Relationships of P53 and Bak with EPO and EPOR in Human Colorectal Cancer

MAREK BALTAZIAK[1], MARIUSZ KODA[1], ANDRZEJ WINCEWICZ[2], MARIOLA SULKOWSKA[1], LUIZA KANCZUGA-KODA[2] and STANISLAW SULKOWSKI[1]

Departments of [1]General and [2]Medical Pathomorphology, Medical University of Bialystok, Collegium Pathologicum, Medical University of Bialystok, 15-269 Bialystok, Poland

Abstract. *Background: EPO (erythropoietin) counteracts p53-dependent apoptosis. EPO, which acts via its receptor EPOR, protects cells and inhibits apoptosis in normal cells and some cancer tissues by severe down-regulation of Bak. We aimed to investigate the relationship between p53 and Bak expression and EPO and EPOR in human colorectal carcinomas. Materials and Methods: The expression of p53 was compared with Bak, EPO and EPOR in 96 colorectal carcinomas by means of immunohistochemistry. Results: Purely nuclear p53 was significantly higher expressed in the moderately differentiated cancers in comparison with the poorly differentiated ones (p=0.007). P53 expression did not correlate with cytoplasmic markers: Bak, EPO and EPOR, but EPO and EPOR were significantly associated with Bak expression (p<0.001, r=0.524 and p<0.001, r=0.455, respectively). p53 expression was not associated with disease-free survival during the 3 years and 9 months long follow-up. Conclusion: A complete disruption of association between p53 and Bak could impair of p53-dependent apoptotic pathway that involves Bak. The relationship of Bak with EPO and EPOR is evidence of their co-expression suggesting competition between EPO mediated cell survival and Bak associated apoptosis in colorectal carcinomas.*

Apoptosis is the process of programmed cell death, and the dysregulation of apoptosis is one of the most important features in cancer. The Bcl-2 family proteins interact in the regulation of apoptosis as promotors (Bax (BCL-2-associated X), Bak (Bcl-2 homologous antagonist killer)) or inhibitors (Bcl-2 (B-cell leukemia/lymphoma 2), Bcl-xl (B-cell leukemia/lymphoma extra long), Mcl-1 (myeloid cell leukemia-1 protein)). The balance of the Bcl-2 family proteins is also regulated by p53 protein via regulation of gene transcription. P53 is responsible for the normal apoptotic function of Bak by the decomposition of the Bak-Mcl1 complex and the oligomerization of Bak which are followed by the release of cytochrome C from the mitochondria (1). Bak is stored as inactive monomers in the mitochondria of healthy cells and must be activated – by means of a conformation change – in order to oligomerize (2).

Erythropoietin (EPO) is a glycoprotein hormone that serves as the primary regulator of erythropoiesis by stimulating growth or preventing apoptosis and inducing differentiation of red blood precursors (3). EPO is produced in the kidney and delivered to the target organ via the circulation. The action of EPO on the responding cells is mediated through its receptor EPOR. Previously, it was thought that EPOR and EPO were only connected with the proliferation of erythroid cells. However recent studies have indicated their presence in normal cells of other tissues (4) and also in carcinomas (5). Rapidly dividing neoplastic cells need sufficient oxygen for growth and expansion. The hypoxic state causes changes in tumor cells that can lead to cell stasis or apoptosis. EPO and EPOR are so called hypoxia – associated markers, which become up-regulated in the hypoxic environment of neoplasms. EPO has been shown to promote cell viability by repressing apoptosis by the down-regulation of Bak (6). Silva *et al*. using a mouse erythroleukemia cell line have shown, that in the absence of EPO, Bcl-xL and Bcl-2 are rapidly down-regulated and this is accompanied by activation of the apoptotic process (7). Batra *et al*. (8) have shown that the addition of exogenous erythropoietin to tumor cell lines increased the expression of the antiapoptotic genes: *bcl-1*, *bcl-xL*, and *mcl-1*. In a previous study (9) we have found very prominent relationship between EPO and EPOR and the antiapoptotic protein Bcl-xL in colorectal cancer, too. The aim of this study was to investigate any relationship between p53 and proapoptotic protein Bak expression with EPO and EPOR in human colorectal cancers.

Correspondence to: Andrzej Wincewicz, MD, Ph.D. Department of Medical Pathomorphology, Medical University of Bialystok, Waszyngtona St 13, 15-269 Bialystok, Poland Tel: +48 857485945, Fax: +48 857485944, e-mail: andwinc@gmail.com

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Materials and Methods

Materials. Our study included 96 colorectal carcinomas classified as adenocarcinoma: 69 tumors were moderately differentiated (G2) and 27 were poorly differentiated (G3). Forty-six patients had lymph node metastases at the time of diagnosis. There were 8 tumors in pT1+pT2 stage and 88 tumors in pT3+pT4 stage.

No individual was given chemotherapy or radiotherapy before sampling of the cancer tissues. The biopsy specimens were fixed in a 10% buffered formalin solution, embedded in paraffin at 56˚C, and then cut into 3 to 5 μm thick slices and stained with hematoxylin and eosin (H+E). Next the diagnosis of colorectal cancers was made and several pathological features were determined including AJCC/UICC (American Joint Committee on Cancer/International Union Against Cancer) TNM stage (Tumor Node Metastasis), histopathological type (HP) and grade of histological differentiation (G). In view of the relatively small groups for statistical analysis, the pT1 and pT2 neoplasms were combined into one group (pT1+pT2) and the pT3 and pT4 carcinomas constituted another group (pT3+pT4).

Staining. The expression of p53 and Bak proteins was detected by immunohistochemical reaction as described previously (10, 11). Monoclonal antibody for P53 (1:100 dilution) (Dako, Glostrup, Denmark) and polyclonal antibody for Bak (1:200 dilution) (Santa Cruz Biotechnology, Santa Cruz, California USA) were applied. Slides were also incubated overnight at 4˚C with antibodies against EPO (rabbit polyclonal, H-162, 1:200 dilution), and EPOR (rabbit polyclonal, C-20, 1:400 dilution) (Santa Cruz Biotechnology). In order to visualize the antigen-antibody reaction, the labeled streptavidin biotin (LSAB) technique was applied for P53, avidin-biotin-peroxidase complex (ABC) staining system for Bak and EnVision method for EPO and EPOR, using diaminobenzidine (DAB) as a chromogen.

The primary antibodies were omitted in the negative controls, whereas samples of colorectal cancer tissue, which showed strong positive p53, Bak EPO, and EPOR immunoreactions, were used as the positive controls. The immunostaining of p53, Bak EPO and EPOR was assessed in 10 different tumor fields using a magnification of ×20. The mean percentage of tumor cells with positive staining was scored. The sections were classified as positive, if at least 10% of cells expressed the studied antigens. The blind counting was used for the rate of positive malignant cells in every representative microscopic slide of each carcinoma.

Statistical analysis. The correlations of p53 with Bak, EPO and EPOR and associations of Bak with EPO and EPOR were analyzed in regard to patient age, grading, staging and lymph node metastases. The significance of those relationships was determined using Spearman correlation analysis for the 3 grade scoring scale (0-negative or below 10% of positive cells, 1-10 to 50% postive cells, 2- above 50% of positive cells in a case). Apart from that the Chi-square Pearson's test served to highlight any statistically significant differences in p53 expression between analyzed groups. Kaplan-Meier analysis was used to determine any significant difference in disease-free survival of the patients with p53 positive or negative tumors during the 3 years and 9 months long follow-up. Pearson's test and Kaplan-Meier analysis based on 2 grade scoring 0-negative tumors (below 10% of positive cells), 1-positive tumors (over 10% of positive cells). *P* values ≤0.05 were regarded as statistically significant. This study was guided in concordance with the revision of the Declaration of Helsinki of 2004 and approved by the local ethical committee at the Medical University of Bialystok.

Results

Patterns of staining. Immunohistochemical analysis of the colorectal cancer sections revealed a nuclear localization of p53 proteins and a cytoplasm localization of Bak, EPO and EOR proteins (Figure 1). The expression of p53 was abundant, evident, granular and restricted only to the nuclei of the colorectal cells. Forty three tumors were p53 negative and fifty three were p53 positive. The expression of Bak was microgranular, the antibodies labeled the cytoplasm of the cancer cells and the adjacent colorectal mucosa also revealed a positive but relatively decreased immunostaining for this protein. Forty seven tumors were Bak negative and forty nine were Bak positive. The expression of EPO and EPOR was evenly distributed in a finely granular pattern in the cytoplasm. Twenty seven tumors were EPO negative and sixty nine were EPO positive. Linear perinuclear, coarse granular anti-EPOR staining was also observed in the cytoplasm of the colorectal cancer cells in the vicinity of necrosis. Seventeen tumors were EPOR negative and seventy nine were EPOR positive. The pattern of staining was described in detail for each of the studied proteins in our previous reports (9, 10, 11, 12) (Figure 1). In the immunohistochemical analysis of EPOR, in order to avoid scoring of non specific antibody- antigen binding, only the granular immunexpression in the cytoplasm was taken into account and a diffuse immunoreactivity in the cytoplasm was not included (12).

Relationships of p53 with anatomoclinical features and disease-free survival. P53 expression was not associated with any of the clinical and pathological features except for grading. P53 expression was significantly higher expressed in the moderately differentiated cancers (G2) in comparison with the poorly differentiated ones (G3) (p=0.007). P53 expression did not correlate with Bak, EPO or EPOR (Table I). There was no significant difference in disease free-survival between p53 positive and p53 negative colorectal carcinomas during the 3 years and 9 months long follow-up of the patients (Figure 2).

Comparisons of P53 and Bak with EPO and EPOR. P53 and EPO expressions were only correlated in the subgroup of patients younger than 60 years (p=0.030, r=0.389) and there were no other significant associations of p53 with Bak, EPO or EPOR in the selected clinical and pathological subgroups (Table 1). Both EPO and EPOR expressions were significantly associated with Bak expression (p<0.001, r=0.524 and p<0.001, r=0.455, respectively) in all the

patients together and the subgroups of patients except for EPO expression in the individuals with shallower cancer invasion (pT1+T2) (Table I).

Discussion

This is the first report showing a significantly higher expression of P53 in moderately differentiated cancers compared to poorly differentiated carcinomas. In agreement with our previous study (13) in a smaller group, no association was revealed between p53 protein expression and tumor histological type or site or the age or sex of the patients (13). Also similar significantly increased immunorectivities of Bak, EPO and EPOR in better differentiated carcinomas have been presented previously (10, 12). 5-Fluorouracil (5-FU)-induced apoptosis was accompanied by the increased expression of Bax and Bak proteins in a human colon cancer cell line with wild-type p53 and if p53 was mutated, a huge increase of Bak was observed (14). The loss of p53 function, due to mutation may result in the decreased activation of Bak as the oligomerization of Bak is hindered in the absence of functional p53. Impairment of Bak activity could result in higher demands for Bak and consequently up-regulation of Bak to achieve oligomerization by higher intracellular concentration. An abundant expression of Bak was observed in the present study. However, a complete loss of association between p53 and Bak expression was also discovered in our work. This could indicate disruption of co-operation between these proteins and subsequent apoptosis impairment as a consequence of the frequent p53 mutations in colorectal carcinomas. In our earliest study of proliferation and apoptosis markers in papillomas and carcinomas of conjunctiva and eyelid a lack of correlation between P53 and Bak was also noted in our previous study on proliferation and apoptosis markers in papillomas and carcinomas of conjuctiva and eyelid (15).

Lin *et al*. described the prolongation of G1 cell cycle phase arrest after p53 activation as a consequence of EPO action (16). EPO inhibited such apoptotic events as escape of cytochrome C from the mitochondria or the resultant activation of caspases, in that study. In addition, the population of examined cells had a very peculiar molecular profile being a Friend virus-transformed erythroleukemia cell line that produced a temperature-sensitive p53 allele (16). Despite the altered pathway of EPO signaling, EPO was proved to prevent apoptosis (16). Inhibition of apoptosis was also observed during erythropoiesis, in which EPO switched on expression of Bcl-xL and mediated the differentiation of erythroid progenitor cells (17). In contrast to the antiapoptotic role of EPO, EPO failed to affect p53 but was found to be correlated with accelerated Bax/Bak mediated mitochondrial apoptosis in EPOR-positive human renal carcinoma cell lines and the myelomonocytic leukemia cell line U937 (18). In that study, EPO maintained the chemosensitization of the malignant cells by the impairment of NF-kappaB (nuclear factor-kappaB) activation. NF-kappaB supported the survival of colorectal cancer cells by the recruitment of anti-apoptotic proteins (19). The regulators of apoptosis were obviously very much involved in EPO-mediated signaling and the present correlations between EPO and Bak confirmed this involvement.

The surprising positive linkage between apoptosis mediating Bak and cell survival favoring EPO and EPOR could indicate their co-expression thus implicating competition between simultaneous EPO-mediated cell rescue processes and apoptotic mechanisms that appear to be p53 independent but Bak-associated in our the present study. Such an explanation became more convincing with the observation that the EPO expression was distributed in the necrotic foci of the colorectal cancer tissue in the present study in a similar manner to that described in endometrial cancer (20). Such a perinecrotic pattern of staining inferred that the cells were sensitive to cell death mechanisms. On the other hand, correlations between Bak and EPO or EPOR could result from EPO sensitization of malignant cells to apoptotic signaling in EPOR- positive cells by disruption of NF-kappaB activation (18).

EPOR mediated signaling abolished p53-dependent apoptosis, which was induced by gamma-irradiation in the DA3 murine myeloid cell line in which EPO stimulated Jak2 kinase (Janus 2 kinase) and expression of Bcl-2 or Bcl-xl to overcome apoptosis and growth arrest (21). Putting aside single and probably accidental association between EPO and p53 in the younger patients, the predominant lack of correlations between p53 and EPO or EPOR could have resulted from the completely adverse actions of those proteins from the perspective of cell survival and death.

P53 has been considered to possess prognostic significance in various tumors *e.g.* for overall survival in thyroid carcinomas (22). Moreover, p53 and Ki-67 could be detected in growing numbers of epithelial cells during progression of precancerous lesions and in carcinoma of the oral cavity (23). Indices of mutant p53 and Ki-67 increased simultaneously in the colorectal adenoma-adenocarcinoma sequence and were regarded as poor prognostic pathological markers (24). Both Ki67 and p53 combined were adversely linked with disease-free survival in male germ cell tumors (25). Similarly, in colorectal carcinomas p53 and EGFR co-expression tended to be significantly associated with overall survival, but P53 alone was not (26). Similarly, the present study also failed to uncover any significant difference in the disease-free survival between the p53 positive and P53 negative colorectal tumors. However, Wiksten *et al*. found p53 to be an unfavorable prognostic factor in survival analyses in gastric cancers (27). Additionally, p53 has often been considered as a key protein and factor of prognostic

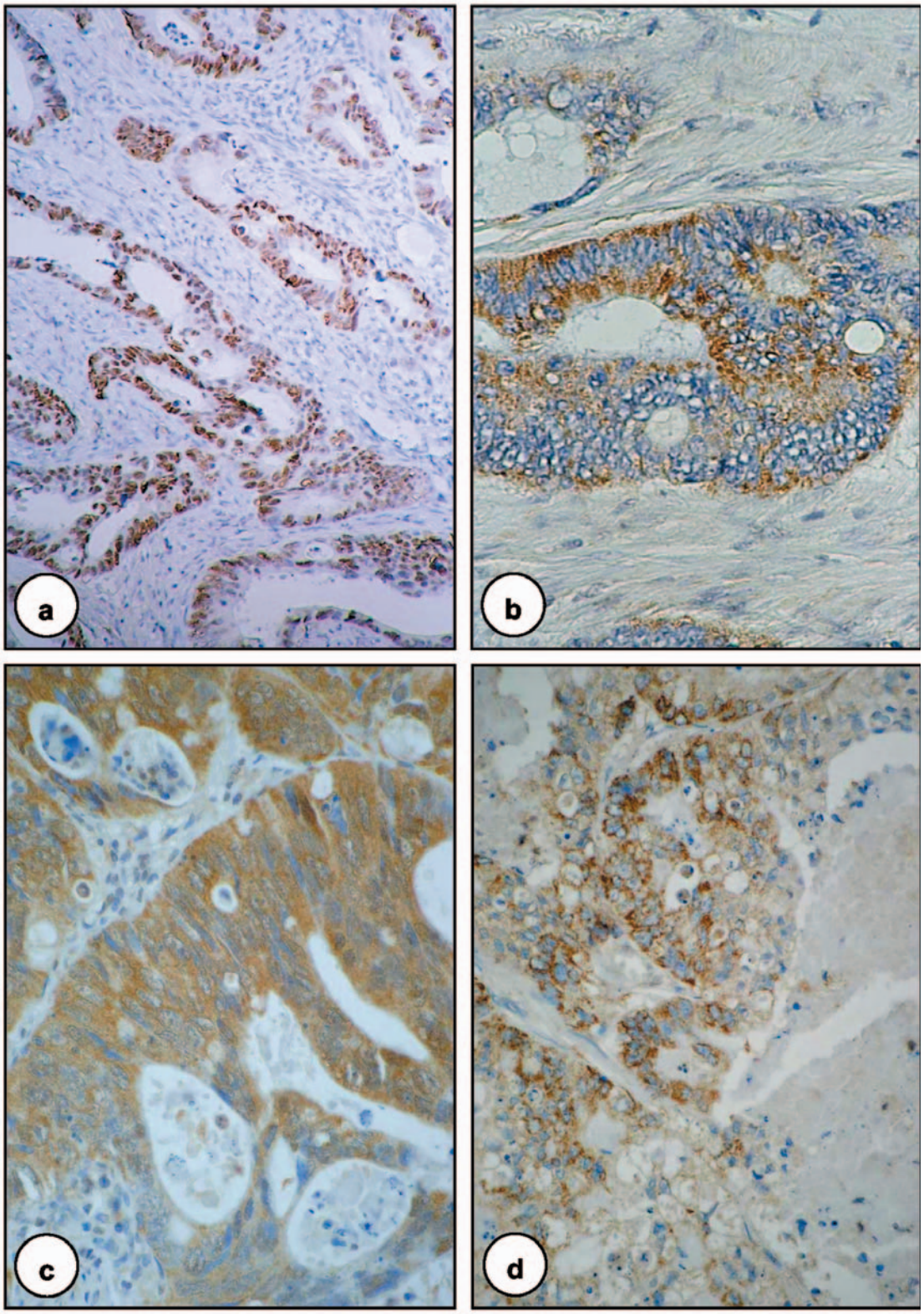

Figure 1. *Immunostaining for p53, Bak, EPO and EPOR in colorectal carcinoma tissues. a. Nuclear distribution of p53 shown as coarse granules. Magnification ×100. b. Granular pattern of staining for Bak in the cytoplasm. Magnification ×200. c. Cytoplasmic, evenly distributed finely granular immunoreactivity to EPO. Magnification ×400. d. Linear perinuclear, coarse granular anti-EPOR staining in the cytoplasm in the vicinity of necrosis. Magnification ×400.*

Table I. *Associations of P53 with Bak and EPO and EPOR expressions. Spearman's correlation rank test.*

Patients characteristics	Number of patients	P53-EPO		P53-EPOR		P53-Bak		Bak-EPO		Bak-EPOR	
		p	r	*p*	r	*p*	r	*p*	r	*p*	r
All patients	96	N.S.	0.106	N.S.	0.038	N.S.	0.040	<0.001	0.524	<0.001	0.455
N											
N-	46	N.S.	0.190	N.S.	0.216	N.S.	0.038	<0.001	0.583	<0.001	0.641
N+	50	N.S.	0.025	N.S.	–0.123	N.S.	0.040	<0.001	0.505	0.008	0.365
pT											
pT1+T2	8	N.S.	<0.001	N.S.	0.179	N.S.	–0.258	0.017	0.802	N.S.	0.435
pT3+T4	88	N.S.	0.102	N.S.	0.019	N.S.	0.066	<0.001	0.517	<0.001	0.503
G											
G2	69	N.S.	0.076	N.S.	0.033	N.S.	0.130	<0.001	0.496	<0.001	0.460
G3	27	N.S.	–0.126	N.S.	–0.049	0.051	–0.380	0.002	0.566	0.004	0.524
age											
≤60	31	0.030	0.389	N.S.	0.180	N.S.	0.172	0.001	0.580	0.002	0.544
>60	65	N.S.	–0.034	N.S.	–0.042	N.S.	–0.013	<0.001	0.492	<0.001	0.420
Gender											
male	47	N.S.	0.160	N.S.	0.137	N.S.	0.183	0.001	0.448	0.006	0.335
female	49	N.S.	0.057	N.S.	–0.051	N.S.	–0.102	<0.001	0.598	<0.001	0.567
Tumor site											
rectum	41	N.S.	<0.001	N.S.	0.122	N.S.	0.181	<0.001	0.559	0.027	0.346
colon	55	N.S.	0.171	N.S.	–0.035	N.S.	–0.056	<0.001	0.505	<0.001	0.547
HP type											
Adc	82	N.S.	0.066	N.S.	–0.017	N.S.	0.062	<0.001	0.519	<0.001	0.434
Adc muc.	14	N.S.	0.149	N.S.	0.176	N.S.	–0.244	N.S.	0.447	0.052	0.529

N, lymph node involvement: N(-), negative, N(+), positive; pT, depth of tumor intramural growth: G, grading of histological differentiation; HP type, histopathological type; Adc adenocarcinoma; Adc muc., mucinous adenocarcionoma. N.S., not significant. Only $p \leq 0.05$ presented.

significance in colorectal cancer (28, 29). In particular, cytoplasmic expression of p53 was associated with advancement of Duke's stages and poor survival in colorectal cancer (29). Immunohistochemical evaluation of prognostic significance seems to be somewhat less reliable for p53 in gastrointestinal malignancies especially colorectal cancers in the light of such different results. Thus, the analysis of p53 gene polymorphism seems to be much more promising, providing detailed evaluation of the prognostic impact of p53 gene variants in Dukes' B stage patients (30).

In conclusion, a lack of association between p53 and Bak could indicate a disturbance of p53-dependent apoptosis in colorectal cancer. Positive correlations between the apoptosis mediator Bak and cell survival proteins EPO and EPOR could be explained by their co-expression resulting probably from simultaneous competitive up-regulation of cell rescue processes and cell death mechanisms in colorectal cancer cells.

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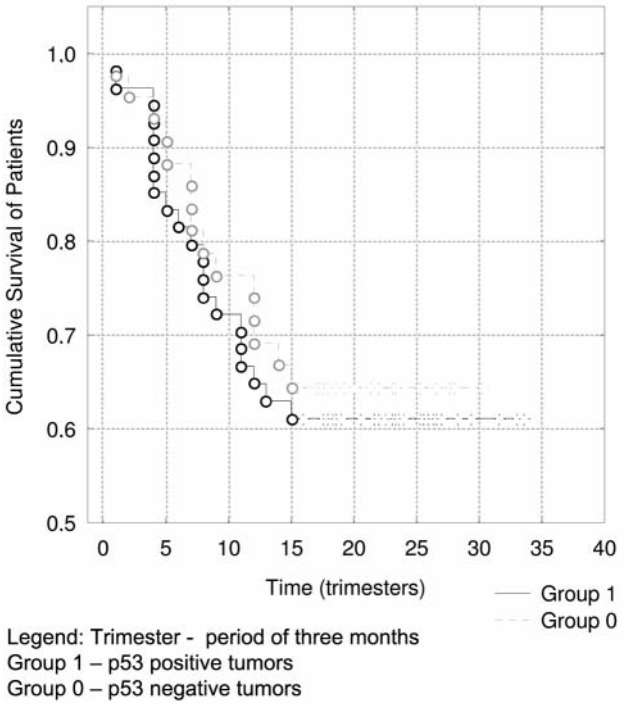

Figure 2. *Disease-free survival of patients in p53 positive and negative tumors.*

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