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p73 isoforms affect VEGF, $VEGF_{165}b$ and PEDF expression in human colorectal tumors: $VEGF_{165}b$ downregulation as a marker of poor prognosis

Raquel Díaz[1], Cristina Peña[1], Javier Silva[1], Yolanda Lorenzo[1], Vanesa García[1], José M. García[1], Antonio Sánchez[1], Pablo Espinosa[1], Rosario Yuste[2], Félix Bonilla[1] and Gemma Domínguez[1]*

[1]*Department of Medical Oncology, Hospital Universitario Puerta de Hierro, 28035 Madrid, Spain*
[2]*Department of Pathology, Hospital Universitario Puerta de Hierro, 28035 Madrid, Spain*

The secreted mitogen vascular endothelial growth factor, VEGF, plays a pivotal role in angiogenesis. Hypoxia, inactivation of p53 and oncogenic K-Ras induce VEGF expression. Other factors such as p73 may also affect VEGF levels. Curiously, p73 may also regulate angiogenesis by affecting the expression of the pigment epithelium-derived factor, PEDF. Additionally, VEGF might harbor additional functions through the activation of E2F transcription factors. Recently, a new VEGF variant formed by alternative splicing, $VEGF_{165}b$, has been described as exerting anti-angiogenic activity. We study here whether p73 isoforms levels -TAp73 and ΔTAp73- and p53 and K-Ras status affect the expression of the above-mentioned angiogenesis-related genes (through the correlation between their expressions), the prognostic value of $VEGF_{165}b$ and PEDF and the correlation between VEGF and E2F-1 levels. Tumor and normal tissue of 112 colorectal cancer patients was analyzed to evaluate: (*i*) levels of TAp73, ΔTAp73, VEGF, $VEGF_{165}b$, PEDF and E2F-1 by quantitative real-time RT-PCR, (*ii*) p53 status by immunohistochemistry and (*iii*) mutations in the first exon of *K-Ras* by PCR-SSCP. Tumor characteristics were examined in each patient. Associations were observed between: (*i*) specific p73 isoforms and VEGF and $VEGF_{165}b$ expression; (*ii*) ΔEx2p73 variant and downregulation of PEDF; (*iii*) VEGF and PEDF expression; (*iv*) inactive p53 and $VEGF_{165}b$ levels; (*v*) oncogenic *K-Ras* and PEDF downregulation; (*vi*) E2F-1 and VEGF expressions; (*vii*) $VEGF_{165}b$ downregulation and poor prognosis parameters of tumors. We conclude that the levels of p73 isoforms could affect the expression of VEGF, $VEGF_{165}b$ and PEDF. This scenario becomes complicated because a feedback between VEGF and PEDF may exist. VEGF may activate the E2F-1 factor. Mutations in *K-Ras* could negatively regulate PEDF expression. p53 inactivation might result in compensatory mechanisms such as over-expression of $VEGF_{165}b$. Our data support the role of $VEGF_{165}b$ as a tumor suppressor factor in colorectal carcinogenesis and its possible prognosis value.

Key words: p73 isoforms; angiogenesis; VEGF; $VEGF_{165}b$ and PEDF

As tumors enlarge and hypoxia develops, the induction of new blood vessels becomes critical to sustaining neoplastic proliferation.[1] Angiogenesis depends on the local balance between positive and negative effectors, whose production can be regulated by oncogenes and tumor suppressor genes. The secreted mitogen, vascular endothelial growth factor A, VEGF-A (commonly referred to as VEGF), seems to play a pivotal and irreplaceable role in the regulation of physiological and pathological angiogenesis.[2] Various events stimulate VEGF expression, such as hypoxia.[3–6] In addition, several specific transforming alterations may also induce VEGF expression, such as inactivating mutations of *p53*[7] and oncogenic mutations of *K-Ras*.[8] Interestingly, VEGF may promote proliferation by affecting the expression of E2F family transcription factors.[9]

An increase in VEGF mRNA expression has been identified in almost all known tumors.[10] Specifically, VEGF is found overexpressed in both advanced colon cancers and pre-malignant colonic adenomas.[11] Positive correlation between tumor VEGF expression and tumor vascularity has been described, and in many studies a correlation with prognosis has been shown.[12–16] Currently, different anti-VEGF therapies are being given[17] and improved efficacy on metastatic colorectal cancer without increasing toxicity has been reported.[18]

Exon splicing of the VEGF pre-RNA results in multiple isoforms that show angiogenic properties ($VEGF_{189,165,121,206,183,145,148}$),[10,19–22] although $VEGF_{165}$ appears to predominate quantitatively and functionally in most angiogenic states.[23] Recently, Bates *et al.*[24] identified $VEGF_{165}b$, a new variant formed by differential splicing from the end of exon 7 into the 3′ untranslated region of the VEGF mRNA. $VEGF_{165}b$ has been described as acting as an endogenous inhibitory form of VEGF and, therefore, has a putative anti-angiogenesis role.[23] Other $VEGF_{XXX}b$ forms have been described, but the only one of these isoforms for which there is any functional information is $VEGF_{165}b$. Further experiments showed that $VEGF_{165}b$ inhibited $VEGF_{165}$-induced angiogenesis in the rabbit cornea and the rat mesentery, and inhibited tumor growth in xenotransplanted tumors in mice.[23] $VEGF_{165}b$was found down-regulated in renal and prostate human cancers,[23,24] and its absence has been recently described to predict metastatic spread in patients with primary melanoma.[25]

Because of its essential role in cancer angiogenesis, and eventually in metastasis, there is growing interest in identifying additional tumor suppressor proteins and/or onco-proteins which may regulate VEGF mRNA and protein levels. Some controversial results regarding the putative role of the p53-related protein p73 in regulating VEGF expression have been published. While some of these data show the plausible involvement of p73 as a VEGF repressor,[26] other observations support its role as a VEGF inducer.[27,28] The *p73* gene gives rise to a complex number of isoforms with both tumor suppressor—TAp73—[29] and oncogenic properties—ΔTAp73.[30] As described for the other p53-family member, p63, TAp73 forms might repress the expression of VEGF while the ΔTAp73 forms might activate its transcription.[31] This remains unclear.

Curiously, p73 seems to play a role in angiogenesis not only by possibly regulating the transcription of VEGF, but also by targeting other angiogenesis-related proteins such as the pigment epithelium-derived factor, PEDF.[32] In cancer cells PEDF expression has been observed to be induced by p73.[32] PEDF is a secreted glycoprotein expressed in many tissues[33,34] and acts as a neurotrophic factor and a natural angiogenesis inhibitor in prostate, pancreas, eye, hepatocellular carcinoma cells and melanoma.[33–37] Loss of PEDF could be involved in glioma progression[38] and in pancreatic adenocarcinoma, PEDF-positive expression was an independent favorable prognostic factor.[39] In VEGF-/- adult fibroblast, it has been observed that Ras oncoprotein downregulates PEDF, giving rise to highly tumorigenic and angiogenic fibrosarcomas.[40] Interestingly, in osteosarcoma cells, PEDF inhibits VEGF expression at mRNA and protein levels,[41] while, in oral squamous cell carcinoma cells, VEGF induced mRNA PEDF expression and its secretion.[42]

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***Correspondence to:** Department of Medical Oncology, Hospital Universitario Puerta de Hierro, C/San Martín de Porres 4, 28035-Madrid, Spain. Fax: +34-91-3445190. E-mail: domingg@hotmail.com
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To explore the angiogenesis process *in vivo*, we address here the real-time quantification of VEGF, $VEGF_{165}b$, PEDF, E2F-1 and TAp73 and ΔTAp73 variants and evaluate the mutational status of p53 and *K-Ras* in a series of 112 patients diagnosed with colon carcinoma. We analyze which p73 variants, oncogenics and/or tumor suppressors could regulate the expression of VEGF, $VEGF_{165}b$ and/or PEDF, whether PEDF levels could affect VEGF expression or vice-versa, the plausible prognostic value of $VEGF_{165}b$ and PEDF expression levels in colon tumorigenesis, and the involvement of inactivated p53 and oncogenic *K-Ras* in the induction of VEGF and PEDF expression. Our data suggest that p73 isoforms could affect the angiogenesis process by regulating the expression of VEGF, $VEGF_{165}b$ and PEDF. A feedback between VEGF and PEDF may exist. In addition to its angiogenic role, VEGF could show other functions through the activation of the E2F-1 factor. Inactivation of p53 and/or activating mutations in *K-Ras* might also modify VEGF and PEDF levels. The association between $VEGF_{165}b$ downregulation and poor pathological parameters strongly supports its possible tumor suppressor function and its potential as a prognostic marker in clinic.

Material and methods

Tumor samples and extraction of nucleic acids

The present study, approved by the Research Ethics Board of our hospital, was conducted on a consecutive series of 112 patients undergoing surgery for colorectal cancer between January 1998 and January 2003. All colorectal cancer patients were considered sporadic cases on the basis that no clinical antecedents of family adenomatous polyposis were reported and those meeting the clinical criteria for hereditary non-polyposis colorectal cancer (Amsterdam criteria) were excluded. Both tumor and normal counterpart tissues were obtained sequentially, immediately after surgery, snap-frozen in liquid nitrogen and stored at −80°C until processing.

All tumor specimens underwent histological examination by a pathologist to confirm the diagnosis of adenocarcinoma, verify the presence of tumor, select those samples with at least 75% tumor tissue and establish the pathological stage.

RNA and DNA were extracted from about 30 mg of colon tumor and normal tissue samples using the RNeasy Mini Kit and QIAmp DNA Mini Kit, respectively (Quiagen Inc., Hilden, Germany). Following extraction, RNA was treated with RNase free DNase (Ambion). Nucleic acids were quantified spectrophotometrically.

Primer design and real-time PCR

Primer sets for ΔEx2p73, ΔEx2/3p73, ΔNp73, TAp73 and E2F-1 have been previously described.[30] Three different pairs of primers were used to quantify VEGF variants. The first set of primers was designed to amplify all of the VEGF isoforms and is named throughout the text as VEGFtotal, F: 5′CACTGAGGAGTC CAACATCACC3′ and R: 5′CTGCATTCACATTTGTTGTGC3. The second set amplifies specifically those forms containing exon 8 and showing angiogenic properties and is named throughout the text as VEGF, F: 5′GATCCGCAGACGTGTAAATGTTC3′ and R: 5′TCACCGCCTCGGCTTGTCACAT3′. The $VEGF_{165}b$ antiangiogenic variant was quantified with the following pair of primers: F: 5′GAGATGAGCTTCCTACAGCAC3′ and R: 5′TT AAGCTTTCAGTCTTTCCTGGTGAGAGATCTGCA3′.[23] In addition, PEDF was amplified using the following set of primers, F: 5′AATCCATCATTCACCGGGC3′ and R: 5′ACAAAGCTG GATTTTATGCGC3′. The housekeeping genes, TATA binding protein (*TBP*), succinate dehydrogenase complex subunit A (*SDHA*) and ubiquitin C (*UBC*), were used to normalized gene expression results.[30]

mRNA levels were calculated in the normal and tumor counterpart samples by a relative quantification approach in which target amounts are expressed in relation to the geometric average of the three reference housekeeping genes, as described previously.[30] The relative concentrations of target and reference genes were calculated by interpolation using a standard curve of each gene generated from a serial dilution of a cDNA prepared from the RNA of an individual expressing the specific gene analyzed. The expression level of the target gene in a patient was calculated as a ratio: target in tumor tissue/target in normal tissue (T/N). For the synthesis of the first strand of cDNA, 400 ng of total RNA was reverse-transcribed using the Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Real-time PCR was performed in a Light-Cycler apparatus (Roche Diagnostics, Mannheim, Germany) using the LightCycler-FastStart DNA Master Plus SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany). The conditions for each reaction are described elsewhere.[37,19] Amplicon size (base pairs) and annealing temperature for VEGF, VEGF-Ex8 and PEDF are as follows: 103 bp and 65°C, 106 bp and 64°C, 163 bp and 63°C, respectively.

Mutational status of K-Ras Exon 1

PCR amplification of *K-Ras* was carried out in a 25 μl reaction volume with a final concentration of 1× PCR buffer, 1.5 units of Ampli *Taq* DNA polymerase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA), 200 μM dNTPs mix, 0.6 μM of each primer, 2.5 mM $MgCl_2$, 100 ng of genomic DNA as template and distilled water to reach the total volume. For amplification, each sample was denatured at 94°C for 5 min and subjected to 35 cycles of PCR (94°C for 30 sec, 58°C for 40 sec, and 72°C for 30 sec) followed by a final 7 min extension at 72°C. The amplified products of *K-Ras* amplification were denatured by mixing with 15 μl of stop solution, containing 98% formamide, 0.02% xylene cyanol and 0.02% bromophenol blue, heated to 95°C for 6 min and then rapidly cooled on ice. Electrophoresis was performed on non-denaturing 12% polyacrylamide gels at 250 V for 12 hr at room temperature. The allelic band intensity on the gels was assessed non-radioisotopically using a commercially available silver-staining method.[43] Primers used for amplification of exon 1 of *K-Ras*, which contains codons 12 and 13 were as follows: F: 5′ GACTGAATATAAACTTGTGGTAGT 3′ and R: 5′ CTATTGTTG GATCATATTCGTCC 3′. The bands that displayed a different mobility shift pattern were sequenced in an ABI Prism™ 377 DNA sequencer (PE Applied Biosystem, Foster City, CA).

VEGF, $VEGF_{165}b$ and p53 immunohistochemistry

VEGF, $VEGF_{165}b$ and p53 immunophenotypic analysis in colon samples was performed according to standard procedures,[44] with overnight incubation in the presence of the following primary antibodies: (*i*) a mouse monoclonal VEGF antibody (VEGF antibody [14-124], Abcam, Cambridge Science Park, Cambridge, UK, diluted 1/100) epitope corresponding to the NH_2-terminal region. This antibody recognizes $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$; (*ii*) a mouse monoclonal $VEGF_{165}b$ antibody (MRVL56/1, Abcam, Cambridge Science Park, Cambridge, UK, diluted 1/100) developed against the 9 amino acid C-terminal sequence of $VEGF_{165}b$; (*iii*) the cl1801 mouse monoclonal antibody (Oncogene Sciences, Manhasset, NY). cl1801 mouse monoclonal antibody was used because of its ability to detect up to 89% of *TP53* point mutations.[45] Immunodetection was performed with peroxidase-labeled streptavidin biotin (LSA; DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as substrate. For VEGF staining, sections were previously microwave-heated in 10 mM trisodium citrate (pH 6.0) for antigen unmasking. All immunostaining was performed using the TechMate 500 (DAKO) automatic immunostaining device.

Data analysis

The following parameters were obtained from the medical records of the 112 colorectal cancer patients: age, tumor size,

TABLE I – CHARACTERISTICS OF THE COLORECTAL PATIENT SERIES

Colorectal series Characteristics	Total (%)
Patients	112
Median age	70.5 ± 10.8
Sex	
Male	60 (54)
Female	52 (46)
Tumor side	
Left	47 (42)
Right	37 (33)
Rectum	28 (25)
Tumor Stage	
I	11 (9.8)
II	62 (55.35)
III	33 (29.5)
IV	6 (5.35)
Vascular invasion	
No	67 (60)
Yes	45 (40)
Polyps	
No	80 (71.4)
Yes	32 (28.6)
Lymph Node Metastases	
Negative	75 (67)
Positive	37 (33)
Tumor differentiation	
Well	67 (59.8)
Moderate	38 (34)
Poor	7 (6.2)
p53	
Negative	31 (28)
Positive	81 (72)
K-Ras	
Negative	78 (70)
Positive	34 (30)

lymph node metastases (LNM), pathological stage, histological grade, vascular invasion (VI) and existence of polyps (defined by the presence of polyps in the surgical specimen). Pathological stage was assessed using the tumor-node-metastases (TNM) classification. Presence of lymph node metastases was evaluated by optical microscopy (Table I).

As the values of gene expression (T/N ratio) displayed a non-normal distribution (Kolmogorov-Smirnov test, Lilliefors correction), the data were normalized by $\log_{10}$ transformation. For the same reason, we used the geometric rather than the arithmetic average of the T/N ratio to describe the gene expression data. The variables were contrasted by ANOVA or the Pearson correlation coefficient. When the distribution was not normalized using $\log_{10}$ transformation, as with the E2F-1 data, the Spearman coefficient correlation was used to contrast the variables. In all statistical tests, two-tailed p values ≤ 0.05 were considered statistically significant. Statistical analysis was performed using the SPSS package version 11.0.

Results

Correlation between expression of VEGF, VEGF$_{165}$b, PEDF and p73 isoforms levels

The mRNA expression levels of VEGFtotal, VEGF, VEGF$_{165}$b, PEDF and individual p73 variants (ΔEx2p73, ΔEx2/3p73, ΔNp73 and TAp73) were examined in 112 colon tumor and normal counterpart tissues (65 tumor-normal matched pairs for *VEGF$_{165}$b*). The median, minimum and maximum, and 25 and 75 percentiles for VEGFtotal, VEGF, VEGF$_{165}$b, PEDF, ΔEx2p73, ΔEx2/3p73, ΔNp73 and TAp73 expression are shown in Table II. Additionally, histograms illustrating the quantitative expression levels of the aforementioned mRNAs in each individual distributed in relation to the median are showed in Figure 1.

Direct correlations between the expression levels of the different p73 variants were observed (Table III). Subsequently, we analyzed whether specific levels of p73 variants could differentially affect the mRNA expression of the 3 above-mentioned angiogenesis-related factors, VEGF, VEGF$_{165}$b and PEDF. Direct statistically significant correlations were found between the expression levels of VEGF and ΔNp73 and TAp73 forms (Table III, Figs. 2*c* and 2*d*). A positive trend was also observed for ΔEx2p73 variant (Table III, Fig. 2*a*). Similarly, significant direct correlations were observed between VEGF$_{165}$b and ΔEx2p73, ΔEx2/3p73, ΔNp73 and TAp73 expression levels (Table III, Figs. 2*e*–2*h*).

No significant correlations were observed between PEDF levels and the expression of the different p73 variants. But when cases were divided by tertiles in 3 groups for ΔEx2p73 expression levels (ΔEx2p73-1—T/N values lower than 0.51–, ΔEx2p73-2—from 0.51 to 1.34 T/N—and ΔEx2p73-3—T/N values higher than 1.34), inverse correlation between ΔEx2p73 and PEDF was observed in the group ΔEx2p73-1 ($p = 0.04$, $r = -0.33$).

VEGF and VEGF$_{165}$bprotein expression

Twenty and 10 colon cancer cases were analyzed for VEGF and VEGF$_{165}$b protein expression, respectively, by immunohistochemistry. All tumors showed positive cytoplasmic immunostaining for VEGF (from weak-moderate to strong intensity) (Figs. 3*b* and 3*d*). Normal counterpart colon tissue showed positive staining (theoretically due to the presence of VEGF$_{165}$b in the normal mucosa) (Figs. 3*a* and 3*b*). Direct trends were observed between VEGF protein expression and VEGFtotal and VEGF mRNA levels ($p = 0.1$ and $p = 0.18$, respectively). The geometric average VEGFtotal mRNA level in patients with weak-moderate (35%) and strong (65%) VEGF protein staining was 0.4 and 1.6, respectively, and 0.7 and 2.1, respectively, for VEGF mRNA level. Similarly, direct trends were observed between VEGF immunostaining and ΔEx2p73, ΔEx2/3p73 and TAp73 mRNA levels ($p = 0.19$, $p = 0.09$ and $p = 0.1$, respectively). The geometric average ΔEx2p73, ΔEx2/3p73 and TAp73 mRNA levels was 0.15, 0.04 and 0.54, respectively, for patients with weak-moderate VEGF protein staining and 0.6, 0.7 and 1.6, respectively, for patients with strong VEGF protein staining.

VEGF$_{165}$b protein expression correlated with mRNA quantification in 7 of the 10 colon cases. VEGF$_{165}$b protein expression was localized in the cytoplasm of both tumor and normal counterpart tissue (Figs. 3*e* and 3*f*). No significant VEGF$_{165}$b immunostaining was detected in the stroma (Figs. 3*e* and 3*h*).

Correlation between VEGF and PEDF expression levels

We evaluated whether levels of VEGF could alter mRNA expression of PEDF and/or vice-versa through the analysis of their correlation. A direct statistically significant correlation was observed between VEGF and PEDF mRNA levels in the colon cancer series ($p = 0.04$, $r = 0.2$) (Fig. 4*a*). Obviously, this association was not found when VEGF expression levels were analyzed with the set of primers that amplified both VEGF$_{xxx}$ and VEGF$_{xxx}$b variants (VEGFtotal set of primers). Most previous studies evaluating the expression levels of VEGF used sets of primers that amplified both type of variants, probably leading to confusing results.

Subsequently, we analyzed the effect of p73 levels on the correlation between VEGF and PEDF. Cases were divided by tertiles into 3 groups for expression of p73 isoforms: ΔEx2p73-1 (T/N values lower than 0.09), ΔEx2p73-2 (from 0.09 to 0.66) and ΔEx2p73-3 (T/N values higher than 0.66); ΔEx2/3p73-1 (T/N values lower than 0.085), ΔEx2/3p73-2 (from 0.085 to 1.24) and ΔEx2/3p73-3 (T/N values higher than 1.24); ΔNp73-1 (T/N values lower than 0.07), ΔNp73-2 (from 0.07 to 1.3) and ΔNp73-3 (T/N values higher than 1.3); TAp73-1 (T/N values lower than 0.28), TAp73-2 (from 0.28 to 1.7) and TAp73-3 (T/N values higher than 1.7). Direct correlation between VEGF and PEDF expression was observed in the group ΔEx2p73-3 ($p = 0.005$, $r = 0.463$) (Fig. 4*b*). Positive trends were also found in the group

TABLE II – MEDIAN, MINIMUM AND MAXIMUM, AND 25TH AND 75TH PERCENTILES, OF VEGF, $VEGF_{165}b$, PEDF, ΔEx2p73, ΔEx2/3p73, ΔNp73, TAp73 AND E2F-1 EXPRESSION LEVELS FOR COLON CANCER SAMPLES EXPRESSED IN LOG_{10}

	VEGFtotal	VEGF	$VEGF_{165}b$	PEDF	ΔEx2p73	ΔEx2/3p73	ΔNp73	TAp73	E2F-1
Median	0.06	0.07	0.23	−0.06	−0.6	−0.5	−0.64	−0.2	0.25
Maximum	1.71	1.49	1.04	1.86	1.85	3.07	2.6	2.6	3.38
Minimum	−2.12	−1.64	−0.9	−1.7	−2.8	−3.7	−5	−4	−4.5
Percentiles									
25	−0.36	−0.33	−0.1	−0.4	−1.2	−1.2	−1.5	−0.85	−0.5
75	0.47	0.41	0.45	0.32	0.08	0.66	0.43	0.63	0.84

VEGFtotal, contains all of the VEGF isoforms; VEGF, contains those forms presenting exon 8 and showing angiogenic properties; $VEGF_{165}b$, contains specifically the $VEGF_{165}b$ variant.

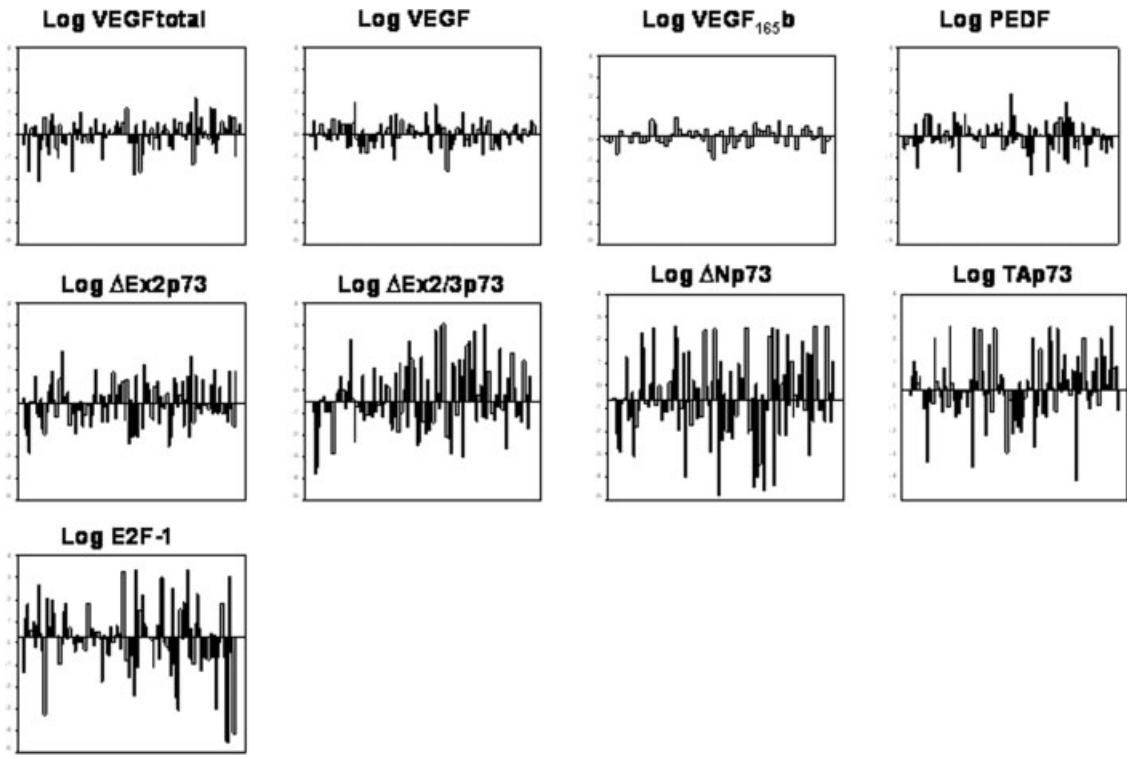

FIGURE 1 – Quantitative expression levels of VEGFtotal, VEGF, $VEGF_{165}b$, PEDF, ΔEx2p73, ΔEx2/3p73, ΔNp73, TAp73 and E2F-1, log(T/N), in the different individuals in our colon cancer series distributed in relation to the median.

TABLE III – CORRELATIONS BETWEEN EXPRESSION OF ALL p73 ISOFORMS, AND VEGF, VEGF165b, PEDF AND E2F-1 LEVELS FOR COLON CARCINOMA CASES

	ΔEx2p73	ΔEx2/3p73	ΔNp73	TAp73	E2F-1
VEGF	$p = 0.07$ ($r = 0.2$)	ns	$p = 0.05$ ($r = 0.2$)	$p = 0.002$ ($r = 0.3$)	$p = 0.03$ ($r^{\dagger} = 0.2$)
$VEGF_{165}b$	$p = 0.037$ ($r = 0.3$)	$p = 0.035$ ($r = 0.3$)	$p = 0.037$ ($r = 0.3$)	$p = 0.005$ ($r = 0.4$)	ns
PEDF	$p = 0.04$ ($r = -0.33$)*	ns	ns	ns	ns
ΔEx2/3p73	$p < 0.0001$ ($r = 0.52$)				na
ΔNp73	$p < 0.0001$ ($r = 0.63$)	$p < 0.0001$ ($r = 0.51$)			na
TAp73	$p < 0.0001$ ($r = 0.32$)	$p = 0.027$ ($r = 0.2$)	$p = 0.006$ ($r = 0.23$)		na

VEFG, contains those forms presenting exon 8 and showing angiogenic properties; $VEGF_{165}b$, contains specifically the $VEGF_{165}b$ variant. p is calculated by analysis of variance. r and $r^{\dagger}$ are the Pearson and Spearman coefficients, respectively. ns, no significant statistical correlation; na, no analyzed.

*Association found when cases were divided by tertiles into three groups for ΔEx2p73 expression levels in the group ΔEx2p73-1.

ΔEx2/3p73-3 ($p = 0.09$, $r = 0.3$) and ΔNp73-3 ($p = 0.08$, $r = 0.343$) (Figs. 4*c* and 4*d*).

Association between molecular transforming events and expression levels of VEGF, $VEGF_{165}b$ and PEDF

It has been suggested that specific events, such as inactivating mutations of the tumor suppressor gene *p53* and oncogenic activation of *K-Ras*, affect VEGF and PEDF expression levels. Positive p53 immunostaining (nuclear), suggesting *p53* mutations, was observed in 81 out of 112 colon patients (72%). Interestingly, in our series, an association was found between positive p53 staining and high levels of $VEGF_{165}b$ ($p = 0.02$) (Fig. 5*a*). The geometric average for $VEGF_{165}b$ levels was 0.95 when p53 staining was negative (no nuclear immunostaining) and 1.8 when positive. A similar association was not observed for either VEGF or PEDF.

K-Ras mutation at codons 12 and/or 13 was observed in 34 out of 112 patients (30%). A statistical association between *K-Ras* mutations and low mRNA levels of PEDF was observed ($p =$

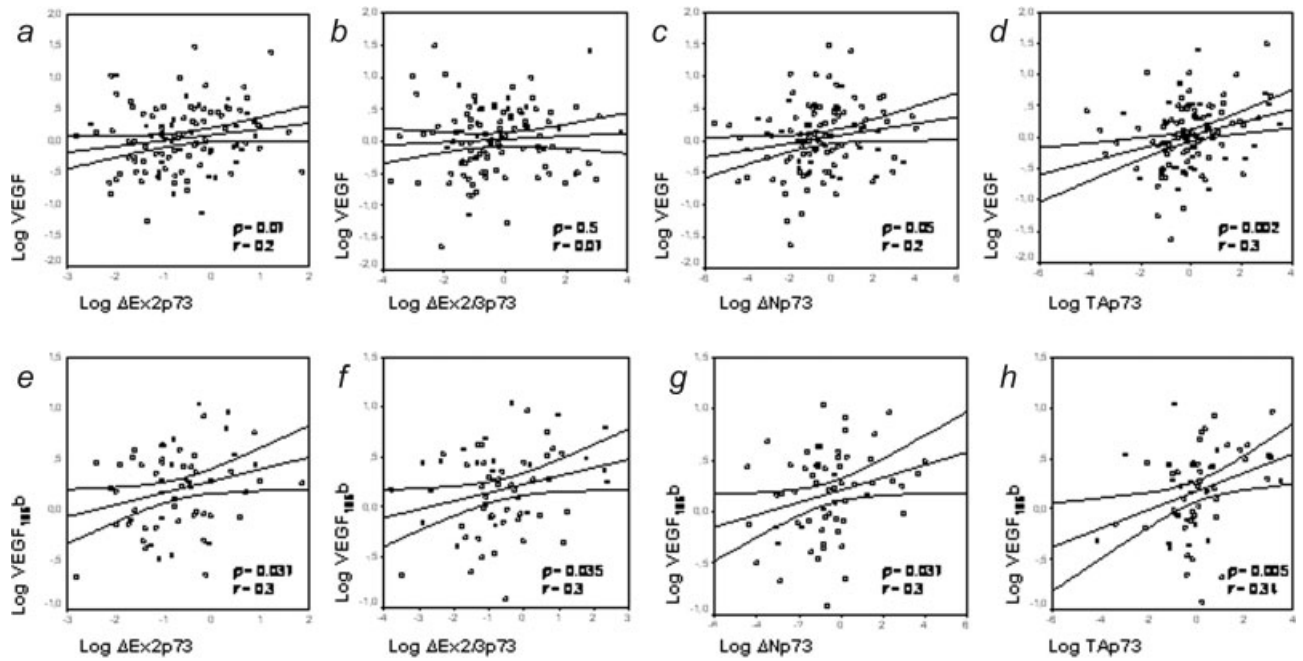

FIGURE 2 – Correlations between VEGF and p73 isoforms (*a–d*) and $VEGF_{165}b$ and p73 isoforms (*e–h*) in the colon cancer series. *p* was calculated by ANOVA test; *r* is the Pearson coefficient. Note: $VEGF_{165}b$ was analyzed in 65 tumor-normal matched pairs (112 pairs for the remaining genes).

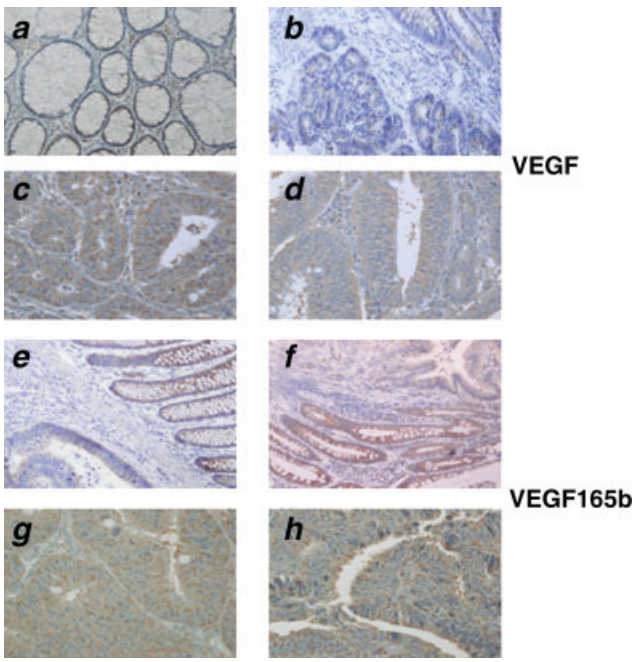

FIGURE 3 – VEGF (*a–d*) and $VEGF_{165}b$ (*e–h*) immunohistochemistry in human colon adenocarcinomas. Representative VEGF expressing normal cells (*a*), positive expression in tumor and adjacent normal tissue (*b*), and tumor cells showing strong immunostaining (*c*), and weak-moderate immunoreactivity (*d*). Representative $VEGF_{165}b$ immunostaining showing stronger positive expression in adjacent normal cells than in tumor tissue (*e*, *f*), and cytoplasmic staining in tumor cells (*g*, *h*).

0.04) (Fig. 5*b*). The geometric average for PEDF levels was 0.6 when *K-Ras* was mutated and 1.1 when wild type. Additional associations with VEGF and $VEGF_{165}b$ were not found.

Association between VEGF mRNA levels and pathological parameters

Clinicopathological characteristics of the series are listed in Table I.

Analysis of the relationship between VEGF expression levels and pathological parameters revealed some significant associations in colon cancer patients. An association was observed between over-expression of VEGF and presence of polyps in the surgical specimen ($p = 0.013$). The geometric average VEGF level in patients with absence (71.4%) and presence (28.6%) of polyps was 0.9 and 1.7, respectively.

VEGF expression levels were associated with advanced stages ($p = 0.01$). The geometric average for the expression of this factor was 0.6 in Stage I and 1.31 in Stage IV.

Association between $VEGF_{165}b$ expression levels and colon tumor pathological characteristics

Clinicopathological characteristics of the series are listed in Table I.

This is the first report analyzing the involvement of $VEGF_{165}b$ in colon carcinoma. Analysis of the relationship between the expression levels of $VEGF_{165}b$ and the pathological data revealed significant associations. One was between expression levels of $VEGF_{165}b$ and tumor stage when cases were classified in 2 groups, those harboring tumor Stage I or II (I + II) and those harboring Stage III or IV (III + IV). $VEGF_{165}b$ expression was significantly lower in those cases in stages III + IV ($p = 0.02$), with geometric averages of 1 for Stage III + IV and 1.8 for Stage I + II (Fig. 6*a*).

Low mRNA levels of $VEGF_{165}b$ were significantly associated with vascular invasion ($p = 0.02$). The geometric average $VEGF_{165}b$ level of the 41 out of 65 patients (63%) who did not show vascular invasion was 1.7; the remaining 37% with vascular invasion had a geometric average expression of 1 (Fig. 6*b*).

Presence of lymph node metastases was associated with down-regulation of $VEGF_{165}b$ ($p = 0.01$). The geometric average for the expression of this variant in 21 out of 65 patients (32%) harboring

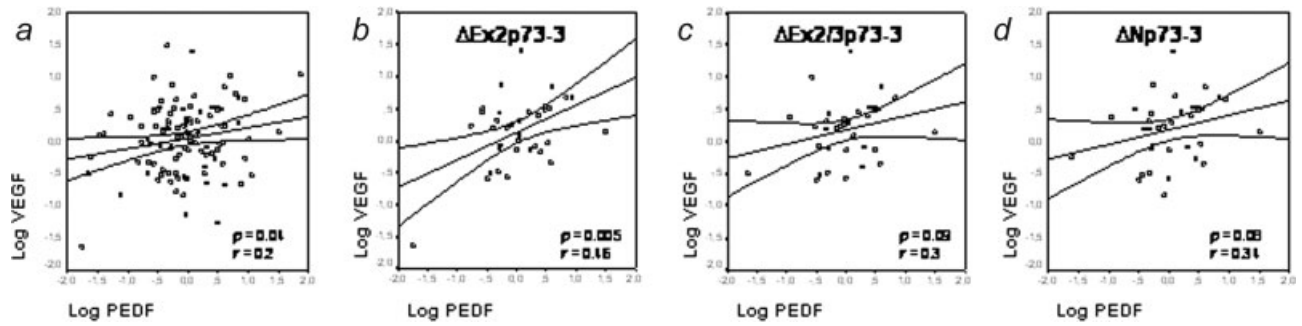

Figure 4 – General correlation between VEGF and PEDF (*a*) and when population was divided in tertiles according to p73 isoforms expression levels (*b–d*). Correlation between VEGF and PEDF in the group of patients with the highest levels of ΔEx2p73 (ΔEx2p73-3) (*b*), the group of patients with the highest levels of ΔEx2/3p73 (ΔEx2/3p73-3) (*c*) and the group of patients with the highest levels of ΔNp73 (ΔNp73-3) (*d*).

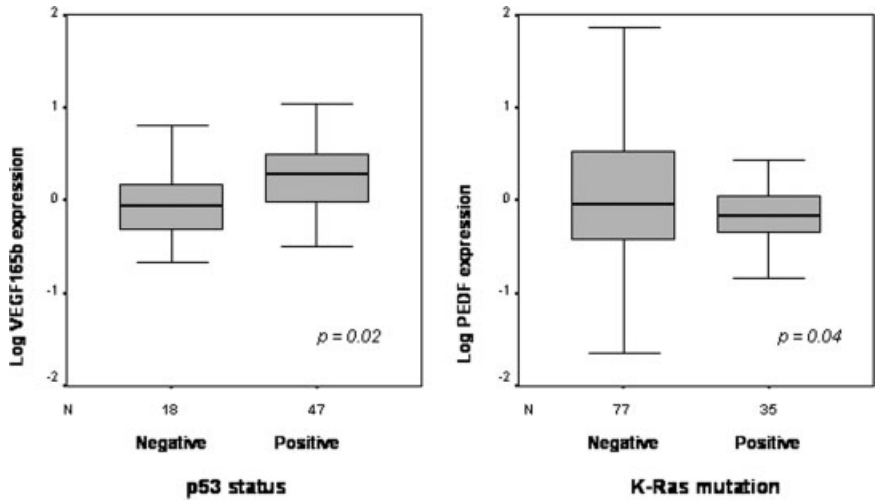

Figure 5 – Association between expression levels of $VEGF_{165}b$ and p53 mutational status (*a*), and between PEDF expression levels and *K-Ras* mutations (*b*). Data on $VEGF_{165}b$ and PEDF were normalized by a $\log_{10}$ transformation. The graphs show the 25th, 50th and 75th percentiles and values lower than 1.5 box lengths. *N*, number of patients.

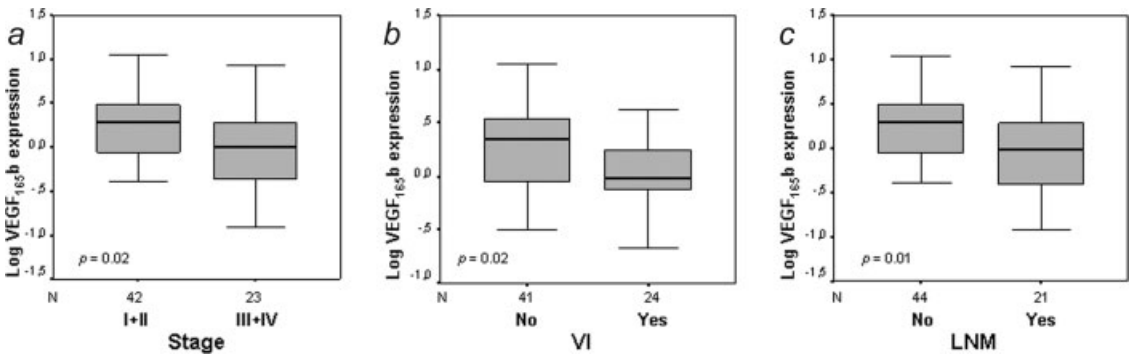

Figure 6 – Association between expression levels of $VEGF_{165}b$ and tumor stage (*a*), vascular invasion (*b*) and lymph node metastases (*c*). Data on $VEGF_{165}b$ were standardized by a $\log_{10}$ transformation. The graphs show the 25th, 50th and 75th percentiles and values lower than 1.5 box lengths. *N*, number of patients; VI, vascular invasion; LNM, lymph node metastases.

lymph node metastases was 0.9; and in those without lymph node metastases (44 out of 65, 68%), it was 1.8 (Fig. 6*c*).

Association between PEDF expression levels and pathological features

Clinicopathological characteristics of the series are listed in Table I.

This is the first report evaluating the involvement of PEDF in human primary tumors. Analysis of the relationship between the expression levels of PEDF and the pathological parameters revealed significant associations. Curiously, and similar to what we have reported elsewhere for p73 variants,[30] a significant association was observed between tumor location and PEDF expression levels ($p = 0.05$). The geometric averages for PEDF levels were 1.5, 0.71 and 0.68 for rectum, right colon and left colon location, respectively.

Interestingly, a trend was observed between expression levels of PEDF and age at diagnosis of the disease. Specifically, downregulation of this anti-angiogenic factor was observed in those patients diagnosed under the age of 50 ($p = 0.06$).

Curiously, when cases were divided into 2 groups for PEDF expression, with the median as the cut-off: PEDF-1 (T/N values lower or equal to 0.87) and PEDF-2 (T/N values higher than 0.87), an interesting association was found between low expression levels of PEDF and vascular invasion in the PEDF-1 group ($p = 0.028$). The geometric average for PEDF level of 22 (39%) of 56 patients of this group with vascular invasion was 0.1; the remaining 61% patients who did not show vascular invasion had a geometric average expression of 0.4.

Induction of E2F-1 by VEGF

The median, minimum and maximum, and 25 and 75 percentiles for E2F-1 expression are shown in Table II. A histogram illustrating the quantitative expression levels of E2F-1 in each individual distributed in relation to the median is showed in Figure 1. VEGF affects neuronal proliferation through the upregulation of E2F family transcription factors.[9] In our series a direct correlation between VEGF and E2F-1 expression levels was observed ($p = 0.03$, $r = 0.2$) (Table III). Similar associations with $VEGF_{165}b$ or VEGFtotal were not found.

Discussion

Controversial data on the role of the p53-related protein p73 in the regulation of VEGF levels have been reported. Thus, some results point to p73 as a VEGF repressor,[26] while other publications confer on p73 a VEGF inducer function.[27,28] As the p73 gene is translated into different variants with opposing functions, we hypothesized here that the tumor suppressor variants may repress VEGF expression and the oncogenic ones may induce it, but our results do not support the initial hypothesis. We observed that both tumor suppressor and oncogenic isoforms directly correlate with VEGF expression levels (Table III, Figs. 2*a* and 2*d*), suggesting that p73 isoforms could act as VEGF inducers. It is possible that the presence of ΔTAp73 isoforms, even at low levels, suppresses the transactivating activity of TAp73 variants, and consequently their anti-tumorigenic function. Although few cases were analyzed, it seems that the same correlations could be observed when considering VEGF protein expression instead of mRNA levels. As VEGF and $VEGF_{165}b$ arise from the same promoter, we analyzed whether $VEGF_{165}b$ might also be regulated by p73 variants. Direct correlation between $VEGF_{165}b$ and the different p73 variants was found (Table III, Figs. 2*e*–2*h*), suggesting that p73 forms could also induce the anti-angiogenic isoform of VEGF. No previous data describing this *in vivo* or *in vitro* have been reported. Interestingly, $VEGF_{165}b$ immunostaining was observed in the cytoplasm of both tumor and normal adjacent tissue. No significant stroma immunoreactivy was observed (Figs. 3*e*–3*h*).

PEDF has been described in some biological systems as an angiogenesis inhibitor[33,35–37] and its downregulation has been involved in tumor progression.[38,39] PEDF expression could be induced by p73.[32] In our colon cancer series we observed an inverse correlation between ΔEx2p73 and PEDF in the group of patients harboring the lowest levels of ΔEx2p73 (Table III), which suggests that this specific p73 variant could negatively regulate PEDF levels. It seems that this regulation could be ΔEx2p73 level-dependent.

Interestingly, PEDF inhibition of VEGF expression in osteosarcoma cells [41] and VEGF induction of PEDF in oral squamous cell carcinoma cells have been described.[42] The functional connection between the 2 proteins may be tissue-specific. Our data showed a direct correlation between both mRNA expression levels (Fig. 4*a*), suggesting that VEGF levels could be induced by PEDF or viceversa. Positive feedback could also exist. The fact that this correlation is only observed in the groups of patients showing higher levels of p73 variants supports the idea that both mRNA expressions could be modulated by the levels of the p73 variants (Figs. 4*b*–4*d*).

Inactivation of p53 has been associated with VEGF overexpression.[7] Although such an association was not observed in our series, an interesting association between altered p53 and $VEGF_{165}b$ over-expression was found (Fig. 5*a*). $VEGF_{165}b$ overexpression could be a mechanism to compensate p53 inactivation and to control cell growth. Other oncogenic events such as K-Ras activation could also lead to VEGF over-expression or PEDF downregulation.[8,40] Our data corroborate that oncogenic K-Ras could negatively regulate PEDF expression *in vivo* contributing to the angiogenesis process (Fig. 5*b*).

VEGF could affect neuronal proliferation through the upregulation of E2F family transcription factors.[9] Additional data supporting this in other scenarios have not been reported. We observed in our colorectal cancer series direct correlation between VEGF and E2F-1 expression levels (Table III), suggesting that in the carcinogenic process VEGF might also promote cellular proliferation through E2F-1 upregulation.

The association between over-expression of VEGF and poor tumor prognosis parameters is well documented.[12–16] In our series a correlation between over-expression of VEGF and presence of polyps in the surgical specimen and advanced stage was observed. Remarkably, although downregulation of $VEGF_{165}b$ has been previously observed in renal and prostate human cancers,[23,24] its association with poor prognosis is badly documented in the literature. Only very recent data described the association between $VEGF_{165}b$ downregulation and tumor spread in patients with primary melanoma.[25] Therefore, studies evaluating this association could highlight the role of $VEGF_{165}b$ as a tumor suppressor protein and a tumor prognosis marker. In our colon cancer series we observed an association between downregulation of $VEGF_{165}b$ and advanced tumor stages, vascular invasion and lymph node metastases (Fig. 6). Classically, these 3 parameters are the most robustly associated with a poor outcome in colorectal cancer patients and consequently $VEGF_{165}b$ could be a sensitive marker of tumor spread and metastasis.

Although interesting associations were also observed for PEDF and the pathological parameters, the associations found for $VEGF_{165}b$ were stronger, indicating that $VEGF_{165}b$ could be a better marker of poor prognosis.

As most studies analyzing VEGF levels were done before the discovery of isoforms with putative anti-angiogenic properties, they used primers quantifying both angiogenic and anti-angiogenic variants. We suggest utilizing specific primers for the variant subject of study based on the differences we observed.

Outstandingly, this is the first series in human specimens analyzing the correlation between p73 and VEGF expression levels which take into account the different functional isoforms of both genes. Previous studies evaluating such as correlations did not consider that fact.[26–28] In summary, p73 isoforms could modify *in vivo* the expression levels of angiogenesis-related factors such as VEGF, $VEGF_{165}b$ and PEDF. The VEGF/$VEGF_{165}b$-PEDF ratio may be critical for tumor angiogenesis capability. However, this scenario becomes complicated, since a feedback between VEGF and PEDF may exist. VEGF may show additional functions through the activation of the E2F-1 factor. We also observed that oncogenic stress, like mutations in *K-Ras*, could downregulate PEDF, as previously described. Other oncogenic signals such as p53 inactivation might result in compensatory mechanisms such as over-expression of $VEGF_{165}b$. Evidence is presented here on the role of $VEGF_{165}b$ as a tumor suppressor factor and its prognosis value in colorectal carcinogenesis.

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