P53 correlates positively with VEGF in preoperative sera of colorectal cancer patients

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Diversity of P53 impact on tumor angiogenesis is due to the fact that wild-type P53 decreases expression of vascular endothelial growth factor (VEGF), but mutant P53 upregulates it. Therefore, we aimed at uncovering relations between preoperative serum levels of VEGF and P53 in colorectal cancer (CRC) patients. Preoperative blood samples of 125 CRC patients and 16 control healthy volunteers were examined with an ELISA-kit for serum P53 levels and VEGF. P53 did not correlate with VEGF in the whole group of CRC patients. However, P53 associated with VEGF in case of colorectal cancer patients, whose serum values of VEGF were higher than in controls (VEGF{H} >5.9333 pg/ml) ($r=0.274$, $p<0.009$). We revealed a positive correlation between P53 and VEGF{H} in subsets of poorly differentiated (G3) cancers ($p<0.02$), lymph node positive ($p<0.007$), pT3 or pT4 patients ($p<0.004$) without analogous relation in moderately differentiated (G2) tumors, node negative patients or pT1 or pT2 patients. P53 and IGF-I negatively correlated in all CRC patients ($p<0.04$) and VEGF{H} individuals of pT3 or pT4 ($p<0.05$) without any significant linkage in tumors of pT1 or pT2. The positive correlation between serum P53 and VEGF points at mutation of P53 and is a highly probable sign of poor prognosis in colorectal cancer. For now it can not be excluded that the binary analysis of serum P53 and VEGF could help select CRC patients endangered by rapid growth and lymph node metastases.

Key words: colorectal cancer, P53, VEGF, serum

Vascular endothelial growth factor (VEGF) is a major agent that is responsible for sprouting new capillaries and their subsequent differentiation into venules and arterioles that can extend to larger dimensions in a process called angiogenesis [1]. VEGF was proved to be a valuable prognostic marker of cancer progression and its role was also uncovered in colorectal carcinoma [2]. Preoperative and postoperative values of VEGF levels go up in serum of colorectal cancer patients in comparison with healthy volunteers and are even more increased in cases of colorectal cancer metastases [3]. Moreover, its preoperative levels were assumed as the only parameter that significantly corresponds with length of disease-specific survival and disease-free survival [3]. VEGF is a key upregulator of tumor angiogenesis and its expression can reflect progression of colorectal cancer [4]. The roles of P53 and VEGF in neoplastic growth have been elucidated for a long time. Their relationship is not clear in an aspect of their serum levels during development of colorectal cancer, though. P53 as an inhibitor of uncontrolled proliferation is called a good guardian of human genome, what reflects its indispensable function in repairing of cellular defects and later if repairs fail, P53 induces apoptosis of abnormal cells [5]. However, if P53 is mutated it looses its proapoptotic function, but programmed cell death can occur independently in colorectal cancer [6]. Tissue expressions of P53 and VEGF were correlated with each other, bcl-2, microvessel density and node involvement [7], but comparisons between P53 and VEGF were not reported in blood serum at all. Their relationship was not clear in an aspect of their serum levels during development of colorectal cancer as well. It was also presumed that wild-type P53 decreased VEGF expression and had powerful impact on angiogenesis [8], but mutated P53 inversely upregulated VEGF [9]. With regard to these findings we aimed at uncovering relations between levels of VEGF and P53 in sera of patients before surgical treatment of colorectal cancer.

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Material and methods

Patients. Preoperative blood samples were obtained from 125 colorectal cancer patients (51 female and 74 male patients with CRC) qualified for tumor resection. All subjects did not have any radiotherapy or chemotherapy before collection of the samples. Population of patients was divided into group of age older than 60 years and younger ones or at the age of 60. Conventional histopathologic parameters (including AJCC/UICC TNM stage, tumor type and grade of histologic differentiation (G) were assessed by two independent pathologists. According to guidelines of World Health Organization (WHO), primary tumors of 81 patients were classified to grade G2 (moderately differentiated) and 43 individuals were at G3 (poorly differentiated). A diagnosis of adenocarcinoma was established in 106 investigated cases and other 19 tumors turned out to be mucinous carcinomas. We split patients in two groups according to location of tumor: 58 cases were found in rectum and the 67 cancers were in colon. We also divided all subjects in two groups according to pT classification: there were 10 subjects with pT1 or pT2 (pT1+pT2) and 115 primary cancers grew up to dimensions of pT3 or pT4 (pT3+pT4). Cancer metastasized to local lymph nodes in 61 cases (N+) and 64 patients were node negative (N-). Donors of control blood samples were 16 healthy volunteers.

ELISA analysis – evaluation of serum levels of the proteins. The levels of serum p53 were measured with an ELISA kit according to the manufacturer's protocol (all reagents of the ELISA were supplied by Roche Diagnostics, Germany). The samples were coagulated and spinned at room temperature. Serum samples were diluted in 1:5 manner. Then standard and sample solutions (each in a dose of 100 µl) were inserted into the wells of the ELISA plate. The biotin-labeled antibody, which was attached to streptavidin-covered microtiter plate, bound with P53 molecules from provided serum samples that in next step were also joined with peroxidase labeled, specific antibody for P53 (700 µl of anti-p53-POD prediluted, Roche Diagnostics, Germany) in a routine of one incubation that was performed in 2 hours at room temperature on shaker. Afterwards the incubation buffer was removed thoroughly by tapping off. The wells were rinsed five times with washing buffer. Then peroxidase dyed samples in result of reaction with its substrate – tetramethylbenzidine (TMB), that was added in dose of 200 µl of its solution. The color was developed in 15 minutes of subsequent incubation at room temperature on the shaker for 20 minutes in dark. The reaction was inhibited with the stop solution provided by the manufacturer. The color was then quantified by determination of its absorbance at 450 nm with a spectrophotometer (reference wavelength: 690 nm) against blank solution (distilled water for P53). The photometrically assessed intensity of color was proportionally transposed into values of P53 and VEGF respectively by use of an appropriate calibration curve for each of the studied proteins (provided by the manufacturer).

The levels of VEGF were also evaluated with ELISA kit. Serum samples were diluted in the standard that was recommended by producer (PromoCell GmbH, Heidelberg, Germany). 50 µl of each solution and 50 µl of assay diluent was dispensed into every well. Biotinylated rabbit anti-human VEGF polyclonal antibody was diluted and its dose of 25 µl was placed in every well. The plate was sealed with acetate plate sealer. Then VEGF molecules reacted with the antibody in 3 hour long incubation. Plates were then washed 5 times. Next streptavidin conjugated alkaline phosphatase was added to each well in dose of 50 µl and left at room temperature for 45 minutes. After resealing the plate, the washing was repeated 5 times with the method given by the manufacturer. Later color reagent solution was added at room temperature for incubation which was finished with stop solution after 20 minutes. Phosphatase dephosphorylated NADPH to NADH which served as a cofactor of cycling redox reaction with involvement of alcohol dehydrogenase and diaphorase. In the end, a deep red product (formazan) appeared. Intensity of its color was measured at 492 nm wave. Absorbances of color suit appropriate values of VEGF that were read with the standard curve.

Statistical analysis. As P53 did not correlated with VEGF in the whole group of CRC patients, we selected patients with relatively high levels of VEGF above the average value (5,9333 pg/ml) for control sera. Correlations were examined for bivariate associations of the serum proteins using Spearman's rank correlation. The level of significance was taken to be $p<0.05$. Determination of highly significant correlation was reserved for coefficient of correlation (r) achieved 0.5 and higher. Therefore we only signalized about the tendency toward it, if r was more than 0.3.

Results

Mean serum levels of the proteins were displayed in the Table 1. Levels of P53 increased in sera of all colorectal patients in comparison with control group of healthy volunteers (p mean control level 16.75±18.44). Similarly, levels of VEGF were significantly higher in sera of all colorectal patients compared to the same controls ($p<0.0001$ mean control level 5.93±1.15). It is very striking that exact values of VEGF and P53 show clear differences among groups of clinicopathological variables with visible increment in number in node-positive cancers, higher grade of differentiation, more advanced age of patients and in colonic location of tumors. Anyway, statistical significance of $p<0.009$ was discovered only by comparison between serum levels of VEGF in rectal and colon locations of tumors. Carcinomatous levels of P53 and VEGF were manifold higher than the ones of healthy volunteers. In our study, the increases of P53 and VEGF levels corresponded with each other and appeared to grow symmetrically. This order is reversed only between groups of pT1+pT2 and pT3+pT4 and both P53 and VEGF serum levels decreased concomitantly.

Table 1. Comparison between P53 protein levels and VEGF in preoperative sera of CRC patients

Groups of patients		Serum levels (pg/ml)			Comparison of serum protein values			
					P53 & VEGF		P53 & VEGF-H	
		P53	VEGF	VEGF {H}	P	r	P	r
Control group		16.75 ± 18.44	5.93 ± 1.15	–	N.S.	–0.423	–	–
All of CRC patients		181.39 ± 382.95	128.35 ± 146.05	164.02 ± 148.07	N.S.	0.137	<0.009	0.274
N	(–)	144.1 ± 312.7	113.47 ± 124.46	152.24 ± 124.23	N.S.	0.185	N.S.	0.172
	(+)	220.5 ± 444.5	144.22 ± 165.63	175.31 ± 168.33	N.S.	0.095	<0.007	0.388
G	2	161.9 ± 276.1	115.73 ± 116.91	149.83 ± 114.10	<0.009	0.942	<0.05	0.752
	3	220.3 ± 532.5	154.67 ± 188.13	188.75 ± 193.12	N.S.	0.207	<0.02	0.436
pT	pT1+pT2	219.7 ± 285.5	158.52 ± 171.34	196.65 ± 171.57	N.S.	–0.087	N.S.	–0.414
	pT3+pT4	178.3 ± 390.6	125.70 ± 144.20	161.05 ± 146.51	N.S.	0.122	<0.004	0.316
HP type	Adenoca	198.4 ± 410.1	131.30 ± 149.23	168.41 ± 151.16	N.S.	0.123	<0.02	0.271
	Ca muc	84.3 ± 120.7	112.04 ± 129.49	140.32 ± 132.25	N.S.	0.295	N.S.	0.117
Sex	Males	170.3 ± 323.1	127.89 ± 146.25	156.30 ± 148.75	<0.0001	0.415	<0.004	0.375
	Females	198.2 ± 462.6	129.03 ± 147.23	176.88 ± 148.12	N.S.	–0.171	N.S.	–0.042
Age	≤60 years	66.7 ± 78.2	106.66 ± 115.26	136.06 ± 115.47	N.S.	0.31	N.S.	0.226
	>60 years	226.1 ± 450.03	138.15 ± 158.78	180.80 ± 160.99	N.S.	0.131	0.29	0.022
Site	Rectum	152.6 ± 289.4	94.95 ± 117.87	135.00 ± 122.49	N.S.	0.102	N.S.	0.079
	Colon	207 ± 451.1	157.70 ± 162.21	184.74 ± 161.80	N.S.	0.135	<0.006	0.378

Relationship of P53 to VEGF in CRC groups of different clinicopathological features. P53 did not correlate with VEGF in the whole group of CRC patients. A positive relationship of P53 to VEGF was revealed in sera of all CRC male patients (r=0.415, p) without any correlation of these proteins in group of female colorectal cancer patients. Among all analyzed CRC patients serum P53 did not correspond significantly to VEGF in groups of different age, histologic type, grade, tumor size (pT), involvement of lymph nodes (N+) and location of cancer.

Relationship of P53 to VEGF{H} in CRC groups of different clinicopathological features. We determined a statistically significant positive correlation between serum P53 count and high levels of serum VEGF (VEGF{H}) in the entire group of all CRC patients (r=0.274, p<0.009). We revealed a positive correlation between P53 and VEGF{H} in subsets of G3 (r=0.436, p<0.02), node positive cases (r=0.388, p<0.004), pT3+pT4 subjects (r=0.316, p) without analogous relation in G2 tumors, node negative or pT1+pT2 individuals. In cases of adenocarcinoma without a mucinous component we drew out a positive correlation between P53 and VEGF{H} (r=0.216, p<0.02), while subjects with mucinous carcinoma did not show any linkage of this kind. P53 positively correlated with VEGF{H} in a group of patients older than 60 years (r=0.29, p<0.03) or if the tumors were localized in colon (r=0.378, p<0.006). A positive relationship of VEGF{H} to P53 also occurred in sera of men (r=0.375, p<0.004), but there was no correlation between these proteins in group of female colorectal cancer patients.

Discussion

P53 and VEGF are present in tumor tissues of colorectal cancer [10, 11], but they also enter circulation [12]. SHIM et al reported that serum levels of mutant P53 were significantly more elevated in colorectal cancers than the quantities of this protein in circulation of patients with adenomatous polyps [13]. Analogously carcinomatous levels of P53 and VEGF in our study were manifold higher than controls. They agree with previous studies of ZUSMAN et al that revealed a greater amount of serum P53 in colon cancer than in benign proliferations and healthy individuals [14]. We present for the first time positive correlations between preoperative serum levels of P53 and VEGF in colorectal cancers. There were immunohistochemical evaluations, in which a poorer prognosis was estimated in case of P53 and VEGF expression in malignant tumors of large intestine [11]. Consequently, in our evaluations, development of cancer is accompanied by positive correlations between serum levels of studied proteins in more advanced stages of the neoplasm, particularly in group of pT3+pT4 and node positive cancers. Similarly, serum evaluation of p53 gene grew up with cancer progression that was marked with a higher degree of Duke's scale [12]. In case of mutant P53 protein, gradual increase of its levels went with more advanced Duke's stage unfortunately without statistical significance [13]. Moreover, mutant P53 levels decreased after surgery and significantly correlated with CA19-9 which is a certain marker of colorectal cancer among other neoplasms of gastrointestinal tract [13]. A prognostic value of serum P53 is also suspected because its serum level was upregulated with levels of carcino-embryonic antigen (CEA) in recurrence and

metastases of colorectal cancer [15]. Detection of two markers of eventual prognostic significance can specify more accurately the outcome of CRC patients as serum postoperative coexistence of both increased CEA and VEGF predicts significantly higher rate of deaths than in case of high serum CEA accompanied with low serum VEGF [16].

Even alone, preoperative high serum levels of VEGF predict a poor survival in colorectal cancer (CRC) [17]. Compared to those of healthy controls, significantly higher preoperative serum VEGF levels associate with extent of local invasion, liver and lymph node involvement in colorectal cancer [3, 18]. That finding is of a great importance for us, because we noticed significant relations in analysis of preoperative P53 and VEGF that included only selected cases of high serum VEGF (VEGF{H}). On the other hand, counts of serum p53 protein were reported to be significantly low in patients with ulcerative colitis and matched directly with colorectal cancer risk factors [19], whereas the serum levels of VEGF were significantly increased in active ulcerative colitis in comparison with the controls [20]. In opposition to findings of KANAZAWA et al [20], mutant P53 increased tumor growth and was overexpressed together with VEGF in human cancer cell lines with constitutive production of nitric oxide synthase 2 (NOS-2) [21]. VEGF augments production of nitric oxide by endothelium, what is associated with proliferation of human endothelial cells [22]. The predominant mutations of P53 in colorectal cancer were transitions (the G:C to A:T transition at CpG dinucleotides) [23]. Most of these transitions appeared at 5-methylcytosine sites in colorectal cancer and they are presumably dependent on dose of NOS2 activity and overproduction of NO in infiltrating mononuclear cells [23]. Furthermore, mutations of p53 accompany overproduction of VEGF and NO that co-operate with each other in angiogenesis [24]. Our data also confirm a positive linkage between elevation of P53 and VEGF in blood serum. Thus, our positive correlation between P53 and VEGF seems to prove that P53 can be a marker of colorectal cancer development, as it was suggested for P53 in comparison between mutant P53 and CA19-9 in this neoplasm [13].

Further researches uncovered that transfection of mutant P53 into murine NIH 3T3 cells resulted in protein kinase C-dependent overexpression of VEGF [9]. These findings provide an explanation for our positive correlation between serum P53 and VEGF. In front of previous works that show dependence of upregulation of VEGF on mutations of P53 [10, 25], we are encouraged to recognize a significant relationship of the investigated serum proteins as an indirect evidence for presence of P53 mutation in the suitable individuals. A question arises about a nature of immunologic response to P53. Mutations of P53 induce a generation of specific antibodies that were characterized as IgG1-reactive anti-p53 Abs in colorectal cancer [26]. However, in breast malignancies P53 antibodies are directed against amino- and carboxyl-terminal regions that are the same in mutant and wild forms of P53 [27]. Additionally, in gastrointestinal cancer serum levels of autoantibodies were not correlated with mutations of P53 plasma DNA [28]. Therefore it is an idea that humoral response is not directly provoked by changes of P53 conformation, but is rather due to accumulation of P53. Nevertheless, storage of inactive P53 occurs in case of this protein mutation in colorectal cancers [29]. In our work we observed serum accumulation of P53. Immunization to P53 can help construct anti-idiotypic vaccines in therapy of colorectal cancer [26]. Efficiency of a immunotherapy with P53 specific antibodies is questioned. There was considered the possibility that this treatment would affect mostly mutant and inactive molecules of P53 but the immunotherapy would impair beneficial function of wild type forms of this protein. Thus, it is very important to determine a significance of increased levels of P53 in development of colorectal cancer. That can be partly accomplished by comparison of P53 with other prognostic factors like VEGF. As for VEGF, mutations of p53 abolished the expression of TPA (12-O-tetradecanoylphorbol-13-acetate) inducible genes but boosted TPA induction of VEGF [10]. This influence of mutant P53 on induction of VEGF seems to be confirmed in esophageal cancer [30]. Lately scientists have constructed vaccine that contain recombinant canarypoxvirus (ALVAC) that encodes wild-type human p53 [31]. When ALVAC has been administered to patients with advanced colorectal cancer, normal function of P53 has not been resumed as there was no P53 dependent proliferation or production of cytokines. Anyway, this vaccine has induced T cell derived IFN-γ response against both ALVAC and P53 [31]. At present we do not know, how efficient is that action in the fight against tumor cells overloaded with mutant P53. A similar immunotherapy is being administered with use of anti-VEGF agents. After a construction of bevacizumab – a recombinant humanized murine monoclonal antibody to VEGF, it has been established as a standard therapy to add bevacizumab (AvastinTM) to irinotecan, 5-fluorouracil and leucovorin in metastatic colorectal cancer [32]. Thus, we hope that studies on P53 will result in analogous clinical implications.

We revealed that tumor progression goes with serum accumulation of P53 in VEGF{H} patients. Overexpression of P53 was usually observed when this protein was inactive due to its mutation [8, 12, 30]. That is why, we suspect that binary analysis of serum P53 and VEGF would be useful in a selection of patients to eventual immunotherapy against mutated P53 in cases that show associated increase of VEGF level.

The positive correlation between serum P53 and VEGF points at mutation of P53 and is a highly probable sign of poor prognosis in colorectal cancer. In front of these findings, it can not be excluded that the binary analysis of serum P53 and VEGF could help select CRC patients that are endangered by rapid growth and lymph nodes metastases. Those patients could be qualified to eventual combined immunotherapy against VEGF and mutated P53 in cases with elevated serum proteins.

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