Original Article

P16 and p27 Immunohistochemical Expression in Colorectal Cancer: Analysis of 128 Patients

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Abstract

P16 and p27 are inhibiting proteins of cyclin-dependent kinases (CDKIs) that act in the restriction points of the cellular cycle, and it avoids its progression to DNA verification and repair by the cellular apparatus. This way, there should be, physiologically, an inverse relation between the expression of these proteins and cellular proliferation. However, what is really observed are changeable amounts of p27 in normal and tumor tissues. P16 participation in tumorigenesis is controversial. The expression of p16 and p27 as a prognostic factor in colorectal cancer (CRC) patients is controversial. **Objetive:** To establish a correlation between p16 and p27 immunohistochemical expressions with clinical and anatomopathological variable from patients with CRC. **Material and methods:** descriptive and retrospective study, with 128 CRC patients, treated surgically between 2000 and 2004, with available material for immunohistochemical analysis through standardized methods. The association between categorical variables was done using Chi-square, Pearson or Fisher's Exact tests, and the continuous variables were analyzed by t-Student. Global survival and disease-free period were calculated according to Kaplan-Meier method and the associations through log-rank test. **Results:** The average follow-up time of patients was 35 months. Positivity of p16 was detected in 100% of cases. Negativity of p27 in 6.3% (n=8) of cases, with a significant association (p30.05) between p27 negative and tumors located in right colon (62.5%, n=5) and mucinous (62.5%, n=5). The average global survival was 54.8 months, and the significant clinical and pathological variables associated to survival were: better for curative surgeries; better for early stages; better for well-differentiated tumors; worse for cases with sanguineous or vascular lymphatic invasion; worse for perineural invasion. **Conclusions:** p27 negative is more frequent in right colon (ascending and transverse) and mucinous tumors. No association was found between p16 and p27 immunohistochemical expression and other clinical and anatomopathological variables or survival.

Keywords: Survival. Immunohistochemistry. Colorectal neoplasms Prognosis. Genes p16.

Introduction

The proteins p16 and p27 (gene *P27* or *Kip1*) are representative of the two inhibiting protein classes of cyclin-dependent kinases (CDKIs), INK4/ARF and Cip/Kip[1], respectively, that group CDKIs with similar structures and target proteins. In INK4/ARF class, p16, p15, p18 and p19 inhibitors bind to cyclin-dependent kinases 4 and 6 (CDK-4 and CDK-6) and in Cip/Kip class, p21, p27 and p57 inhibit cyclin-dependent CDK2 and regulate CDK-D positively.[2]

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Their CDK-inhibition property allows p16 and p27 to act in the restriction points, moments when the cellular cycle stops and other proteins search for errors on the genetic material and try to correct them. Thus, p16 and p27 have the function to stop cycle progression so that DNA-verification and repair cell apparatus has time to act, acting thus, respectively, at G1/S and G2/M restriction points.

In the normal cell division cycle, the passage to a new stage requires the availability of certain proteins, synthesized for the activation of transcription factors that bind to DNA. In the G1 phase, the binding of transcription factor E2F (active) to DNA is necessary for protein synthesis essential to DNA response in the S phase.[3] In the quiescent cell, E2F is inactive, forming a complex with hypophosphorilated Rb protein (retinoblastoma protein).[4] E2F release only happens with Rb protein phosphorilation by the action of complexes cyclin D[1-3)]-CDK4,6, which dissociate proteins and activate E2F. This mechanism represents what would be a control to *bind* and *unbind* the cycle as Rb protein phosphorilation eliminates the main barrier to the cycle progression and allows replication.[5]

Thus, the property of p16 of binding to complexes cyclin D-CDK4,61 inhibits the activity of these sets, keeping Rb protein hypophosphorilated and therefore E2F inactive. The cycle starts again with the stimulation of growth factors, which increase cyclins concentration, that form complexes with CDK4,6 and phosphorilate Rb protein.[5]

G2/M passage is initiated by cyclin transcription mediated by factor E2F that binds CDK2 and begins to regulate events critical to the transition, such as microtubule stability reduction, centromeres separation and chromosome condensation.[5] p27 binds to CDK2, preventing it from forming complexes with cyclin B and E.[4]

Once known p16 and p27 mechanisms in the cell cycle, one expects to find an inverse relation between the expression of these proteins and cell proliferation, as some studies show[6-7] However, this expectation is broken when one observes an increase of p27 expression in the absence of genic mutation in primary colon and breast cancers.[8] Doubts regarding the altered pattern of p27 expression persist before its changeable amounts in normal and tumor tissues and the absence of statistical differences for the average values of p27 in the two types of sample, [8-9] although p27 total amount is reduced in tumors.[8]

There are also doubts regarding protein p16, with a disputable participation in tumorigenesis[10] and divergences regarding the most common alteration in tumors. While Ohhara et al.[11] considers that the gene P16 inactivation is not a common phenomenon and the activation do is, Tomlinson et. Al.[12] and Ahuja et. Al.[13] observe the opposite, with a loss of heterozygosis in locus P16 in 38% of cases and hypermethylation of the gene in 34%.

Regarding the impact of p16 and p27 in CRC patients' survival, controversies persist. Some studies do not find association between survival and p16[14] and p27[15] expression, as well as descriptions of the reduction or absence of p16[16] and p27[17] expressions correlated to a worse prognostic, emphasizing that this same pattern of p27 expression is also associated to a higher survival.[18]

Controversies regarding what p16 and p27 altered expression would be like, the possible roles played in tumorigenesis, the possible impact in survival and the possibility of reverting alterations of the expression with chemopreventive agents[19] encourage research on these proteins.

This work aims to correlate p16 and p27 immunohistochemical expression with clinical and anatomopathological variables of CRC patients.

Material and Methods

This is a descriptive and retrospective study. 128 patients operated for CRC in A.C.Camargo Hospital were analyzed who had available surgical parts and no preoperative treatment between 2000 and 2004. From the initial sample with 196 cases, 68 were excluded for the following reasons: 51 were inadequate material; 17 had no material available.

Immunohistochemistry

The process begins by marking areas with tumor cells in the blades with HE (hematoxilin/eosine) staining, which guide the withdrawal of material conserved in paraffin. From each clinical case 4 samples are removed from the block in two different sections. The specimens removed are enclosed in a new paraffin block according to tissue-microarray (TMA) methodology. Immunohistochemical reactions are carried through in blade mounted from TMA block according to immunohistochemical protocol. The reagents used for p16 and p27 are, respectively, F12 (Sc 1661), Santa Cruz Biotechnology (U.S.A.), and SX53G8 (M7203), Dako Ltd, Ely, UK. F12 reagent is submitted to a 1:100 dilution, recovered in a electric pressure pan with citrate and ph

6.0 and the detention is done with StreptABC Complex and HRP Duet. Reagent SX53G8 is diluted in a 1:50 ratio, recovered in a way similar to F12 recovering and detected by LSAB and HRP system.

Tissue-microarray (TMA) blades analysis was done by only one pathologist doctor, considering the global immunohistochemical expression of the 4 samples of each case. Positivity was defined as at least 10% of cell nuclei presenting a brownish coloration.

Statistical Analysis

For studying he association between protein positivity and negativity and the categorical variable Chi-square, Pearson or Fisher's Exact tests and, for the continuous variable, t-Student.[20] Global survival and disease-free period were calculated according to Kaplan-Meier method, and the associations with immunohistochemical results were verified through log-rank test.[21]

Results

The average follow-up time of patients was 35 months, and the median was 32 months. The characteristics and survival rates of the 128 patients studied are described in Table 1. Table 2 shows the relationship between the selected characteristics of the sample and protein p27 immunohistochemical expression. There was a significant association (p30,05) to localization of primary tumors and histological type: p27 negative is more frequent in right colon tumors (both ascending and transverse) and mucinous ones. There was no statistically significant association between the average age at diagnosis and immunohistochemical results.

Global survival average was 54.8 months (I.C. 95%), and the variables presenting statistically significant differences were: surgery character (curative or palliative); TNM staging; differentiation degree; vascular and blood vessel lymphatic invasions; perineural invasion. P27 immunohistochemical expression presented no significant relation to survival.

Table 1 - Selected characteristics from 128 patients and survival

Variables	Categories	N	%	3-year survival (%)	p
Age	< 50 years	25	19.5	70.0	0.365
	> 50 years	103	80.5	77.8	
Sex	Female	79	61.7	75.2	0.296
	Male	49	38.3	72.7	
Tumor site	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
	Paliative	23	18.0	7.0	
T	T1-T2	39	30.5	91.1	0.007
	T3-T4	83	64.8	69.5	
	Ignored	6	4.7		
N	N0	63	49.2	87.6	0.004
	N1-N2	61	47.7	66.6	
	Ignored	9	3.1		
M	M0	94	73.4	91.4	<0.001
	M1	34	26.6	33.8	
Histological type	Tubullovilous	108	84.8	77.0	0.899
	Mucinous	20	15.6	69.0	
Differentiation degree	Well	22	17.2	85.6	0.045
	Moderately	94	73.4	75.7	
	Pour	7	5.5	53.6	
	Ignored	5	3.9		
Vascular Invasion	No	113	88.3	81.9	<0.001
	Yes	10	7.8	48.0	
	Ignored	5	3.9		
Lymphatic invasion	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	
	Ignored	11	8.6		
p27	Positive	120	93.7	54.4	0.659
	Negative	8	6.3	49.6	
p16	Positive	128	100.0	-	-
	Negative	0	0		

Discussion

All patients selected were operated in A.C.Camargo Hospital, a criterion used to avoid possible bias mainly concerning clinical evaluation, surgical treatment and anatomopathological evaluation. Patients who had preoperative treatment were also excluded for avoiding the influence of radiotherapy or chemotherapy in tumor cells and consequent alterations in immunohistochemical results.

Table 1 - Selected characteristics from 128 patients and survival

Variables	P27 negative		P27 positve		p
	N	%	N	%	
Age					
< 50 years	3	37.5	22	18.3	0.187
> 50 years	5	62.5	98	81.7	
Sex					
Female	2	25.0	47	39.2	0.346
Male	6	75.0	73	60.8	
Tumor site					
Right colon	5	62.5	32	26.5	0.044
Left colon and rectum	3	37.5	88	73.4	
Type of surgery					
Curative	6	75.0	99	82.5	0.634
Paliative	2	25.0	21	17.5	
T					
T1-T2	1	20.0	39	33.1	1.000
T3-T4	4	80.0	79	66.9	
N					
N0	2	40.0	61	51.3	0.677
N1-N2	3	60.0	58	48.7	
M					
M0	4	50.0	90	75.0	0.207
M1	4	50.0	30	25.0	
Histological type					
Tubollovilous	3	37.5	5	87.5	0.002
Mucinous	5	62.5	15	12.5	
Differenciation degreee					
Well	0	0	22	18.8	0.286
Moderately	5	83.2	89	76.1	
Pour	1	17.8	6	5.1	
Vascular invation					
No	4	80.0	9	92.4	0.350
Yes	1	20.0	9	7.6	
Lymphatic invasion					
No	3	60.0	88	75.9	0.597
Yes	2	40.0	28	24.1	
Perineural invasion					
No	4	80.0	91	81.2	1.000
Yes	1	20.0	21	18.8	

The significant relations among survival and surgery character, N stage and lymphatic vascular invasion are coherent with those found in Russo et al. metanalysis.[22] Worse survival rates were found for characteristics known in clinical practice: surgeries with a palliative character, little differentiated tumors, T3 and T4, N1 and N2, M1 stages as well as vascular invasions and perineural invasion.

As immunohistochemical positivity of p16 was found in all studied cases, immunohistochemical reaction was repeated. The new results confirmed p16 positivity in all cases. This is maybe due to the use of a polyclonal reagent (antibody F12 Santa Cruz), which allows more heterogeneous reactions than monoclonal ones. However, a high incidence of p16 positive expression in tumors is frequently described[23] Palmqvist et al.[6] found p16 positive expression in 81.52% (N=92) of cases, against 100% of p16 negativity in normal tissue samples (N=8). This association between p16 positive and tumors seems to be inconsistent with evidences of the crucial role p16 has in cell proliferation control. 23 There are a few studies characterizing the expression of p16 in CRC, and that makes controversial this seeming inconsistence.[6]

Tumor cells express p16 both in the cytoplasm and the nucleus, what motivated the search of a possible correlation between protein localization and tumor evolution in some studies. Zhao and Talbot[24] demonstrate that p16 superexpression in the cytoplasm and the absence of its expression in the nucleus are considered important factors in the evolution of normal epitheliums to carcinoma. In the present study, we choose nuclear expression as the relevant parameter.

Given the results of 100% of positivity of p16 immunohistochemical expression, it was not possible to find associations among clinical and anatomopathological factors and survival. Nevertheless, some studies seem not to establish such correlations[6,14] although p16 negativity may be associated to little differentiated tumors.[6] An interesting fact to mention is the association of p16 negative with the weak expression or absence of p27 (p=0,02), suggesting an associated expression inhibition.[6]

Regarding protein p27, the first difficulty is establishing what would be its modified expression. In a way similar to p16, we found a high diversity of described correlations between tumor alterations and p27 negativity and positivity. This situation can be an effect of the changeable amounts found both in normal and tumor tissues and the absence of statistical differences for p27 average values in the two sample types.[8-9] Even so the superexpression is observed in tumors, positive expression

is considered the normal pattern, based on evidences of a reduction of cell proliferation with the use of substances that increase the expression of these proteins[1] and reports of a reduction of p27 and p16 expression in tumors.[25-30] Moreover, in contrast with other tumor suppressors, some affirms that p27 action seems to directly depend on its absolute levels[31]

The finding of 6.3% of p27 negativity is near the 11% (N=89) in tumors with a very low expression of the protein described by Palmqvist et al.[17] However, the number is below the 30.5% observed by Sarli et al.[18] 33% by Ogino et al.[32] and 51% of Zhang and Sun.[9]

The association of p27 negative with tumors located in right colon (p=0,046) corresponds to the observations of Palmgvist et al.[17] (p=0.026) and Sarli et al.[18] (p30,005), which also found a correlation with the mucinous histological type (p30,001).

The absence of a statistically significant correlation of p27 expression to age, sex, differentiation degree and lymphatic vascular infiltration corresponds to the literature.[9,17]

As regards survival, the available studies in the literature are heterogeneous, with controversial results.[14-18] The absence of association of p27 immunohistochemical expression with survival verified in the present study was also observed by Rosati et al.[15] and Read et al.[33]

Important prognostic factors such as traditional anatomopathological parameters, TNM staging and complete tumor resection have a high impact in survival, a result not found for p27 immunohistochemical expression separately.

In this study, the curative character of surgery, TNM staging system, differentiation degree, vascular blood and lymphatic invasion and perineural invasion were significant prognostic factors for survival. CRC localization was associated with p27 negative expression, more frequent in right colon tumors of the mucinous type.

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