C with the Mab 110 (1:200 in TBS). The inmunodetection was performed with alkaline phosphatase-anti-alkaline phosphatase (APAAP; Dako, Carpinteria, CA) and Fast Red as chromogen. Internal negative controls included preincubation with a 10-fold excess of specific blocking peptide (Santa Cruz) for the polyclonal antibody against Epo-R. Slides of kidney were used as positive control. An built-in negative control was done in each case by omission of the primary antibody. The specificity of the antibodies were confirmed previously [8, 14, 15].

Microscopical Analysis

Two investigators independently evaluated Epo-R staining under a ligth microscope at a magnification of 40×. Five images of representative areas were acquired for each specimen. Cases without valid internal controls were excluded. Those cases presenting 100% staining of membrane/cytoplasm of tumor cells within the five fields examined were considered positive. A part from the adenocarcinoma mucosa, Epo-R expression was examined in non-tumoral mucosa and metastatic nodes in cases N1, N2, and N3.

In tumor mucosa, three patterns for Epo-R immunoexpression were defined by their similarity to staining intensity shown by the different cell subtypes of the the normal mucosa [16]: *Pattern A,* 100% of tumor cells stained with weak intensity (+) similar to that observed for mucosa secretory cells; *Pattern B,* 100% of cells stained with moderate intensity (++) comparable to that of normal mucosa parietal cells; *Pattern C:* 100% of cells stained with strong intensity (+++) equivalent to that found in chief cells of the mucosa.

Immunoexpression of p53, c-erb-B2, COX-2, β-catenin, E-cadherin, and proteins involved in carcinogenesis and gastric progression was analyzed both qualitatively (membrane, cytoplasm and nucleus) and quantitatively.

p53 Qualitative analysis allowed defining the staining pattern of both, normal and tumoral tissues. The number of positive cells was determined by counting 100 cells within five areas. The result was considered as being

negative when less than 10% of nuclei became stained. It was regarded positive when more than 10% of nuclei became stained.

C-erb-B2 The criterion for positivity for this antibody was as follows: positive membrane staining but with no staining in cytosol. When 25% of cells in gastric mucous membrane showed a positive staining, it was considered to be positive. Positive cell number was determined by counting 100 cells within 5 areas. Slides of breast carcinoma with an intense staining of HER-2 (3+, FISH positive) was used as positive control [17, 18].

COX-2 During the quantitative analysis, the positive percentage was classified into five subgroups as follows: 0 (no staining), 1 (focal or less than 10%), 2 (10–30%), 3 (30–50%), and 4 (>50%). Furthermore, staining intensity was assessed with a 0–4 scale, in, which 0 as no staining and 4 as intense staining. The final score for staining was determined by the product of intensity by positive percentage. Thus, 0 was considered negative, 1–4 weak, 5–8 moderate, and 9–16 intense. In order to facilitate the data analysis, two groups were established: 1) negative or weak staining, and 2) moderate or intense staining. It was stablished that there was overexpression when staining was either moderate or intense.

β-catenin and E-cadherin Positive immunostaining was considered when more than 50% of mucous membrane cells were positive in both cases. Intensity expression was not evaluated because it was similar in all cases. Positive cells number was determined by counting 100 cells within five areas. E-cadherin and β-catenin conserved expression in gastric carcinoma mucosa was used as positive controls [19].

Statistical Analysis

Data statistical analysis was performed by means of the computer program SPSS 13.0. Epo-R expression was correlated with relapse-free survival (RFS) with Kaplan Meier method. Log-rank and Wilcoxon tests were used for comparisons between the different expression patterns. The correlation between expression patterns and the different clinicopathological variables (i.e., stage, differentation grade, histologic subtype) was determined by univariate analysis by applying χ^2test. Epo-R expression predictive value was determined by means of the interaction test between the type of immunohistochemical staining and DFS. Multivariate analysis for DFS was carried using the Cox proportional hazard model, in, which parameters with $p<0.05$ were included.

Results

1. Patients population and tumours

Table 1 shows a summary of clinicopathological features of 44 patients with gastric adenocarcinoma included: 33 males and 11 females, aged from 27 to 74 years. According to histological stage: 2 IB, 11 II, 16 IIIA, 13 IIIB and 4 IV A. Various pathological features as histological subtype (Lauren clasiffication), differentation grade (OMS classification) and proximal or distal site.

Table 1 Clinico-pathological features

Variable	N cases	%
Age		
-Median	62.5 years	
-Interval	(27 a 74)	
ECOG		
-0	32	2.7
-1	10	22.7
-2	2	4.5
Sex		
-Male	33	75
-Female	11	25
Node dissection		
-D1	29	65.9
-D2	15	34.1
N metastatic nodes		
-<4	15	34
-4–8	18	41
->8	11	25
Stage		
Early		
-IB	2	4.5
-II	11	25
Advanced		
-IIIA	16	36.6
-IIIB	13	29.5
-IVA	2	4.5
Lauren classification		
-Intestinal	22	50
-Mixed	13	29.5
-Diffuse	9	20.4
Differentation (OMS classification)		
-G1	0	0
-G2	9	20.4
-G3	35	79.5
Location		
-Corpus	27	61.3
-Cardia	8	18.1
-Pyloro	6	13.6
-Stump	3	6.8
Relapse after 2 years	15	34
-Locally	5	33.3
-Distance	10	66.6

2. Inmunostaining

- Epo-R expression in adjacent non-tumoral gastric mucosa

All gastric mucous cells showed positive membrane and cytoplasmatic immunostaining patterns with different intensity: Mucus-secreting cells exhibited weak staining (+), and parietal cells were moderately (++) stained, whereas chief cells showed strong immunostaining (+++) (Fig. 1a and b). Macrophage cells and fibroblasts were not stained for this antibody. Endothelial cells and cytosol neurons displayed moderate stain for Epo-R similar to parietal cells (Fig. 2). So, as previously mentioned, we defined three patterns of stain in order to elaborate a comparision with cancer samples (Table 2).

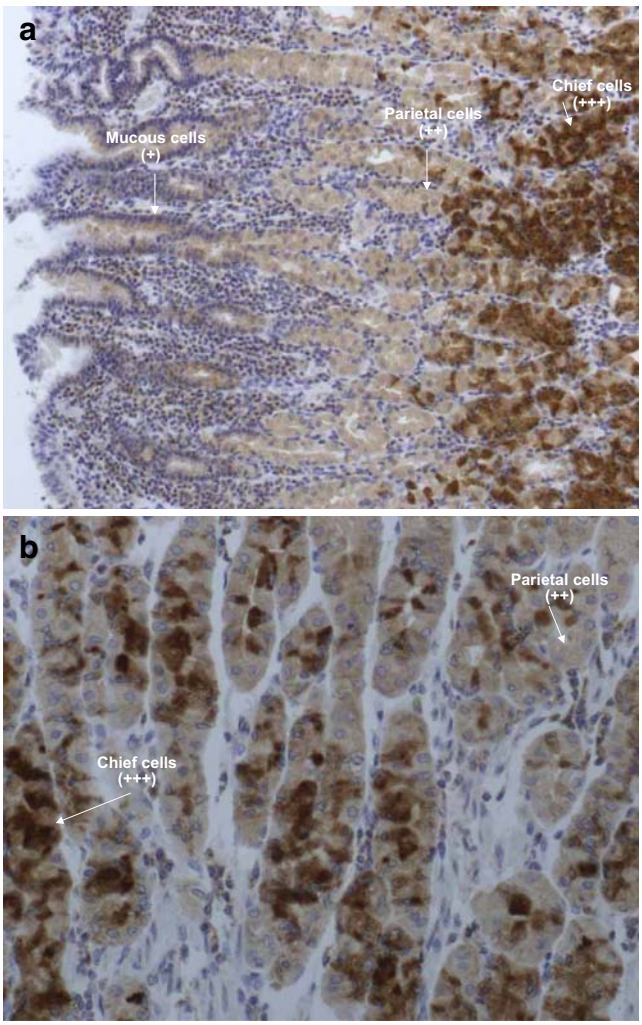

Fig. 1 a, **b** Gastric mucous cells showed positive membrane and cytoplasmatic inmunostaining patterns with different intensity: Mucus-secreting cells exhibited weak staining (+), and parietal cells were moderately (++) stained, chief cells showed strong inmunostaining (+++)

- Epo-R expression in gastric carcinoma samples

In the tumor specimens we analyzed, three immunohistochemical expression patterns were defined based on similarity to stain intensity showed by the different cell subtypes of the normal mucosa: *Pattern A*; this pattern was assigned when tumor mucosa became uniformly stained with weak staining intensity (+), similar to that showed by mucosal secretory cells. *Pattern A* was seen in eight tumor specimens (Fig. 3a). *Pattern B*; was assigned when cells became homogenously stained with moderate intensity (++) comparable to that encountered in normal mucosal parietal cells. Most cases (30 tumor specimens) showed pattern B (Fig. 3b). *Pattern C*; was assigned when an intense stain was observed (+++), comparable to that seen in normal mucosal chief cells. Six tumoral cases showed the pattern C (Fig. 3c). The results of the inmunohistochemical assays of gastric adenocarcinoma are summarized in Table 3.

- Epo-R expression in node metastases of gastric adenocarcinoma

The results of the inmunohistochemical assays of node metastases of gastric adenocarcinoma are summarized in Table 3. Among the selected cases, we found only 39 patients with ganglionar involvement. In these cases, immunohistochemical analysis for Epo-R was carried out in the involved nodes. There were only 7/39 (17.9%, IC 95% 14, 3–19.5) with *pattern A* expression. However, 27/39 (69.2%, IC 95% 60.2–71.3) showed *pattern B* expression and 5/39 (12.8%, IC 95% 9.3–15.2) presented inmunostaing

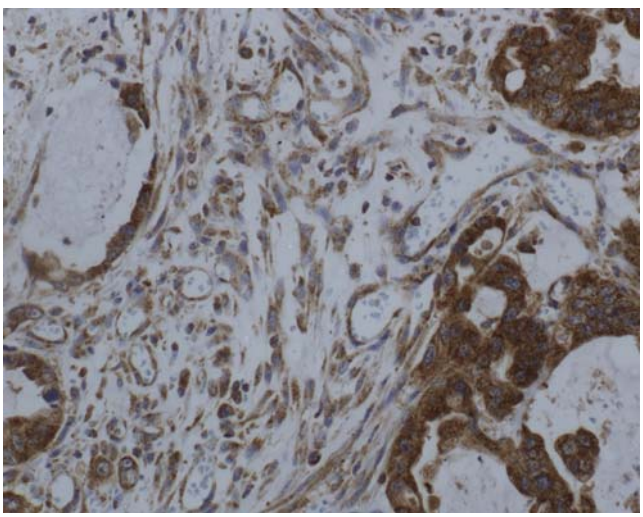

Fig. 2 The picture shows endothelial cells and cytosol neurons displayed moderate stain for EPO-R similar to parietal cells (++)

Table 2 EPO-R patterns and clinical-pathological features correlation

Pattern of inmnunochemical expression of EPO-R	Clinical-patological feature	Inmunohistochemical expression			P
	Stage	Early		Advanced	0.08
A		2 (15.3)		6 (19.3)	
B		7 (53.8)		23 (74.1)	
C		4 (30.7)		2 (6.4)	
	Histological subtype	Intestinal	Mixed	Diffuse	0.02
A		0 (0)	1 (12.5)	7 (87.5)	
B		18 (81.8)	10 (76.9)	2 (22.2)	
C		4 (18.2)	2 (15.3)	0 (0)	
	Differentiation	G1	G2	G3	0.1
A		0 (0)	0	8 (22.8)	
B		0 (0)	5 (55.5)	25(71.4)	
C		0 (0)	4 (44.4)	2 (5.7)	
	Location	Proximal		Distal	0.3
A		1 (12.5)		7 (19.4)	
B		6 (75)		24 (66.6)	
C		1(12.5)		5 (13.8)	
	H. Pylori infection	Positive		Negative	0.12
A		1(12.5)		7 (19.4)	
B		5 (62.5)		25 (69.4)	
C		2 (25)		4 (11.1)	
	Relapse	Yes		No	0.1
A		1 (6.6)		7 (24.1)	
B		10 (66.6)		20 (68.9)	
C		4 (26.6)		2 (6.8)	

similar to *pattern C*. Differences between Epo-R expression in tumor *vs.* metastatic nodes were not found.

3. Survival and Epo-R pattern expression

The median follow-up in our serie was 25.5 months (range: 7–48 months). Median overall survival (OS) was not reached with this median follow-up. Median RFS was 38 months (CI 95%: 33–43) and 15 months (IC 95%: 3–27 months) in the groups *B* and *C*, respectively. In the group *A*, median DFS was not reached.

RFS analysis in relation to Epo-R expression pattern was carried out by means of the Kaplan-Meyer method. Survival curves for the three cases were compared each other by the Log-rank test, which showed that the longest DFS was that for the group *A*, though this difference did not reach statistical significance ($p=0.06$) (Fig. 4a).

4. Survival and clinical-pathological features

As far as stage is concerned, although the early-stage group was found to have a more marked trend to a longer DFS relative to the advanced-stage group, this difference did not reach statistical significance ($p=0.30$ and $p=0.06$, respectively) (Fig. 4b). When histologic subtype was analyzed, we noted that, despite the intestinal variant showed a more marked trend to a longer DFS relative to the other variants, this difference did not reach statistical significance either ($p=0.15$ and $p=0.13$, respectively) (Fig. 4c).

Multivariate analysis revealed that Epo-R expression pattern, stage and subtype all were independent factors with prognostic value that was independent of RFS ($p=004$, $p=0.034$, $p=0.054$, respectively).

5. Epo-R inmunoexpression and clinicopathological features

Table 2 shows Epo-R inmunoexpression related to clinical and selected pathological features. While we did not find predominance of staining *pattern B* in the advanced stage relative to the other stages (19.3%, CI 95%: 17.8–22.5 *A*, 74.1%, CI 95%: 69.2–78.5 *B* and 6.4% CI 95%: 3.9–9.1 C, respectively), the differences did not reach statistical significance ($p=0.08$). With regard to histologic subtype, we noted that most of diffuse cases showed weak staining (*pattern A*) in the intestinal variant (87.5% *vs.* 0%, respectively), while in the intestinal variant most of cases showed *pattern B* (81% *vs.* 22.2%, respectively) ($p=0.02$). We did not find a conclusive relationship

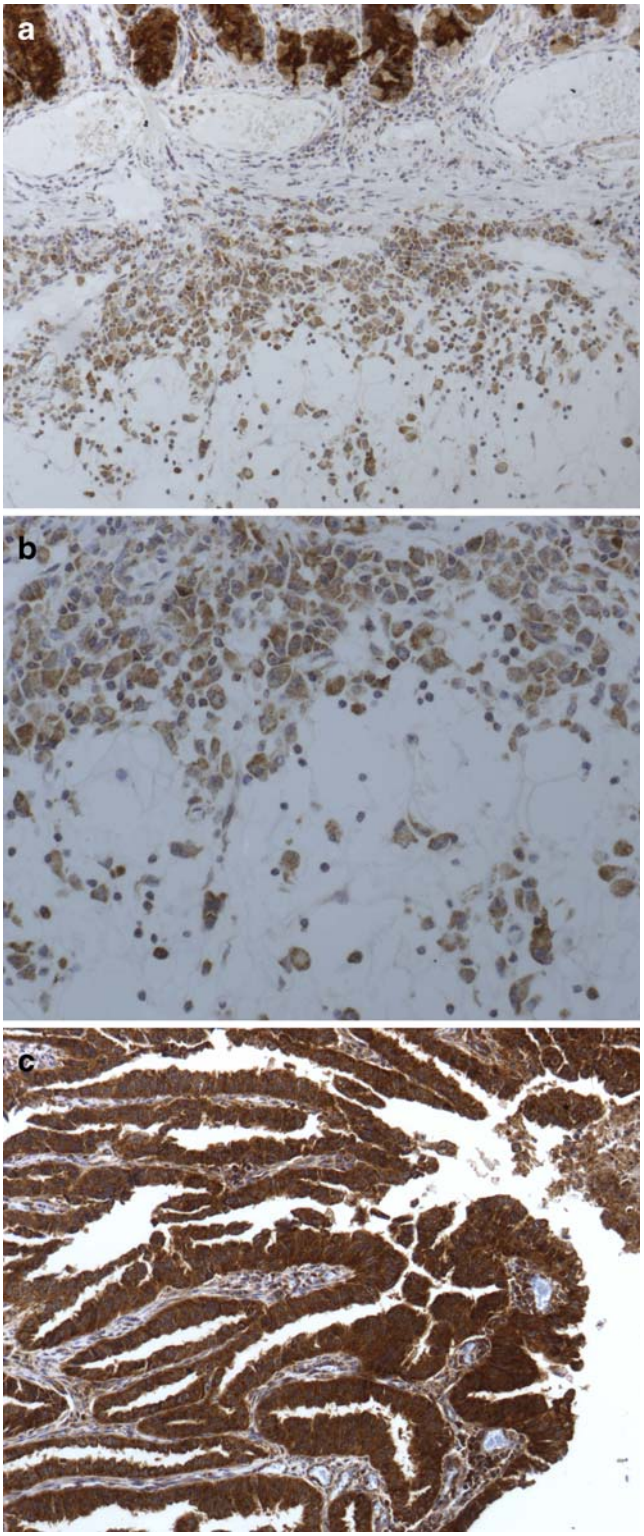

Fig. 3 **a** Weak staining intensity (+) similar to that showed by mucosal secretory cells (*pattern A*). **b** The picture shows moderate intensity comparable to that encountered in normal mucosal parietal cells (*pattern B*). **c** The picture shows intense staining comparable to that seen in normal mucosal chief cells (*pattern C*)

Table 3 Quantitative analysis of EPO-R inmunoexpression

Inmunoexpression pattern of EPO-R	*N* cases/% Primary Tumour	*N* cases/% Node metastases
Positive	44(100)	39(100)
A	8(18.1)	7(17.9)
B	30(68.1)	27(69.2)
C	6(13.6)	5(12.8)
Negative	0(0)	0(0)

between Epo-R staining pattern and the remaining clinicopathological variables under study (Table 2).

6. Epo-R inmunoexpression and some of gastric carcinogenesis proteins

We were also interested in analizing the relationship between Epo-R expression and other protein involved in gastric carcinogenesis: p53, COX-2 and molecular adhesion proteins as E-cadherin and β-catenin. We did not find a statistical association beetwen previous mentioned proteins and Epo-R expression in gastric adenocarcinoma (p= 0.15) (Table 4).

Discussion

Currently, there are no studies available assessing Epo-R expression role in progression or RFS in GC. To our knowledge, only Ribatti has studied Epo-R expression by means of immunohistochemical techniques in 40 specimens of GC, and established the relationship of Epo-R expression to the stage, differentation grade and the tumoral angiogenesis [20]. In addition, there are few studies carried out with cell cultures aimed to determine Epo-R role in tumoral progression. We found two studies based on cell cultures from CG in the rat transfected with Epo-R. In these studies, the authors noted that the addition of recombinant erythropoeitin acted as a trophic and mitogenic factor [21, 22]. Recently, a number of published studies have characterized by the use of microarrays that try to define different expression profiles with prognostic value and useful for predicting treatment response. However, among the selected genes, Epo or Epo-R gene have not been included in these studies [22–28].

On the other hand, Epo-R immunoexpression has been previously studied in other tumors, and a positive relationship between the expression of this protein and the stage has been found for breast [5], leukemia [29], endometrial [8], melanoma [30], head and neck [9], and transitional bladder cancers [31]. Nevertheless, some studies have yielded conflicting results, and one of them attributes positive prognostic value for papillary thyroid cancer to the overexpression of this protein [32].

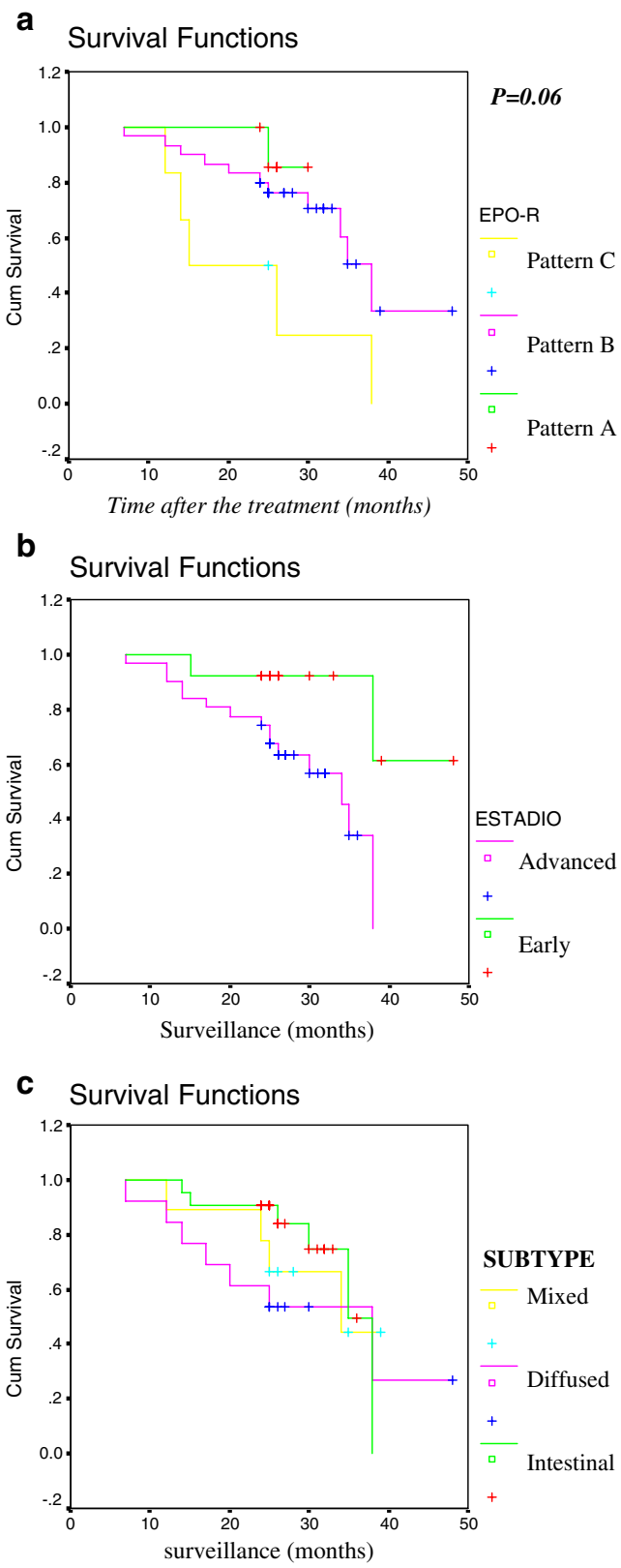

Fig. 4 **a** Survival curves show that the longest DFS was that for the group A, though these differences did not reached statistical significance (p=0.06). **b** Survival curves show that the early staged group has a marked trend to a longer DFS compared to advanced staged group but the difference did not reached statistical significance (p=0.06). **c** Survival curves show that intestinal variant has a marked trend to a longer DFS compared to the other variants, but the difference did not reached statistical significance (p=0.15)

Taking into account the shortage of studies in the medical literature on the role of Epo-R in predicting failure in GC and knowing that a number of earlier investigations had been published reporting that Epo-R immunoexpression might be of certain prognostic value for tumors other than GC [7, 29, 33, 35], we decided to analyze the role of Epo-R expression in 44 specimens of CG. Firstly, we noted that Epo-R staining for GC corresponded to three differential patterns: *Pattern A*, staining comparable to that of epitelial cells of non-tumoral mucosa; *Pattern B*, staining similar to that of parietal cells; and *Pattern C*, intense staining similar to that seen in the chief cells. To our knowledge, the present investigation is the first one that analyzes the relationship between RFS and Epo-R expression patterns in GC patients undergoing adjuvant treatment after surgery. In these patients, we have seen a nearly statistically significant (p=0.06) potential association between Epo-R expression pattern and RFS. Thus, patients presenting *type A* staining did not reach the median RFS after a median follow-up of 26 months. The median follow-up was 38 and 15 months in the *type B* and *C*, respectively. The selected patients had been treated with radiation therapy and chemotherapy after surgery, according to the scheme described by MacDonald [11]. Our patients reached a median time to failure of 35 months, which is slightly longer than that achieved by MacDonald (29 months). Theoretically, the benefit for RFS from the administration of this adjuvant scheme may have biased our results, which suggests that it may be necessary to extend the present investigation to include treatment-naive patients with early or advanced GC. Another factor that may influence the interpretation of the results from the present study is the presence of anemia. Most patients included in our study presented multifactorial anemia in some time point along their clinical course, in part due to tumor bleeding of variable severity either before operation or during the perioperative period. While the effect of acute anemia on intratumoral hypoxia has been well recognized [36], the relationship between chronic anemia and intratumoral hypoxia in GC patients remains unclear [37–39].

As secondary aims of our study, we were interested in determining whether or not Epo-R expression is related to a number of clinicopathological variables, such as stage, differentation grade and histologic subtype. As far as stage is concerned, we found a trend to a longer DFS in the early

Table 4 Correlation between EPO-R expression and other proteins involved in gastric carcinogenesis

	Bcl-2	P53	ki-67	COX-2	c-erb-B2	Beta-catenine	E-cadherine
EPO-R expression (*p* value)	0.3 (NS)	0.09 (NS)	0.09 (NS)	0.2 (NS)	0.3 (NS)	0.08 (NS)	0.08 (NS)

relative to the advanced stage, though this association did not reach statistical significance (p=0.30). On the other hand, we found no association of Epo-R expression to stage (p=0.08). In the literature review, we have found only one study addressing the correlation between this protein immunoexpression and cancer stage [20]. The authors analyzed microvascular density and Epo-R expression in the primary comparatively with the stage (I–IV) of the 40 patients participating in the study. They noted that Epo-R positivity for the primary and endothelial cells was higher and higher as the tumoral stage increased, this difference being statistically significant for stages II, III and IV relative to stage I. Different criteria for interpretation of Epo-R immunohistochemical staining were used in the Italian study and ours, which may be one of the reasons for differences in results from both studies. Thus, while Ribatti considered as being positive those cases in, which more than 50% of tumoral cells became stained, in our study the staining was uniform and homogenous between the different tumor specimens, and 100% of tumoral cells became stained, even though with varying intensity dependant on the specimen selected. Additionally, while the authors of the Italian study established an intensity gradation scale ranging from weak (1+) to intense (3+), which relied on the observer's judgment, in the present report we considered the different cellular components of non-tumoral gastric mucosa of each case as both an internal control and a reference for staining intensity of the corresponding tumoral specimen. Also, differences in methodology, which in part may be due to the shortage of studies in this field published in the medical literature, may account for discrepancies between findings in the Italian study and ours. It would be of interest to replicate these findings in further studies using the same criteria for staining as defined in the present investigation; however, the sample size should be larger and study design should include strategies to extend the investigation to other settings, such as patients with irresectable advanced disease.

To our knowledge, our investigation is the first that analyzed Epo-R differential expression according to Lauren classification (intestinal and diffuse) and the subtype described by Carneiro (mixed) [13]. Although there seemed to be a longer DFS for patients with intestinal tumor relative to the two other subtypes, this difference did not reach statistical significance (p=0.18 and 0.4, respectively). Still, we did note that most of diffuse tumors (7/9, 87%) showed *pattern A* staining, and we found no cases of intestinal tumor presenting this pattern. However, the latter subtype did present predominance of *pattern B* staining (18/22, 81%) (p=0.02). In other words, nearly the totality of diffuse tumors seemed to present weak staining unlike intestinal tumors, which presented predominantly more intense staining. It is necessary to carry out further investigations to determine whether or not intestinal tumors with weak expression (type A) present a clinical evolution better than that of intestinal tumors presenting type B o C staining. This finding may be explained by the fact that the intestinal variant has better preserved protein synthesis machinery, and this may be related to a higher accumulation of Epo-R relative to that of diffuse tumors, which contain a simple and more degenerated architecture. In relation to differentation grade, we found no association of this variable to DFS, OS or Epo-R expression. Again, this finding contrasts with those in the Italian study, in, which authors concluded that positive expression of this antibody was more strongly associated with indifferentiated, aggressive, and most often diffuse, tumors [20]. It is likely that the small size of the sample in each subgroup (we did not find any G1 cases in our series) has hindered the achivement of conclusive results.

Another objective in the present investigation was to determine whether Epo-R staining correlate with the immunohistochemical expression of other proteins that are undoubtedly involved in the pathogenesis and progression of GC, such as p53, c-erb-B2, COX-2, E-cadherin, and β-catenin. However, we found no significant association between immunohistochemical staining of these proteins and that of Epo-R.

Therefore, on the basis of our results we suggest that it is likely to exits a relationship between Epo-R expression pattern and RFS. Results from the present investigation may be useful, along with the stage and histologic subgtype of GC patients operated on who then undergo adjuvant treatment, for prognosis determination. Nontheless, our results should be interpreted under the following considerations:

1. The present investigation is a retrospective study, in, which the clinical records from 44 GC patients were selected. All 44 patients had been operated on, and had undergone adjuvant treatment (MacDonald scheme). We took clinicopathological data from the aforementioned clinical records. Follow-up lasted at least 2 years.
2. A priori, one can consider that our sample size was too small, which would challenge our results. However,

when reviewing the medical literature in this field, one observes that our sample size is similar to that in most studies. This holds true not only for GC [20], but also for other neoplasms [9, 34]. Furthermore, because there are no planned studies addressing the influence of Epo-R expression on GC patients' survival, our sample size may be considered as being appropriate as an initial sample that can be extended in the future in accordance with findings.

3. On the other hand, as said above, all selected patients had undergone adjuvant treatment based on MacDonald scheme. According to MacDonald, this adjuvant scheme improves overall survival and extends time to progression; thereby, the prognostic impact of the different Epo-R expression patterns may have been biased owing to the administration of the aforementioned treatment.
4. Other limiting factor in our study is the unspecificity of the polyclonal antibody used, despite it is a valid antibody that has been utilized in the immunohistochemistry works we have reviewed [5, 9, 10, 20, 30, 40]. However, this antibody does not show a specific staining for membrane or cytoplasm, and it is not infrequent for the antibody to yield a dual staining pattern. The reason for this may be the fact that the antibody binds to both the intracellular and extracellular portion of Epo receptor. In fact, a study has been recently published that questions this antibody reliability in quantifying Epo-R expression [41]. In addition we are working on confirming our results with another analysis like western blotting or functional cells cultures studies.
5. On the other hand, there is no uniformity in the immunohistochemical interpretation of the different tumors, and it exists several classification systems for staining intensity. Therefore, it is hard to extrapolate results from one study to the next. For our work, we have devised a new classification system for Epo-R immunostaining in the tumor specimens by analogy with the differential staining presented by the different types of non-tumoral mucosa cells. Further investigation to validate such a semiquantitative interpretation method for Epo-R is warrant.

In summary, there is scanty scientific evidence for the role played by Epo-R expression in GC. In the present study, we have analyzed Epo-R immunoexpression in GC by relying on the interpretation of staining intensity comparatively with the expression shown by the different normal mucosa subtypes. This is an original method that has not been previously described in the medical literature, and we feel that it is more objective than others. Our findings suggest that there may be a relationship between Epo-R expression and DFS in the patients with GC resected followed by adjuvant standard treatment. Thus, the patients showing *type A* staining seemed to have a RFS longer than those showing *type C* staining, though such a difference didi not reach statistical significance ($p=0.06$). Therefore, it is necessary to carry out further investigation addressing the role played by Epo-R in the clinical course of GC patients. The ultimate goal should be to devise a method to distinguish GC patients with better and worse prognosis.

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