Histol Histopathol (2014) 29: 207-216
DOI: 10.14670/HH-29.207

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Differential expression of p53 family proteins in colorectal adenomas and carcinomas: Prognostic and predictive values

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Summary. We studied the contribution of *p53* family proteins and their isoforms to the development and progression of colorectal carcinoma in relation to VEGF. Methods: *p53, p63, p73* and *VEGF* proteins were assessed in 45 colorectal adenomas (CRAs), 80 carcinomas (CRCs) and 36 normal colonic tissue samples (NCT) by immunohistochemistry. Different *p63* and *p73* isoforms were assessed by RT-PCR. Aberrant protein and RNA expressions were correlated to patients' characteristics, disease free and overall survival (DFS& OS).

Results: *p53, p63, p73* and *VEGF* proteins were detected in 22.2%, 73.3%, 33.3%, 46.7% CRAs; in 68.8%, 38.8%, 62.5%, 62.5% CRCs and 16.7%, 83.3%; 13.9%, 27.8% NCT ($p<0.05$ except for VEGF). Commonest isoforms were *TAp63α, ΔNp63, TAp73α in CRA* and *ΔNp63, TAp63α, ΔNp73, TAp73β* in CRC. Significant correlations were found between aggressive tumor phenotypes and aberrations in *p73, p53, p63, VEGF*. DFS correlated with advanced stage, *p73* and *VEGF* aberrations. While advanced stage, positive lymph nodes, *p73* and *p53* correlated with OS. Prognosis was worse in patients with aberrant *p63*& *p73* than in those with normal *p63*& *p73* expression regardless of *p53* gene status ($p<0.05$).

Conclusions: *p53* family proteins and *VEGF* play a pivotal role in colorectal carcinogenesis. *p53* prognostic potential is augmented by *p73* and p63 aberrations indicating a synergistic effect between the three family members. Nodal status, stage, *p73, VEGF* and *p53* could be used as predictors of DFS and OS.

Key words: Colorectal carcinoma, Adenoma, p53, VEGF, Prognosis

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Introduction

Colorectal cancer (CRC) is the third most common tumor in Western countries and the fifth in Egypt. Patients with similar disease characteristics can exhibit varied survival outcomes (Mokhtar, 2002; Guan et al., 2003). This could be improved by measuring biological markers, which have key roles in tumor progression. Therefore, it is essential to identify new biological prognostic and predictive factors that might help in better management of patients (Toumi et al., 2010; Soldevilla et al., 2011).

The *p53* belongs to a family of proteins which includes *p63* and *p73*. These are transcription factors sharing significant homologies in their structural organization, especially in the DNA-binding domain, the NH2-terminal trans-activation and COOH-terminal oligomerization domains. They recognize the same DNA sequences and thus they can regulate the expression of a large number of *p53* transcriptional target genes. Consequently, they might share some molecular functions in the cell (Levrero et al., 2000; Harms et al., 2004).

Although the importance of *p53* as a tumor suppressor is undisputed, the roles of *p63* and *p73* are less clear, due to their distinct and opposing biological

effects. This could be attributed to the complexity of their genomic structure, which is translated into different isoforms (Stiewe and Putzer, 2002). Both genes produce two opposed protein classes through alternative promoters and exon splicing: the transactivation domain (*TAp63, TAp73*), and the inhibitory proteins lacking TA domain (*ΔNp63* and *ΔNp73*), which retain the DNA binding and tetramerization competence and act as powerful dominant-negative inhibitors of *p53*. Moreover, they can undergo extensive COOH-terminal splicing producing different species; named α, ß, γ….etc (Zaika et al., 2002; Spiesbach et al., 2005).

Previous studies demonstrated that *p53* inactivation causes an imbalance between angiogenic and antiangiogenic factors with angiogenic switch in several tumor types (Guan et al., 2003; Soldevilla et al., 2011). The vascular endothelial growth factor (*VEGF*) and its receptors are of utmost importance in this process. Guan et al., 2003 mentioned *TP73* as an angiogenic factor being significantly associated with VEGF expression and Dominguez et al (2006) demonstrated an association between *ΔNp73* and poor prognosis in CRC patients. However, the contribution of other *p53* family members to CRC development and progression is still controversial.

We studied the contribution of *p53* family proteins (combined) and their isoforms to the development and progression of CRC in relation to *VEGF*, standard prognostic factors and survival.

Materials and methods

Patients

This prospective study included 45 CRA and 80 CRC cases. Normal colonic mucosal tissues (NCT) were obtained from areas adjacent to removed benign colonic lesions as control. Samples were collected from patients attending the clinics of National Cancer Institute (NCI) and Kasr Al-Aini School of Medicine, during the period from 2007 to 2009. An informed consent was obtained from all patients prior to enrollment in the study and the ethical committees of NCI and Kasr Al-Aini School of Medicine approved the protocol, which was in accordance with ethical guidelines of 2007 Declaration of Helsinki. None of the carcinoma patients received neoadjuvant therapy. Fresh tumor and normal tissues were obtained at surgery or colonoscopy and divided into two parts: the first was put in 10% neutral buffered formalin and embedded in paraffin. From each paraffin block of tumor or normal samples, a hematoxylin and eosin-stained slide was prepared and examined microscopically to confirm diagnosis in tumors or absence of neoplastic cells in NCT and to assure that tumor samples contain ≥75% neoplastic cells. The second part was stored at -80°C for RNA extraction. Cases were diagnosed and graded according to the WHO classification of colorectal tumors and staged according to TNM staging system (Hamilton and Aaltonen, 2000). Patients were followed-up for at least 12 months. Patients' characteristics and survival data were obtained from the clinical records. Overall and disease-free survival rates (OS& DFS) were calculated from the date of diagnosis till the end of the follow-up period.

Treatment protocols

Colectomy, abdomino-perineal resection, posterior pelvic excentration or low anterior resection were performed according to NCI guidelines. Postoperative adjuvant chemo-radiation therapy was applied for recto-sigmoid and rectal tumors with T3-4 and/or lymph nodes positive cases. Irradiation was given in a dose of 50Gy/5 weeks. Six cycles of the Mayo regimen (bolus intravenous fluorouracil 425 mg/m^2 and leucovorin, 20 mg/m^2) were given, for 5 consecutive days every 28 days. For rectal carcinoma, chemotherapy was given as a radio-sensitizer, with bolus intravenous fluorouracil 375 mg/m^2 and leucovorin 20 mg/m^2 during the first and last three days of postoperative irradiation. The Mayo Clinic regimen was continued for 6 cycles immediately after the end of irradiation if laboratory investigations were satisfactory.

Immunohistochemistry

A section was obtained from the paraffin block of each studied case and stained with hematoxylin and eosin. Another 3 (5 μm sections) were cut from each sample and control blocks and placed on positive charged slides. Sections were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method (El-Serafi et al., 2010). The monoclonal antibodies used are: mouse anti-pan *p63* (4A4, 1:200 dilution), anti-*p73* (*p73α/ß* Ab-2, NeoMarkers, USA, 1:50), anti-*p53* (DO-7, 1:25) and anti-*VEGF* (*VEGF[C-1]:sc-7269*, 1:100) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA, except for *p73α/ß*). Biotinylated anti-mouse immunoglobulins (Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 30 minutes after overnight incubation at 4°C, followed by avidin-biotin peroxidase complexes (1/25, Vector Laboratories, Inc.). Diaminobenzidine was used as the chromogen and Mayer hematoxylin as a counter stain. Slides were reviewed by two pathologists (BA& AA) and results were scored by estimating the percentage of tumor cells showing nuclear staining (cytoplasmic for *VEGF*). An arbitrarily defined 10% cut-off categorized data into positive and negative groups. Any cytoplasmic staining was considered positive for *VEGF* (Urist et al., 2002; Guan et al., 2003; Puig et al., 2003; Kaklamanis et al., 2006). A case of invasive breast carcinoma was used as a positive control for *p53, p73, VEGF* and normal prostatic tissue as positive control for *p63*. Negative controls were achieved by replacing the primary antibody by serum.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cases and controls using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to manufacturer's instructions. The RNA (1.0 μg) was amplified in 50 μl volume with *p63* and *p73* isoform-specific primers using the Superscript One-Step RT-PCR Kit with Platinum *Taq* (Life Technologies, Inc.). RT-PCR reactions were performed for 30 minutes at 50°C, 3 minutes at 94°C, followed by isoform-specific PCR. *GAPDH* was used as an endogenous RT-PCR standard. Primer sequences and PCR conditions are illustrated in table 1. The RT-PCR was performed in duplicate and 25 μl of each RT-PCR product were resolved in 2.0% agarose gels (Urist et al., 2002; Puig et al., 2003).

Statistical analysis

SPSS version (12) was used for data analysis. Mean and standard deviation described quantitative data, while percentages described qualitative data. Marker expression was treated as continuous and categorical variables. Difference in tumor prognostic factors between groups according to data from markers expression was analyzed as categorical variables. OS was calculated from the date of diagnosis till the end of follow-up or death while DFS was calculated from the date of surgery to last follow up or occurrence of recurrence. Kaplan Meier estimates survival and log rank test compared curves, Cox regression analysis was done for OS/DFS as outcome (dependent variable) and different prognostic factors including the tested markers to describe independent effect on survival. Odd ratio described likelihood of death or recurrence for a subgroup of patients compared to another group. p value is significant at 0.05 levels.

Results

The mean age of CRA patients was 40.3±4.1 years (range 20-50). The male to female ratio was 1.3:1. Forty two cases were tubular adenomas and three were tubulo-villous adenoma. The mean age of CRC patients was 49.8 years (range, 21-85 years) with a male to female ratio of 1.9:1. Forty two patients had colonic carcinoma and 38 had rectal/recto-sigmoid carcinomas. Thirty patients received postoperative adjuvant chemo-radiotherapy, and 50 received adjuvant chemotherapy only.

Protein expression of the studied markers

A relatively restricted distribution of *p53, p63, p73* and *VEGF* was detected in NCT (16.7%, 83.3%, 13.9% and 27.8%; respectively) mainly in the cells of the crypts compared to 22.2%, 73.3%, 33.3%, 46.7% in CRAs and 68.8%, 38.8%, 62.5%, 62.5% in CRCs; respectively. There was a significant difference between NCT and CRCs ($p<0.001$) as well as between CRAs and CRCs regarding the expression of *p53, p73,* and *p63* (p=0.01,

Table 1. Primer sequences and PCR conditions of p36 and p73 isoforms.

Isoform	Fragment size	Primer sequences	PCR cycles
TAp73	305	5'-TCTCTGGAACCAGACAGCAC-3' 5'-GGGGTAGTCGGTGTTGGAG-3'	40 cycles: 94°C (30s), 56°C (40s), 72°C (30s)
Δ Np73	213	5'-TGTACGTCGGTGACCCCG-3' 5'-GGGGTAGTCGGTGTTGGAG-3'	40 cycles: 94°C (30s), 60°C (20s), 72°C (8s)
TAp73α	306	5'-CTGAAGATCCCCGAGCAGTA-3' 5'-CTCCGTGAACTCCTCCTTGA-3'	40 cycles: 94°C (30s), 56°C (40s), 72°C (30s)
TAp73ß& p73δ	304	5'-GACCGAAAAGCTGATGAGGA-3' 5'-CCCCAGGTCCTCTGTAGGAG-3'	40 cycles: 94°C (30s), 56°C (40s), 72°C (30s)
TAp73γ	213	5'-CGGGATGCTCAACAACCAT-3' 5'-TGCAGGTGGTAAATGCTCTG-3	40 cycles: 94°C (30s), 54°C (4s), 72°C (6s)
TAp63	896	5'-CCCAGAGCACACAGACAAA-3' 5'-CACAGATCCGGGCCTCAAA-3'	2 cycles: 94°C (30s), 57°C (40s), 72°C (30s). 2 cycles: 94°C (30s), 55°C (40s), 72°C (30s), then 36 cycles: 94°C (30s), 53°C (40s), and 72°C (30s)
TAp63α	213	5'-GAGGTTGGGCTGTTCATCAT-3' 5'-AGGAGATGAGAAGGGGAGGA-3'	2 cycles: 94°C (30s), 57°C (40s),72°C (30s), then 38 cycles at 94°C (30s), 55°C (40s), 72°C (30s)
TAp63ß	205	5'-AACGCCCTCACTCCTACAAC-3' 5'-CAGACTTGCCAGATCCTGA-3'	2 cycles: 94°C (30s), 57°C (40s),72°C(30s), then 38 cycles at 94°C (30s), 55°C (40s), 72°C (30s)
TAp63γ	697	5'-ATGCCCAGTATGTAGAAGA-3' 5'-GGGCTTGGAATGTCTAAAG-3'	2 cycles: 94°C (30s), 57°C (40s),72°C(30s), then 38 cycles at 94°C (30s), 55°C (40s), 72°C (30s)
Δ Np63	392	5'-AACAATGCCCAGACTCAA-3' 5'-ACAGGCATGGCGCGGATA-3'	2 cycles: 94°C (30s), 57°C (40s), 72°C (30s). 2 cycles: 94°C (30s), 55°C (40s), 72°C (30s), then 36 cycles: 94°C (30s), 53°C (40s), and 72°C (30s)
GADPH		5'-GAAGGTGAAGGTCGGAGT-3' 5'-GAAGATGGTGATGGGATTTC-3'	

p=0.003, p=0.003; respectively). The expression of VEGF did not differ significantly between the three groups (p=0.09) (Table 2, Figs. 1, 2).

p63 and p73 isoforms in CRC

RT-PCR for TA, ΔN and the various COOH-terminal splice variants of the *p63* and *p73* in CRA and CRC tissues showed that 38 (84.4%) CRAs and 47 (58.8%) CRCs have TA, ΔN, and COOH-terminal *p63* RNA splice variants. In CRA, *TAp63α* was the commonest variant (14 cases) followed by both *TAp63α/ΔNp63* (13 cases), and *ΔNp63* (9 cases). In CRC, 23 cases showed *ΔNp63*, 14 showed *TAp63* (12 *TAp63α* and 2 *TAp63γ*), and 10 expressed both *TA/ΔNp63* isoforms. *p73* RNA was detected in 17 (37.8%) CRAs and in 62 (77.5%) CRCs. Five CRA cases expressed *ΔNp73*, 10 cases expressed *TAp73α*, one case expressed *TAp73β* and

Table 2. Expression of studied markers in normal colonic mucosa, colorectal adenomas and carcinomas

Markers		Colorectal Carcinoma (80)	Colorectal Adenoma (45)	Normal colonic tissue (36)*
p53	Negative	25 (31.3%)	35 (77.8%)	30 (83.3%)
	Positive	55 (68.8%)	10 (22.3%)	6 (16.7%)
p63	Positive	31 (38.8%)	33 (73.3%)	30 (83.3%)
	Reduced/lost	49 (61.3%)	12 (26.7%)	6 (16.7%)
p73	Negative	30 (37.5%)	30 (66.7%)	31 (86.1%)
	Positive	50 (62.5%)	15 (33.3%)	5 (13.9%)
VEGF	Negative	30 (37.5%)	24 (53.3%)	21 (58.3%)
	Positive	50 (62.5%)	21 (46.7%)	15 (41.7%)

*: Number of cases

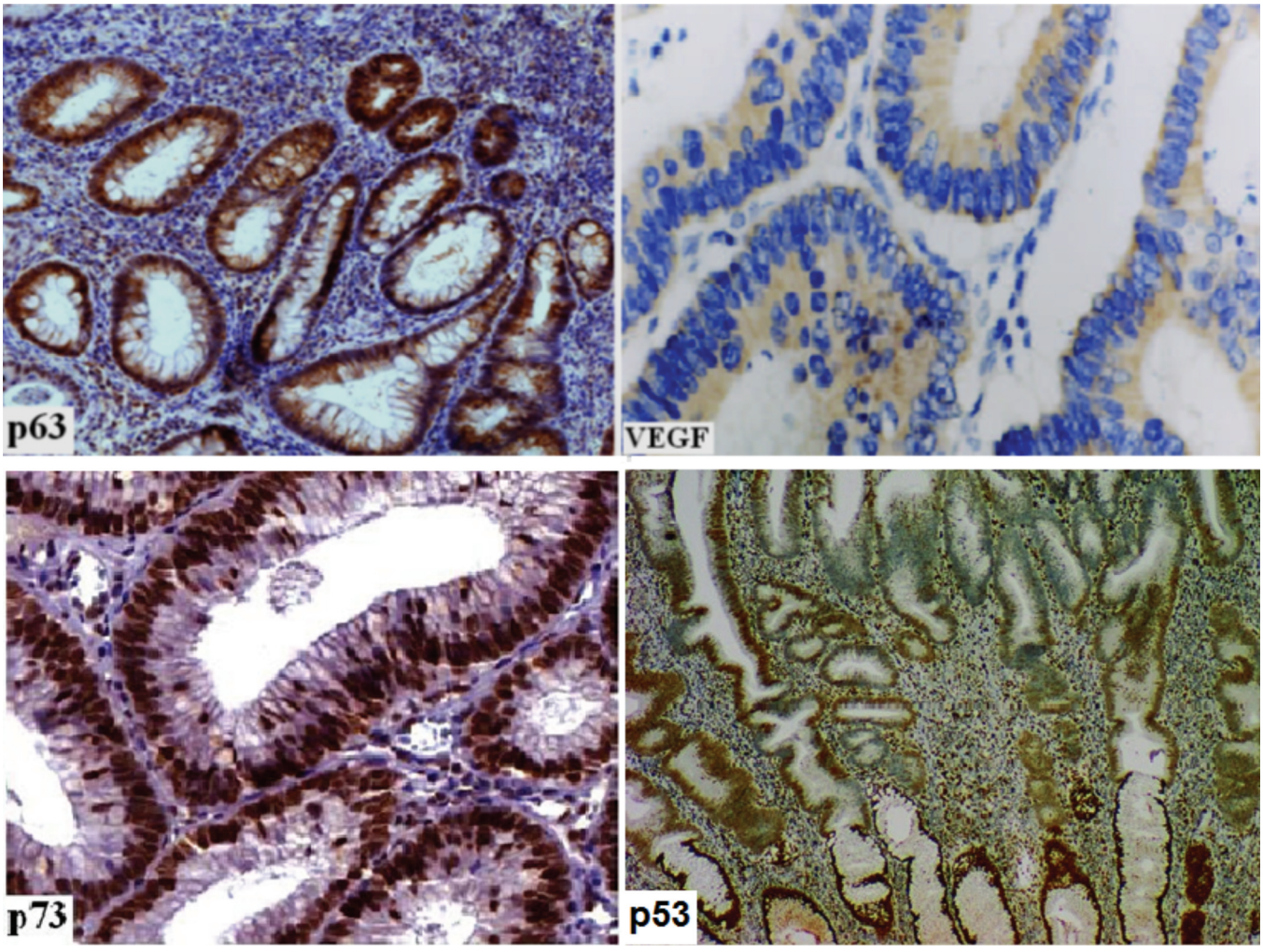

Fig. 1. Colorectal carcinoma cases showing positive nuclear immunostaining of p53 protein, p73 protein, p63 protein and positive cytoplasmic immunostaining for VEGF.

another case expressed both *TAp73α/β*. In CRCs *ΔNp73* was detected in 28 cases with no detectable *TAp73* indicating the absence of any transcriptionally active *p73*. *TAp73* only was detected in 27 cases (10 *TAp73β*, 8 *TAp73α*, 9 *TAp73α/β*) and seven cases expressed both *ΔNp73/TAp73* (Table 3, Fig. 3). We found a significant correlation between *TAp73*-RNA expression and *p73* protein ($p<0.01$), as well as between *TAp63*-RNA and *p63* protein expression ($p<0.05$). The concordance between protein and RNA expressions was 87.5% for *p63* and 90.6 % for *p73*.

Correlation between protein expression of the studied markers

A significant correlations was found in CRC between *p73* and VEGF ($p=0.03$), *p73* and *p53* ($p=0.01$) as well as between *p53* and *VEGF* ($p=0.03$). On the other hand, no significant relation was found between *p63* and any of the studied markers (Table 4). In CRA a

Table 3. 'Expression of p63 and p73 isoforms in colorectal adenoma and carcinoma.

Isoforms	Adenoma (45)*	Carcinoma (80)
p63	38 (84.4%)	47 (58.8%)
TAp63α	14	12
TAp63ß	0	0
TAp63γ	2	2
Δ Np63	9	23
TAp63α&ΔNp63	13	10
p73	17 (37.8%)	62 (77.5)
TAp73α	10	8
TAp73ß	1	10
TAp73α& ß	1	9
Δ Np73	5	7
TA&ΔN	0	7

*: Number of cases

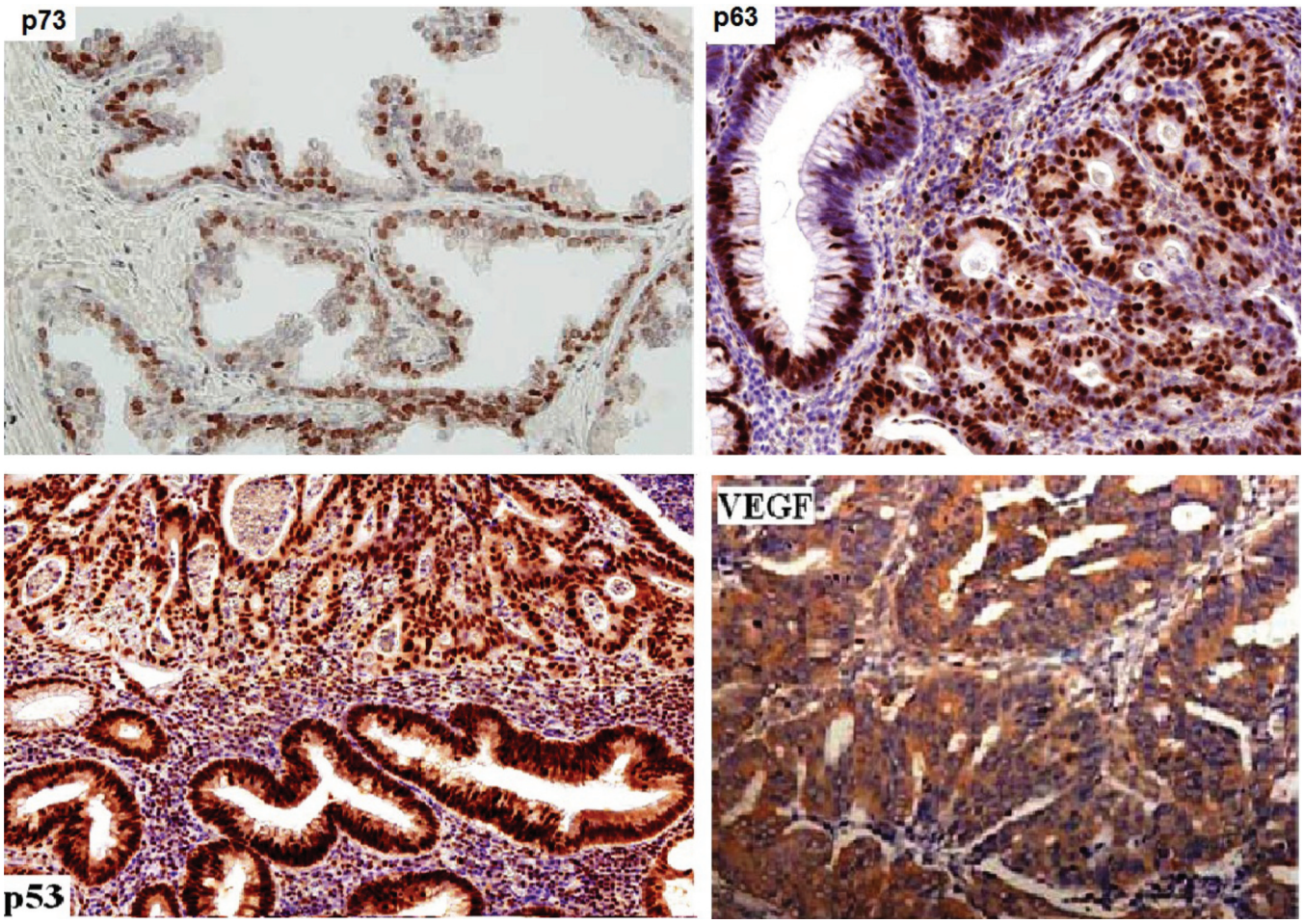

Fig. 2. Colorectal adenoma cases showing positive nuclear immunostaining of p53 protein, p73 protein, p63 protein and moderately positive cytoplasmic immunostaining for VEGF.

significant relation was found between *VEGF* and both *p53* (p=0.03) and *p73* (p=0.02) overexpression In CRA.

Clinical correlations

Significant correlations were found between *ΔNp63* and lymph node status (p=0.01), metastatic recurrence (p=0.01), advanced stage (p=0.03) as well as between *ΔNp73* and poorly differentiated tumors (p=0.02), high incidence of recurrence (p=0.03) and advanced stage (p=0.001). Table 5 shows the relation between marker expression and standard clinicopathologic prognostic factors.

We also examined the correlation between tumor stage [early (I&II) versus late (III&IV)], lymph node status or tumor recurrence and the combined data referring to *p53, p63* and *p73* expression to determine the interaction between the three proteins in relation to tumor aggression. Cases were classified into 4 groups (Tables 6-8). The *first group* [tumors with normal *p53* combined with *p63/p73* aberrations] showed a significant difference in relation to the previously mentioned prognostic factors compared to the *third group* [tumors with normal *p53* and normal *p63/p73*] and the *second group* [tumors with aberrant *p53* and normal *p63/p73*]. Also, there was a significant difference between patients with aberrant *p53* expression and aberrant *p63/p73* (*fourth group*) compared to those with *p53* overexpression and normal *p63/p73* (*second group*).

Table 4. The correlation between aberrant expressions of studied markers.

Markers	p73 positive 50 (62.5%)	p73 negative 30 (37.5%)	p. value
p53 expression			
Normal (25)	6 (24%)	19 (76%)	
Overexpression (55)	44 (80%)	11 (20%)	<0.01
p63 expression			
Normal (31)	17 (54.8%)	14 (45.2%)	
Reduced expression (49)	33 (67.3%)	16 (32.7%)	0.34
VEGF expression			
Normal (30)	5 (16.7%)	25 (83.3%)	
Overexpression (50)	45 (90%)	5 (10%)	<0.001

Table 5. Correlation between markers expression and clinicopathological prognostic factors in colorectal carcinomas.

Parameter		VEGF*(50)	p73*(50)	p63†(49)	p53*(55)
Tumor site	Colon (42)	26	25	30	28
	Rectum (38)	24	25	19	27
		0.54	0.74	0.54	0.81
Tumor size	≤5 (49)	92	30	32	32
	>5 (31)	21	20	17	23
		0.86	0.74	0.32	0.15
Tumor type	Non-mucinous (54)	26	30	33	29
	Mucinous (26)	24	20	16	26
		0.27	0.73	0.18	0.02
Tumor grade	High (26)	21	25	18	20
	Low (54)	29	25	31	35
		0.04	<0.01	0.27	0.04
Tumor stage	Early (34)	15	12	7	10
	Late (46)	35	38	42	45
		0.004	<0.001	<0.001	<0.002
Lymph nodes	Positive (44)	39	39	35	37
	Negative (36)	11	11	14	18
		0.03	0.02	0.03	0.07
Metastatic recurrence	Yes (38)	36	28	21	36
	No (42)	14	22	28	19
		<0.001	0.04	0.81	<0.01

*: Increased expression; †: Reduced expression

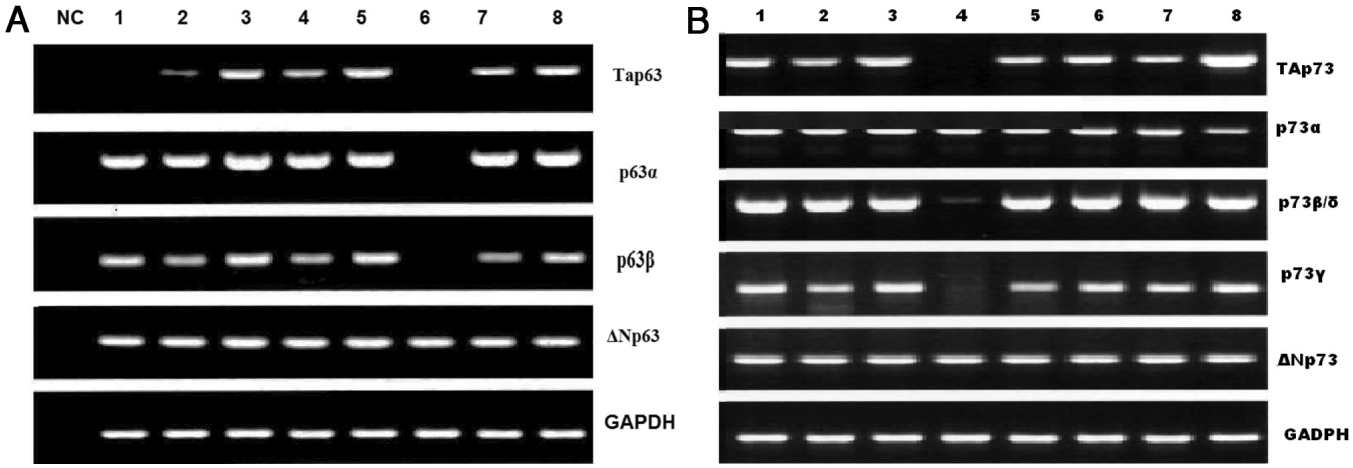

Fig. 3. 2% ethidium bromide- stained gel. **A.** Expression of p63 tissue activating (TA) and N isoforms in colorectal adenomas and carcinomas (lane no. 1: negative control; 2,3,7: adenomas; 4,5,6,8: carcinomas). **B.** Expression of p73 tissue activating (TA) and N isoforms in colorectal adenomas and carcinomas lane no.1: negative control; 2,3,5,7 adenomas; 4,6,8: carcinomas).

The first group showed significantly worse prognosis (in terms of lymph node status, tumor recurrence and advanced disease stage) compared to the second and third groups, signifying the prognostic effect of *p63* and *p73* in conjunction with *p53* aberrations or even regardless of *p53* gene status.

Table 6. Effect of expression of p53 family proteins in relation to recurrence.

	Recurrence					
P value	Total	Negative (43)	Positive 37	p73	p63	p53
0.03	16	5 (31.3%)	11 (68.8%)	Aberrant	Aberrant	Normal
	21	15 (71.4%)	6 (28.6%)	Normal	Normal	Aberrant
	24	17 (70.8%)	7 (29.2%)	Normal	Normal	Normal
0.01	19	6 (31.6%)	13 (68.4%)	Aberrant	Aberrant	Aberrant

Table 7. Effect of aberrant expression of p53 family proteins in relation to stage.

	Stage					
P value	Total	Late 46	Early 34	p73	p63	p53
<0.01	26	21 (80.8%)	5 (19.2%)	Aberrant	Aberrant	Normal
	19	7 (36.8%)	12 (63.2%)	Normal	Normal	Aberrant
	16	6 (37.5%)	10 (62.5%)	Normal	Normal	Normal
0.04	19	13 (68.4%)	6 (31.6%)	Aberrant	Aberrant	Aberrant

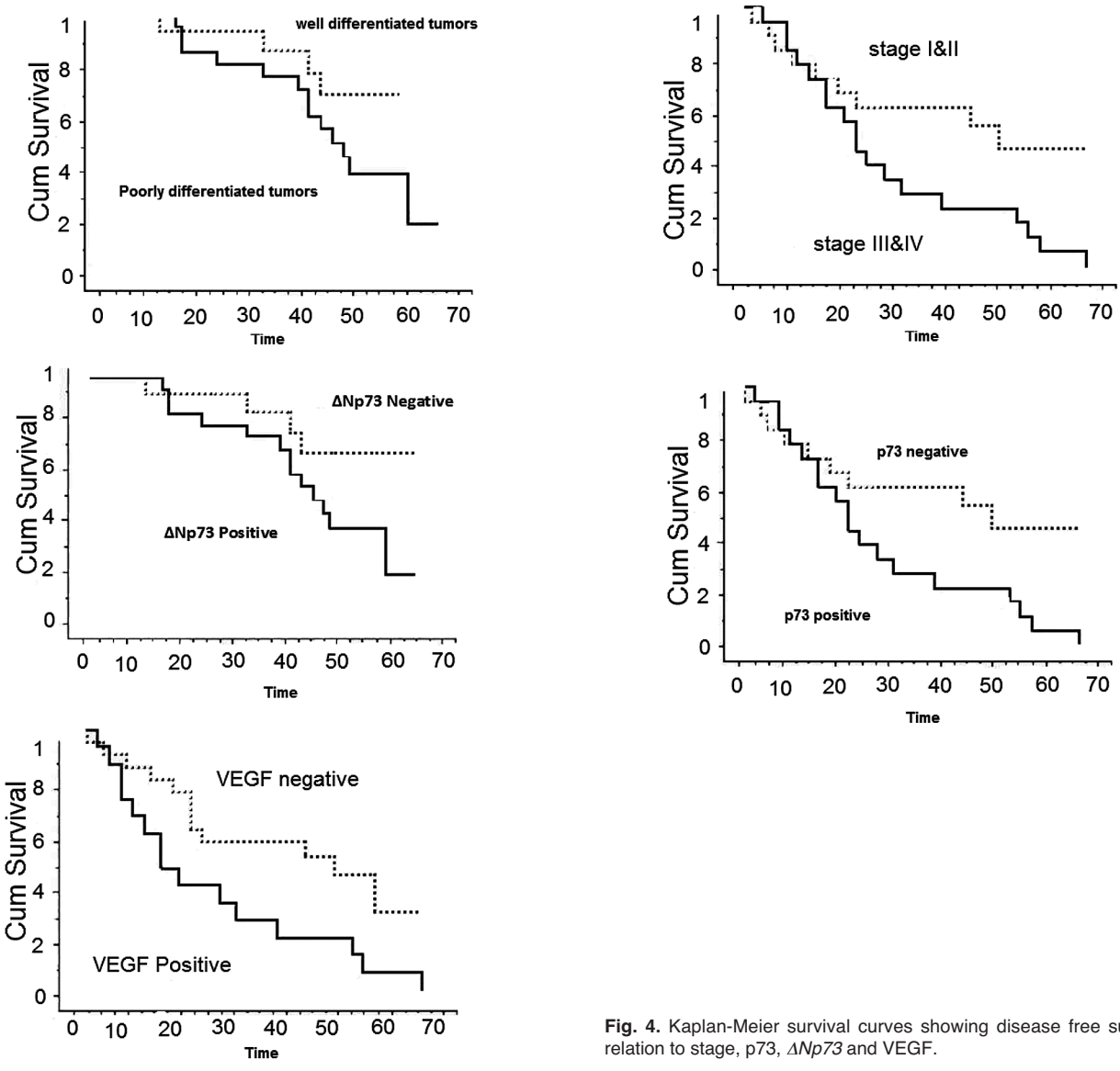

Fig. 4. Kaplan-Meier survival curves showing disease free survival in relation to stage, p73, *ΔNp73* and VEGF.

Survival analysis

The median follow-up period was 34 months (range: 4-59 months). At the end of the study, 22 patients were dead and 58 were alive. Cox regression analysis revealed that positive lymph node status, advanced stage, *p73* and *p53* overexpretion were significant prognostic variables for OS (p=0.01, p=0.032, p=0.04 and p=0.045; respectively) while, advanced stage, *VEGF*, *p73* overexpression and *ΔNp63* expression were significantly associated with DFS (p=0.031, p=0.04, p=0.02, p=0.01, respectively).

On multivariate analysis, reduced DFS associated with poorly differentiated tumors (p=0.04), advanced stage (p=0.01), *VEGF* (p=0.02), *ΔNp73* (p=0.04) and *p73* overexpression (p=0.01). Reduced OS significantly associated with positive lymph nodes (p=0.03), advanced stage (p=0.02), *p53* and *p73* overexpression (p=0.04 and p=0.02) (Figs. 4, 5).

Table 8. Effect of aberrant expression of the p53 family proteins in relation to lymph nodes.

	Lymph node status					
P value	Total	Positive 44	Negative 36	p73	p63	p53
0.031	22	15 (68.2%)	7 (31.8%)	Aberrant	Aberrant	Normal
	20	6 (30%)	14 (70%)	Normal	Normal	Aberrant
	12	6 (50%)	6 (50%)	Normal	Normal	Normal
0.06	26	18 (69.2%)	8 (30.8%)	Aberrant	Aberrant	Aberrant

Discussion

The present study is the first to investigate the role of the three *p53* family members together with their oncogenic and suppressor isoforms in the development and progression of CRC. Our results provide an evident that the *p53* family proteins are significantly involved in the genetic cascade of colorectal carcinogenesis with highly significant correlation between *p53*/*p73* aberrations and *VEGF* over-expression suggesting the involvement of these proteins in the regulation of angiogenesis in colorectal cancer patients.

Our data regarding the angiogenic effect of *p53* family proteins confirms previously published data where Vikhanskaya et al. (2001) mentioned for the first time that *p73* acts as an oncogene in CRC. It regulates angiogenesis via induction of *VEGF* or via reducing *thrombospondin-1* expression leading to enhanced angiogenesis (Guan et al., 2003; Nahor et al., 2005). In addition, the *ΔNp73* may inactivate *TAp73* and *p53* suppressor properties (Dominguez et al., 2006). The *p53* was shown to regulate *VEGF* expression by two mechanisms. In the first, the wild type *p53* acts through repression of *v-Src*-mediated *VEGF* up regulation while in the second,the mutant p53 stimulates VEGF expression by activating *protein kinase C* (Cascinu et al., 2001; Des Guetz et al., 2006).

Alternative mechanisms for *p73* oncogenic effects in CRC have been mentioned including activation of a silent allele, or disruption of *p73* and *p63*- mediated signal transduction pathways with increased insulin

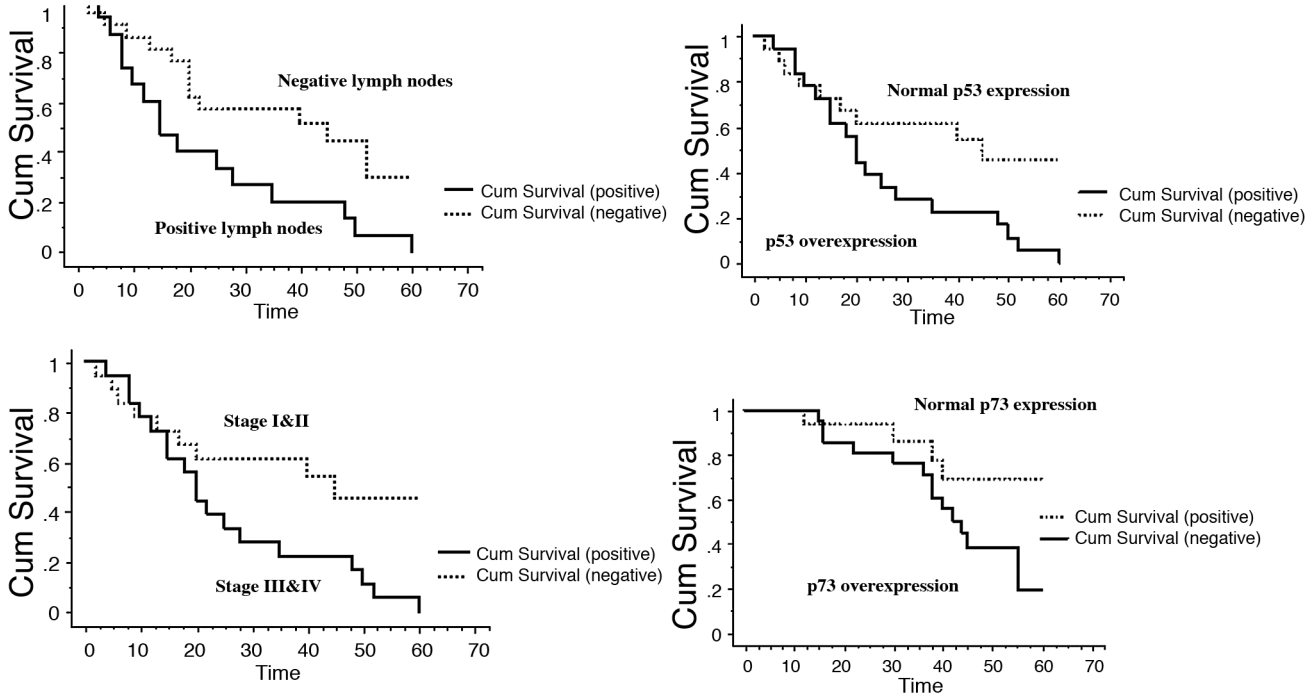

Fig. 5. Kaplan-Meier survival curves showing overall survival in relation to stage, lymph node status, p53 and p73.

growth factor-1(*IGF*) transcription and reduced apoptosis (Vikhanskaya et al., 2001; Nahor et al., 2005).

The increased expression of *p73* protein and RNA reported here is attributed to *ΔNp73/TAp73α* in CRA or *ΔNp73/TAp73*, in CRC, whereas the expression of *TAp73α* was markedly reduced. This differential expression of *p73* isoforms was significantly associated with the acquisition of aggressive tumor phenotypes e.g. positive nodal status, high incidence of recurrence and reduced survival. Accordingly, the oncogenic potential of *p73* could be attributed to the increased oncogenic (*ΔNp73*) relative to suppressor (*TAp73*) isoform with subsequent inactivation of *p53* and *TAp73* suppressor properties, or directly to its angiogenic effect as evidenced by the correlation with *VEGF*. *p73* may also exert both anti-apoptotic and pro-proliferative activities or confer a chemoresistant phenotype irrespective of *p53* status, providing evidence for the prognostic role of other *p53* family members. In this context, previous studies demonstrated significant associations between *ΔNp73* and poor prognostic factors, increased proliferation rate, enhanced angiogenesis, acquisition of drug resistance and reduced OS in CRC patients (Dominguez et al., 2006; Toumi et al., 2010; Soldevilla et al., 2011).

Our study provides, for the first time, a deep insight into the role of *p63* in colorectal carcinogenesis by assessment of *p63* protein and isoforms in CR adenomas and carcinomas. *p63* has been mentioned as a highly specific marker for the diagnosis of anal gland carcinoma (AGC) and as a marker of poor differentiation in CRC (Carneiro et al., 2006; Lisovesky et al., 2007). Herein, we report a significant reduction in *p63* protein expression from CRA to CRC and a relative increase in the *ΔNp63* isoform. This profile was associated with aggressive tumor phenotype. Our data regarding the significant reduction in *p63* protein expression during the cascade of CRC with a predominance of the *ΔNp63* is consistent with Okada et al (2002) who reported an association between p63 overexpression and up-regulation of the *ΔNp63* isoform. Most studies demonstrate an oncogenic potential of *ΔNp63* in some solid tumors (Park et al., 2000), however, Tannapfel et al (2001) verified that *TAp63* isoforms may also be involved in tumor progression at least in cases which lack *ΔNp63*. This difference in the results could be attributed to the difference in the studied tumors (bladder versus gastric cancer). Possible mechanisms for *p63* associated tumorigenesis include a shift toward mesenchymal morphology, which accompanies loss of *p63* expression or the ability of *ΔNp63* to protect the cells from growth arrest and apoptosis, and at the same time acts as a metastasis suppressor by maintaining the epithelial characters of cancer cells (Christopher et al., 2006). In addition, the *TAp63α* doesn't act as a typical *p53* family member in certain tumors as it exerts a dominant negative effect towards other *p53* family members. Consequently, its expression doesn't trans-activate *p53* downstream genes e.g. $p21^{WAF}$, *BAX, MDM2*, and doesn't arrest the cells at the G1. It can also occupy the DNA binding sites of *p53* responsive elements and prevents their occupancy by more transcriptionally active *p53* family members, providing an oncogenic effect similar to *ΔNp63* (Zaika et al., 2002; Nahor et al., 2005).

An interesting and novel finding in the present study is the negative cooperative effect between the three *p53* family members in relation to stage, local recurrence and lymph node status (the main prognostic factors in CRC). We found that tumors with aberrant *p63/p73* and normal *p53* expression exhibit a significantly worse prognosis than those with aberrant *p53* expression and normal *p63/p73* or those with normal *p63/p73* and normal *p53* expression. Similar findings were reported in bladder cancer (Flores et al., 2002; Puig et al., 2003).

Our findings regarding the early involvement of *VEGF* in colorectal carcinogenesis support previous studies in this area, where Kaklamanis et al (2006) reported *VEGF* in 40% and 23% of CRCs and the respective adenomatous part of the tumor. They also demonstrated that 91% of carcinomas arising from *VEGF*-positive adenomas were *VEGF*-positive, whereas 78% of carcinomas arising from *VEGF*-negative adenomas were *VEGF*-negative suggesting early involvement of *VEGF* in colorectal carcinogenesis.

The correlation reported here between *VEGF* overexpression and aggressive tumor phenotypes or reduced DFS confirms the prognostic value of this marker. In this context Ferroni et al (2005) and Cascinu et al (2001) demonstrated that elevated *VEGF* successfully discriminates between early and late stages of CRC. Moreover, patients with *VEGF* positive/high S phase fraction (SPF) tumors have unfavorable outcome compared to those with *VEGF* negative/low SPF tumors. Consequently we recommend utilization of *VEGF* as an independent prognostic factor in CRC patients. Similarly, a meta-analysis of all published studies on *VEGF* (27 studies) relating angiogenesis to DFS (no= 1064) and OS (no=1301) in CRC provided evidence that *VEGF* expression significantly predicted poor relapse free survival (RR=2.84, 95% CI: 1.95-4.16) and OS (RR= 1.65, 95%CI: 1.27-2.14) rates (Perrone et al., 2004).

Therefore, we conclude that *p53* family proteins and their isoforms play a pivotal role in colorectal carcinogenesis via enhanced *VEGF* expression, among other mechanisms. The prognostic value of *p53* is enhanced by *p73* and *p63* aberrations. However, reduced survival rates correlates with *p73*, *VEGF*, *p63* aberrantions. Therefore, these markers could be added to the standard prognostic panel of CRC. Future studies integrating mechanistic approaches, upstream signaling pathways and target genes of *p73* and *p63* are needed to improve our understanding of the interplay between *p53* family members and other genes.

Acknowledgements. This work has been funded by the NCI, CAIRO. *Competing interests.* Authors disclose any financial or non-financial competing interests.

References

Carneiro F., Ramalho L., Britto-Garcia S., Ribeiro-Silva A. and Zucoloto S. (2006). Immunohistochemical expression of p16, p53, and p63 in colorectal adenomas and adenocarcinomas. Dis. Colon Rectum. 49, 588-594.

Cascinu S., Graziano F., Valentini M., Catalano V., Giordani P., Staccioli M.P., Rossi C., Baldelli A.M., Grianti C., Muretto P. and Catalano G. (2001). Vascular endothelial growth factor expression, S-phase fraction and thymidylate synthase quantitation in node-positive colon cancer: relationships with tumor recurrence and resistance to adjuvant chemotherapy. Ann. Oncol. 12, 239-244.

Christopher E., Luo J., Kimberly A. and Jennifer A. (2006). Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. Cancer Res. 66, 7589-7597.

Des Guetz G., Uzzan B., Nicolas P., Cucherat M., Morere J.F., Benamouzig R., Breau J.L. and Perret G.Y. (2006). Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature. Br. J. Cancer 94, 1823-1832.

Domínguez G., García J.M., Peña C., Silva J., García V., Martínez L., Maximiano C., Gómez M.E., Rivera J.A., García-Andrade C. and Bonilla F. (2006). DeltaTAp73 upregulation correlates with poor prognosis in human tumors: putative in vivo network involving p73 isoforms, p53, and E2F-1. J. Clin. Oncol. 24, 805-815.

El-Serafi M., Bahnassy A.A, Ali. N.M, Salem M.E., Mahmoud M.K., Nayera A.A. and Abdel-Rahman N.Z. (2010). The prognostic value of c-Kit, K-ras codon 12, and p53 codon 72 mutations in egyptian patients with stage II colorectal cancer. Cancer 116, 4954-4964.

Ferroni P., Spila A., Martini F., D'Alessandro R., Mariotti S., Del Monte G., Graziano P., Buonomo O., Guadagni F. and Roselli M. (2005). Prognostic value of vascular endothelial growth factor tumor tissue content of colorectal cancer. Oncology 69, 145-153

Flores E.R., Tsai K.Y., Crowley D., Sengupta S., Yang A., McKeon F. Jacks T. (2002). p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature 416, 560-564.

Guan M., Peng H-X., Yu B. and Lu X. (2003). p73 overexpression and angiogenesis in human colorectal carcinoma. Jpn. J. Clin. Oncol. 33, 215-220.

Hamilton S. and Aaltonen L. (2000). WHO Classification of tumors. pathology and genetics of tumors of digestive system. Lyon, IARC Press., pp 103-126.

Harms K., Nozell S. and Chen X. (2004).The common and distinct target genes of the p53 family transcription factors. Cell. Mol. Life Sci. 61, 822-842.

Kaklamanis L., Trichas M., Amarantidis K., Spathari N., Micheil A., Karaviannakis A., Chatzaki E., Georgulias V. and kakolyris S. (2006). VEGF expression in the colorectal adenocarcinoma sequence. Oncol. Res. 15, 445-451.

Levrero M., De Laurenzi V., Costanzo A., Gong J., Wang J.Y. and Melino G. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions. J. Cell Sci. 113, 1661-1670.

Lisovesky M., Patel K., Cymes K., Chase D., Bhuiya T. and Morgenstern N. (2007). Immunophenotypic characterization of anal gland carcinoma: loss of p63 and cytokeratin 5/6. Arch. Pathol. Lab. Med. 131, 1304-1311.

Mokhtar N.M. (2002). National Cancer Institute Registry, In: The National Cancer Registry Newsletter. Ministry of Health and Population. Egypt. March.

Nahor I., Abramovitch S., Engeland K. and Werner H. (2005). The p53-family members p63 and p73 inhibit insulin-like growth factor-I receptor gene expression in colon cancer cells. Growth Horm. IGF Res. 6, 388-396.

Okada Y., Osada M., Kurata S., Sato S., Aisaki K., Kageyama Y., Kihara K., Ikawa Y. and Katoh I (2002). p53 gene family p51 (p63)-encoded, secondary trans-activator p51B (TAp63 alpha) occurs without forming immunoprecipitable complex with MDM2, but respond to gene toxic stress by accumulation. Exp. Cell Res. 276, 194-200.

Park B., Lee S., Kim J., Lee C., Chang S., Park J. and Chi S.G. (2000). Frequent alteration of p63 expression in human primary bladder carcinomas. Cancer Res. 60, 3370-3374.

Perrone G., Vincenzi B., Santini D., Verzi A., Tonini G., Vetrani A. and Rabitti C. (2004). Correlation of p53 and bcl-2 with vascular endothelial growth factor, microvessel density, and clinicopathologic features in colon cancer. Cancer Lett. 208, 227-34.

Puig P., Capodieci P., Drobnjak M., Verbel D., Prives C., Cordon-Cardo C. and Di Como C.J. (2003). p73 expression in human normal and tumor tissues. Loss of p73· expression is associated with tumor progression in bladder cancer. Clin. Cancer Res. 9, 5642-5651.

Soldevilla B., Diaz R., Silva J., Campos-Martin Y., Munoz C., Garcia V., Garcia JM., Pena C., Herrera M., Rodriguez., Gomez.I, Mohamed N., Marques MM., Bonilla F. and Dominguez G. (2011). Prognostic impact of ΔTAp73 isoform levels and their target genes in colon cancer patients. Clin Cancer Res. 17, 6029-39.

Spiesbach K., Tannapfel A., Mossner J. and Engeland K. (2005). TAp63gamma can substitute for p53 in inducing expression of the maspin tumor suppressor. Int. J. Cancer 114, 555-62.

Stiewe T. and Putzer B.M. (2002). Role of p73 in malignancy: tumor suppressor or oncogene? Cell Death Differ. 9, 237–245.

Tannapfel A., Schmelzer S., Benicke M., Klimpfinger M., Kohlhaw K., Mossner J., Engeland K. and Wittekind C. (2001). Expression of the p53 homologues p63 and p73 in multiple simultaneous gastric cancer. J. Pathol. 195, 163-170.

Toumi A.A., El Hadj O.A., Ben Mahmoud L.K., Ben Hamida A.M., Chaar I., Gharbi L. and Bouraoui S. (2010). The prognostic value of p73 overexpression in colorectal carcinoma: a clinicopathologic, immunohistochemical, and statistical study of 204 patients. Appl. Immunohistochem. Mol. Morphol. 18, 128-36.

Urist M., Di Como C., Lu M-L., Charytonowicz E., Verbel D., Crum C.P., Ince T.A., McKeon F.D. and Cordon-Cardo C. (2002). Loss of p63 expression is associated with tumor progression in bladder cancer. Am. J. Pathol. 161, 1199-1206.

Vikhanskaya F., Bani M., Borsotti P., Ghilardi C., Ceruti R., Ghisleni G., Marabese M., Giavazzi R., Broggini M. and Taraboletti G. (2001). p73 overexpression increases VEGF and reduces thrombospondin-1 production: implications for tumor angiogenesis. Oncogene 20, 7293-7300.

Zaika A., Slade N., Erster S., Sansome C., Joseph T, Pearl M., Chalas E. and Moll U.M. (2002). ΔNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J. Exp. Med. 196, 765-780.

Accepted May 29, 2013