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Original Article

p53 and p21 Immunohistochemistry in Colorectal Cancer: Clinical and Pathological Correlation in 128 Cases

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Abstract

Mutations of tumoral suppressor *TP53* gene are present in 75% of colorectal cancer (CRC) cases. Immunohistochemistry is a method capable of demonstrating the abnormal accumulation of p53 protein in the cell. Some studies associate p53 immunohistochemical positivity and a worse prognosis, while others do not confirm this finding. There are controversies regarding the prognostic value of p53 in CRC. The same doubts apply to p21 protein, activated by p53, which is the main responsible for stopping the cell cycle (checkpoints), both for repair or apoptosis purposes. **Objective:** The objective of this study is to correlate p53 and p21 immunohistochemical expression both with clinical and anatomopathological variables and with survival rates of patients with CRC. **Materials and Methods:** This is a descriptive and retrospective study having as research subjects 128 patients affected by CRC and treated surgically from 2000 to 2004, with available surgical specimens for immunohistochemical analysis using standardized methods. The association among categorical variables was done by Pearson chi-square or Fisher exact tests, and the continuous variables were analyzed by t-Student test. Overall survival and disease-free period rates had been calculated according to Kaplan-Meier method and the associations by log-rank test. **Results**: Follow-up average time was 35 months. p53 and p21 alterations had been detected, respectively, in 67.2% and 27. 3% of cases, with a significant association ($p<0.05$) between p53 and tumors located in rectum (76.0%) and left colon (70.7%), and between p21 and right colon (43.2%). p21 positive expression was related to CRC diagnostic at an older median age. Overall survival was 54 months, and the significant clinical and pathological related variables were the following: better for curative surgeries; better for precocious stages; better for well-differentiated tumors; worse for cases with sanguineous or lymphatic vascular embolization; worse for perineural invasion. **Conclusions:** positive p53 is more frequent in rectum and left colon, while positive p21 appears more frequently in right colon, being associated with diagnostic at an older age. p53 and p21 immunohistochemical expression had no association with other clinical and anatomopathological variables or with survival.

Key words: Tumor Supressor Protein p53. Cyclin-dependent Kinase Inibitor p21. Colorectal Neoplasms; Prognosis; Imunohistochemestry.

Introduction

TP53 gene, located in 17p13 chromosome, codifies p53 ubiquitin phosphoprotein, a transcriptional factor present in several cellular proliferation and homeostasis control processes such as apoptosis and DNA repair.[1] p53 protein

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seems not to have a function in normal cells;[2] however, its activation occurs after DNA damages caused by environmental elements such as: ionizing radiation; UV light; mutagenic chemical agents; cellular stress, without necessarily a direct DNA lesion, as occurs for example in hypoxia; senescence;[3] accumulation of metabolites in intracellular medium.[4] p53 activity is observed at specific moments of cell cycle, the "check-points" or periods of transition of G1 to the S phase (G1/S) and of G2 to mitosis (G2/M), when the genetic material checking occurs. When errors in DNA or cellular stress are detected, RAD and ATM proteins activate p53 transcription,[3] and this in turn activate target-genes like *WAF1/CIP* (that codify p21), *GADD45* (that codifies GADD45), *MDM2* (that codifies MDM2), among others. p21 protein, CDKs (cyclines-dependent kinases) universal inhibitor, having its concentration increased, binds to cycline-CDK complexes, which lose the capacity to phosphorylate RB family components. RB hypophosphorylation allows kidnapping E2F transcription factor, necessary for DNA synthesis. GADD45 protein binds to PCNA, inhibiting DNA synthesis.[4] DNA replication impossibility makes the cellular cycle to stop, giving time for repairing proteins to act.[2] Once the abnormality is corrected, cellular blockade is suspended through p53 degradation, promoted by MDM2 activation, carried through, in turn, by p53 [C] itself, in a self-inhibiting circuit.[2] If DNA damages repairing fails, proapoptotic genes (*BID*, from BCL-2 and BAX family) are activated[3] as well as genes related to apoptosis planning (*Fas/CD95*),[5] and there is also the inhibition of anti-apoptotic elements.[4] Apoptosis is thus activated and the modified cell dies. In normal cells, this phenomenon occurs in senescence.[6] Therefore, p53 loss of function can be deleterious for the cell and the organism as a whole, creating a situation of genomic instability [7] *TP53* somatic mutations are found particularly in tumors associated to environmental or occupational exposition to carcinogenic agents.[6] Diet is the most important environmental factor in CRC genesis.[8] *TP53* alterations are found in 75% of CRC cases,[5] more frequently in the form of genic deletions or point mutations, mainly missense.[5] In the carcinogenesis model proposed by Fearon and Volgelstein,[9] alterations in p53 occur at a late stage of the process, after mutations in other genes such as *APC*, *k-ras* and *DCC*.

In the last 20 years, several studies have demonstrated the occurrence of alterations of *TP53* tumoral suppressor gene in half of human tumors. However,[7] little were added to clinical practice,[7] having a controversial impact in CRC prognosis. Some studies demonstrated that *TP53* mutations can be associated to a worse prognostic, but others do not confirm this finding.[10] Immunohistochemistry detects possible *TP53* mutations through p53 protein accumulation in the cell. Mutations provoke conformational alterations of p53, besides increasing its half-life from twenty minutes to some hours. Thus, the anomalous protein binds to the specific antibody during the immunohistochemical process, and becomes detectable.[2]

This study has the objective to correlate p53 and p21 immunohistochemical expression with clinical and anatomopathological variables in patients affected by CRC.

Materials and Methods

This is a descriptive and retrospective study carried through in the Pelvic Surgery and Pathology Departments at Hospital A. C. Camargo. Patients submitted to surgery due to CRC from 2000 to 2004, which had available surgical specimen and had not been submitted to pre-surgical treatment were analyzed. From the initial sample with 196 cases, 68 were excluded by the following reasons: 51 because of inadequate material and 17 because material was not found.

Immunohistochemistry

The process begins with the demarcation of areas with tumoral cells in slides stained with HE (hematoxylin-eosin), which guide the withdrawal of material conserved in paraffin. From each clinical case, one removes four block samples, in two different plans. The removed specimens are enclosed in a new paraffin block, according to the *tissue-microarray* (TMA) methodology. Immunohistochemical reactions are carried out in a slide mounted from the TMA block, according to the immunohistochemical protocol of Hospital A. C. Camargo . The dilution

of p53 is 1:500, with antigenic recovery in a pressure pan and LSAB revelation. The dilution of p21 is 1:20, with antigenic recovery in pressure pan and DUET 1:200 revelation. Reagents used are: p53 – Dako, DO7 clone, M7001 code; p21 – WAF1/CIP, Sx118 clone, M7202 code. *Tissue-microarray* (TMA) slides analysis was carried out by only one pathologist, and considering the combined immunohistochemical expression of each case four samples. Positivity was defined as at least 5% of cells nuclei presenting a brownish coloration.

Statistical Analysis

To study the association between the proteins positivity and negativity and the categorical variables chi-square or Fisher exact tests were used, and the continuous variables were submitted to t-Student test.[11] Overall survival and disease-free period rates had been calculated according to Kaplan-Meier method and the associations by log-rank test.[12]

Results

Patients' average follow-up time was 35 months, with a 32 months median. The characteristics of the 128 studied patients and survival rates are described in Table 1. Table 2 demonstrates the relation between the samples selected characteristics and p53 and p21 proteins immunohistochemical expression. There was a significant association (p>0.05) only as regards primary tumor localization, and positive p53 is more frequent in rectum and left colon, whereas positive p21 is more frequent in right colon. Table 3 demonstrates associations between patients' age when diagnostic occurred and immunohistochemical results, with a significant result for positive p21 and diagnostic at an older age.

Overall survival average was 54 months,

Table 1 - Correlation between selected characteristics of 128 studied pacientes and survival

Variables		N	(%)	Three-year survival (%)	p
Age (years)	<50	25	19.5	70.0	0.365
	> 50	103	80.5	77.8	
Gender	Female	79	61.7	75.2	0.296
	Male	49	38.3	72.7	
Primary Tumor	Right colon	37	28.9	85.7	0.091
	Left colon	41	32.0	62.1	
	Rectum	50	39.1	82.8	
Type of surgery	Curative	105	82.0	89.8	<0.001
	Palliative	23	18.0	7.0	
T Stage	T1-T2	39	30.5	91.1	0.007
	T3-T4	83	64.8	69.5	
N Stage	N0	63	49.2	87.6	0.004
	N1-N2	61	47.7	66.6	
M Stage	M0	94	73.4	91.4	<0.001
	M1	34	26.6	33.8	
p53	Positive	86	67.2	74.9	0.921
	Negative	42	32.8	78.9	
p21	Positive	35	27.3	74.5	0.991
	Negative	93	72.7	76.7	
Histological Type	Tubolovillous	108	84.4	77.0	0.899
	Mucinous	20	15.6	69.0	
Grade	Low	22	17.2	85.6	0.045
	Intermediate	94	73.4	75.7	
	High	7	5.5	53.6	
Vascular embolization	No	113	88.3	81.9	<0.001
	Yes	10	7.8	48.0	
Lymphatic embolization	No	91	71.1	85.7	<0.001
	Yes	30	23.4	53.1	
Perineural invasion	No	95	74.2	85.3	<0.001
	Yes	22	17.2	20.4	

Table 2 - Correlation between selected characteristics of 128 patients and p53 and p21 proteins

Variables	p53 Positive N	(%)	p53 Negative N	(%)	p	p21 Positive N	(%)	p21 Negative N	(%)	p
Age (years)										
< or = 50	16	(64.0)	9	(36.0)	0.813	5	(20.0)	20	(80.0)	0.457
> 50	70	(68.0)	33	(32.0)		35	(32.4)	73	(67.6)	
Gender										
Female	57	(72.2)	22	(27.8)	0.175	21	(26.6)	58	(73.4)	0.840
Male	29	(59.2)	20	(40.8)		14	(28.5)	35	(71.5)	
Primary Tumor										
Right colon	19	(51.3)	18	(48.7)	0.045	16	(43.2)	21	(56.8)	0.029
Left colon	29	(70.7)	12	(29.3)		10	(24.4)	31	(75.6)	
Rectum	38	(76.0)	12	(24.0)		9	(18.0)	41	(82.0)	
Type of surgery										
Curative	69	(65.7)	36	(34.3)	0.625	30	(28.6)	75	(71.4)	0.611
Palliative	17	(73.9)	6	(26.1)		5	(21.7)	18	(78.3)	
T Stage										
T1-T2	25	(64.1)	14	(35.9)	0.503	11	(28.2)	28	(71.8)	1.000
T3-T4	59	(71.0)	24	(29.0)		24	(28.9)	59	(71.1)	
N Stage										
N0	45	(71.4)	18	(28.6)	0.563	19	(30.1)	44	(69.9)	0.692
N1-N2	40	(65.5)	21	(34.4)		16	(26.2)	45	(73.7)	
M Stage										
M0	64	(68.0)	30	(32.0)	0.832	26	(27.6)	68	(71.4)	1.000
M1	22	(64.7)	12	(35.3)		9	(25.0)	25	(75.0)	
Histological Type										
Tubolovillous	72	(69.2)	32	(30.8)	0.118	27	(25.0)	81	(75.0)	0.180
Mucinous	10	(50.0)	10	(50.0)		8	(40.0)	12	(60.0)	
Grade										
Low	15	(68.2)	7	(31.8)	0.805	7	(31.8)	15	(68.2)	0.608
Intermediate	65	(69.1)	29	(30.9)		25	(26.6)	69	(73.4)	
High	4	(57.2)	3	(42.8)		3	(42.9)	4	(57.1)	
Vascular embolization										
No	75	(66.4)	38	(33.6)	0.167	31	(27.4)	82	(72.6)	0.692
Yes	9	(90.0)	1	(10.0)		3	(30.0)	7	(70.0)	
Lymphatic embolization										
No	60	(65.9)	31	(34.1)	0.507	25	(27.5)	66	(72.5)	0.817
Yes	22	(73.3)	8	(26.7)		9	(30.0)	21	(70.0)	
Perineural invasion										
No	64	(67.4)	31	(32.6)	0.800	27	(28.4)	68	(71.6)	1.000
Yes	16	(73.3)	6	(27.3)		6	(27.3)	16	(72.7)	

and variables with statistically significant differences had been character of surgery; TNM Staging System; grade; sanguineous and lymphatic vascular embolizations and perineural invasion. The immunohistochemical expression of p53 and p21 had not presented a survival-related significant importance.

Survival was also evaluated considering only patients treated with a curative purpose and without metastasis at the time of diagnostic, and no significant differences related to p53 e p21 proteins was observed.

Table 3 - Mean age at diagnosis and correlation to proteins expression

		Mean age	Standard deviation	p
Overall		62.21	12.939	
Proteins p53				
	Negative	59.98	13.282	0.145
	Positive	63.55	12.623	
p21	Negative	60.57	12.998	0.011
	Positive	67.21	11.486	

Discussion

Patients submitted to surgery outside the institution were excluded from this study in order to ward off possible bias, mainly the ones related to clinical evaluation, surgical treatment and anatomopathological examination. Patients submitted to preoperative treatment were also excluded so that tumoral cells to be examined were not influenced by radiotherapy or chemotherapy with a consequent alteration of immunohistochemical results.[7]

Variables stratification in this study followed the model proposed by Munro et al.[7] that by means of meta-analysis argue for the necessity of using sub-groups defined by stage, tumor localization and treatment. The categories "right colon" (ascending and transverse colons), "left colon" (descending and sigmoid colons) and "rectum" had been used because they are considered important factors in prognostic determination.[13] The highest frequencies of positive p53 in rectum and left colon (p=0.046) and positive p21 in right colon (p=0.029) are compatible with the description of Soong et al.,[14] where *TP53* mutations are more present in left colon (43%) than in right colon (34%) (p=0.006). In more advanced tumors, a higher p53 positivity occurs in right colon occurs than in left colon (67% versus 40%).[12]

The significant results for correlation between survival and the surgery character, N stage and vascular embolization variables are consistent with results found in Russo et al.[13] Worse survival outcomes had been found for characteristics known in clinical practice: palliative surgeries, low differentiated tumors, T3 and T4, N1 and N2, M1 stages, as well as the presence of vascular embolizations and perineural invasion.

The positive p53 67.2% percentage is a value near the one described by Cascinu et al.[15] (66%), Ahnen et al.[16] (63%), Kawasaki et al.[17] (60,7%) and Caruso and Valentini[18] (60%). Petersen et al.[10] including 28 studies, describes a 50.2% p53 positive average percentage for immunohistochemical studies and 46.9% for cDNA sequencing. A relevant factor is that in works using immunohistochemistry, p53 positivity determination limits present a broad variation, from values next to zero to 30% for nuclei and from values next to zero to 50% for cytoplasm.[10] This suggests that the criterion for examination reading can be the main cause of variation in results frequencies, besides demonstrating the lack of well-established standards for analysis.[7,10] The relatively high frequency of positive results of this study are possibly a consequence of the establishment of a 5% cut value.

No statistically significant association between p53 and the patients' characteristics was found, confirming the results obtained by Soong et al. [14] and Tollenaar and Valentini [19], who observed no correlation of p53 with sex, age, tumor localization, staging, grade, histology, among other characteristics.

As regards survival, available studies in the literature are heterogeneous, with controversial results.[7,10,14-16,20] p53 positive expression can be associated to best prognosis for clinical stage III, with a 56% survival rate in 7 years, against 43% for negative p53 (HR 2.2; 95% CI 1.3-3.6; p = 0.012)[16] and also for tumors in left colon (HR 0.66, 95% CI 0.47-0.93, p=0.017) and for Dukes C stage (p=0.013).[20] Fearon and Volgelstein[9] describe an increasing risk of death with abnormal p53 (immunohistochemistry RR=1.32, 95% CI 1.23-1.42; c-DNA sequencing RR=1.31; 95% CI 1.19-1.45).

As regards p21, Sasaki et al[21] report positivity in 68.6% of CRC cases and Pasz-Walczak et al [22] in 39%, against 27.3% (n=35) verified in the present study. As p21 transcription is activated by p53, positivity rates are parallel in physiological conditions. However, positive p21 is associated with positive p53 in 77.1% of cases. It is worth remembering the possibility of p21 being activated by alternative processes.[21]

There was no significant association between p53 and p21 expression and the characteristics of CRC patients studied, with the exception of age when diagnostic occurred, something that can suggest a more retarded alteration in the carcinogenesis process, after the occurrence of accumulated genetic alterations able to provide alternative activation processes.

Despite the seeming lack of association between the important function of p53 in cellular homeostasis and the characteristics of CRC patients and survival, some hypotheses could try to explain this paradox. The first one relates to the incomplete knowledge of p53 function; moreover, p53 positivity is a common event,[23] and this could mask any potential prognostic value. As regards immunohistochemistry, genotype

indirect study by means of protein expression does not consider that the positive result can mean, besides *TP53* mutation, protein accumulation due to genic superexpression stemming from normal p53 linking to a variety of other proteins[24] or even due to the loss of apoptosis elements.[23] On the other hand, silent mutations not detected by immunohistochemistry can occur.[24,25] Moreover, other proteins, like p73, can take p53 function when this latter is altered.[7]

Thus, the most important prognostic factors are the traditional anatomopathological parameters, TNM staging and the tumor complete resection.

In this study, curative surgery character, TNM staging system, grade, sanguineous and lymphatic and vascular embolization and perineural invasion were significant prognostic factors as regards survival. CRC localization was associated with p53 positive expression, more frequent in tumors of rectum and left colon, and to p21 positive expression, more frequent in right colon. p21 positive expression was associated with more advanced age at the time of CRC diagnostic.

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References

1 May P, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. Oncogene 1999;18:7621-36.

2 Lewin B, Genes. 1st ed. New York: Oxford University Press; 1997. p.1166

3 Robbins SL, Cotran RS, Patologia: bases patológicas das doenças. 7 ed. Rio de Janeiro (RJ): Elsevier;1999. p.300-17.

4 Silva RL, Oncogenes e genes supressores de tumor. In: Ferreira CG, Rocha JC. Oncologia Molecular.1 ed. São Paulo: Atheneu;2004. p.37,40.

5 Mc Dermott U, Longley DB, Johnston PG. Molecular and biochemical markers in colorectal cancer. J Clin Pathol 2001;*54*:206-14.

6 Waddington-Achatz MI, Hainaut P. TP53 and Li-Fraumeni Syndrome. Appl Cancer Res 2005;25:51-7.

7 Munro AJ, Lain S, Lane DP. P53 abnormalities and outcomes in colorectal cancer: a systematic review. Br J Cancer 2005;92:434-44.

8 Vettore AL, Caballero OL, Câncer colorretal. In: Ferreira CG, Rocha JC. Oncologia Molecular.1 ed. São Paulo: Atheneu;2004. p.171.

9 Fearon ER, Volgelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-67.

10 Petersen S, Thames HD, Nieder C, Petersen C, Baumann M. The results of colorectal cancer treatment by p53 status: tretatment-specific overview. Dis Colon Rectum 2001;44:322-33.

11 Almeida Filho N, Rouquayrol MZ. Análise de dados epidemiológicos in Almeida Filho N, Rouquayrol MZ. Introdução à epidemiologia. 3ed. Rio de Janeiro: MEDSI; 2002. p.236-15.

12 Flamini G. Prognostic significance of cytoplasmic p53 overexpression in colorectal cancer: an immunohistochemical analysis. Eur J Cancer 1996; 32A:802-6.

13 Russo A, Bazan V, Iacopetta B, Kerr D, Soussi T, Gebbia N et al. The *TP53* colorectal internacional collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. J Clin Oncol 2005;30:7518-28.

14 Soong R, Powell B, Elsaleh, Gnanasampanthan G, Smith DR, Gab HS et al. Prognostic significance of *TP53* gene mutation in 995 colorectal carcinoma: influence of tumor site, stage, adjuvant chemotherapy and type of mutation. Eur J Cancer 2000; 36:2053-60.

15 Cascinu S, Catalano V, Aschele C, Barnio S, Debernardis D, Gallo L et al. Immunohistochemical determination of p53 protein does not predict clinical response in advanced colorectal cancer with low thymidylate synthase expression receiving a bolus 5-fluorouracil-leucovorin combination. Ann Oncol 2000;11:1053-6.

16 Ahnen DJ, Feigl P, Quan G, Fenoglio-Preiser C, Lovato LC, Bunn PA Jr et al. Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a southwest oncology group study. Cancer Res1998;15;58:1149-58.

17 Kawasaki Y, Monden T, Morimoto H, Murotani M, Miyoshi Y, Kobayashi T et al. Immunohistochemical study of p53 expression in microwave-fixed, paraffin-embedded sections of colorectal carcinoma and adenoma. Am J Clin Pathol 1992;97:244-9.

18 Caruso ML, Valentini AM. Immunohistochemical p53 overexpression correlated to c-erB-2 and cathepsin D proteins in colorectal cancer. Anticancer Res 1996;16:3813-8.

19 Tollenaar RA, van Krieken JH, van Slooten HJ, Bruinvels DJ, Nelemans KM, van der Broek LJ et al. Immunohistochemical detection of p53 and Bcl-2 in colorectal carcinoma: no evidence for prognostic significance. Br J Cancer 1998;77:1842-7.

20 Soong R, Grieu F, Robbins P, Dix B, Chen D, Parsons R et al. p53 alterations are associated with improved prognosis in distal colonic carcinomas. Clin Cancer Res 1997;3:1405-11.

21 Sasaki K, Sato T, Kurose A, Ikeda E. Immunohistochemical detection of p21waf1/cio1/sdil and p53 proteins in formalin-fixed, paraffin-embedded tissue sections of colorectal carcinoma. Hum Pathol 1996;27:912-6.

22 Pasz-Walczak G, Kordek R, Faflik M. p21 (*WAF1*) expression in colorectal cancer: correlation with p53 and cyclin D1 expression, clinicopathological parameters and prognosis. Pathol Res Pract 2001;197:683-9.

23 McGill G, Fisher DE. p53 and cancer therapy: a double-edged sword. J Clin Invest 1999;104:223-5.

24 Bosari S, Viale G. The clinical significanceof p53 aberrations in human tumours. Virchows Arch 1995;427:229-41.

25 Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A 1990;87:7555 -9.