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Expression of P33^{ING1b} Protein in Colorectal Cancer

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ABSTRACT

BACKGROUND

Colorectal cancer (CRC) is the second most common malignancy in the world. However, its mortality rate can be reduced if diagnosed early. P33^{ING1b} is a tumor suppressor protein, which plays a role in growth control and apoptosis. Suppression of p33^{ING1b} is associated with the loss of cellular growth control. However, p33 ^{ING1b} expression in CRC and its correlations with clinicopathological factors have been less studied. The aim of this study was to examine p33^{ING1b} expression in patients with CRC and evaluate its potential correlations with clinicopathological factors.

METHODS

P33ING1b protein expression was examined in 70 cases of CRC tissue samples and their corresponding neighboring normal tissues by immunhistochemistry. Moreover, p33^{ING1b} expression in CRC and its correlations with clinicopathological variables including patients' sex and age, tumor type, location, stage, and differentiation grade were examined.

RESULTS

P33^{ING1b} expression was significantly lower in tumor samples compared with the normal adjacent samples (p<0.002).

CONCLUSION

Low expression of P33^{ING1b} in patients with colorectal cancer, may be an important molecular event in the pathogenesis of colorectal cancer. Our data suggest that reduced expression of ^{p33ING1}b may be contribute to tumor genesis and accompanied by the loss of cellular growth control. In fact cell growth is out of control in lower expression of P33 and dysfunctional program cell death. P33 expression might explain the etiology of CRC for reducing the expression of tumor suppressor proteins.

KEYWORDS
Colorectal Cancer; Clinicopathological factors; Immunhistochemistry

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INTRODUCTION

Colorectal cancer (CRC) is the second most prevalent malignant neoplasm in the world after the breast cancer.[1,2] It is the third most common cancer in men and the second in women; accounting for 8% (n=608,700) of all cancer deaths worldwide. The highest incidence rate of colon cancer is in the Eastern Europe and Asia. CRC is also the third most common cancer in Iranians in except of the skin cancers. It occurs at younger ages with an increasing trend similar to the Asia-Pacific countries.[3]

Often, the first line of treatment is surgical resection followed by chemotherapy because most patients are diagnosed at an advanced stage.[4,5] About one quarter of the sufferers have Dukes' C disease and a 5-year survival rate of 30–40%.[4]

Several risk factors may play a role in CRC development. The incidence is higher in men than women.[6] Prognosis for CRC is affected by a variety of features presented at the time of initial diagnosis including age, sex, duration of symptoms, and tumor location.[7]

There are two major classes of tumor associated genes that have been implicated in tumorgenesis: oncogenes and tumor suppressor genes. Inactivation (by loss or mutation) of tumor suppressor genes plays an essential role in the genesis of many tumors.[8] Inhibitor of Growth 1 (ING1) is a recently cloned novel growth inhibitor and a candidate tumor suppressor gene detected through a method of cDNA subtractive hybridization from normal and cancer cells followed by an in vitro selection assay. ING1 mapped to the human chromosome 13q33-34, a region that has been linked to the progression of various tumors.[9] Several mechanisms of malfunction for ING1 gene have been proposed including: gene malfunctions (mutations, rearrangements, lack of heterozygosity, homozygous loss and DNA CpG island hypermethylation), decreased mRNA expression, reduced protein expression, and protein malformations.[8]

ING1 encodes four protein isoforms: p47^{ING1a}, p33^{ING1b}, p24^{ING1c}, and p27 ^{ING1d},[4] among which p33 ^{ING1b} is the most widely expressed isoform[10] that is involved in growth control and apoptosis as well a role in senescence.[4] The p33 ^{ING1b} protein plays a fundamental role in multiple cellular activities such as growth regulation, apoptosis, senescence, and DNA repair upon UV-induced DNA damage, which are all typical characteristics of tumor suppressors.[11] While suppression of p33 ^{ING1b} is accompanied by the loss of cellular growth control and immortalization, its overexpression arrests cells in the G0/G1 phase of the cell cycle,[10] which inhibits cell growth and induces apoptosis.[4] By contrast, a reduced p33 ^{ING1b} expression has been detected in lymphoid malignancy, and esophageal, gastric, brain, colon, liver, lung, astrocytoma, and breast cancers, suggesting that the reduced expression of p33^{ING1b} might contribute to tumorigenesis.[10]

P33^{ING1b} expression in CRC is low and seems to play an important role in carcinogenesis or progression.[12] Patients with p33 ^{ING1b} protein under expression exhibit an overall shorter survival compared with those with normal p33 ^{ING1b} expression levels.[4] Chen and colleagues have shown that p33 ^{ING1b} expression reduces in patients with CRC, and that the average mRNA expression levels in the cancerous tissues of Dukes' stages C and D were significantly lower than in stages A and B.[12]

However, the correlation between p33 ^{ING1b} expression in CRC and clinicopathological factors has not been studied. In a study by Ahmed and colleagues, it was found that in patients with Dukes' C stage of CRC, p33^{ING1b} protein was under expressed in 32% of samples. It was also reported that patients with p33^{ING1b} under expression had a shorter overall survival in comparison with those with normal p33^{ING1b} expression.[4]

Studies on CRC show that patients with mucinous adenocarcinoma and high stage, with metastasis to the lymph nodes, have a poorer prognosis.[13,14] Here, we aimed to evaluate p33^{ING1b} expression in CRC tumor samples in comparison with normal tissues and further analyze the potential correlation between p33^{ING1b} expression and different clinicopathological variables including sex, age, tumor location, type, stage, and grade of differentiation.

MATERIALS AND METHODS

Human Tissue Samples

In this retrospective study, a total of 70 formalin-fixed paraffin-embedded tissue samples of CRC and their matched normal colorectal tissues (at least 10 cm away from the tumor margins) were obtained from patients by surgical colectomy (Department of Pathology at Al-Zahra Hospital in Isfahan, Iran). The information regarding patients' sex, age, tumor location, stage, and grade of differentiation were obtained from hospital records. All diagnoses were verified by an independent pathologist who did not have any information about stained slides. This project was approved by the Ethics Committee of Isfahan University of Medical Sciences (No:388207).

Immunohistological staining and evaluation

The expression intensity of p33^{ING1b} protein in the paraffin-embedded histological sections was determined using immunohistochemical analysis. One section block was hematoxylin & eosin-stained and another section was prepared and examined for p33^{ING1b} expression. First, sections (4-5 μm thick) were dewaxed in xylene and then rehydrated by immersion in different percentages of ethanol. Endogenous peroxides were blocked by pretreatment of the sections with a 0.5% solution of hydrogen peroxide in methanol for 10 minutes. The slides were then washed under running tap water. A one-minute pressure cooking step in citrate buffer (200 mM citric acid, 500 mM NaOH, pH = 6.0) was used for antigen retrieval. Treated sections were transferred into Tris-buffered saline (TBS) for five minutes and blocked with 10% normal goat serum in TBS for 5 minutes. Excess serum was removed and the sections were incubated at room temperature with the primary anti-ING1 monoclonal antibody (WH0003621M1; Sigma.Aldrich.co) at 1:200 dilutions for 60 minutes. The sections were washed twice with TBS for five minutes each, and then incubated with the secondary biotinylated rabbit anti-mouse antibody at a 1500 dilution for 45 minutes at room temperature. After rinsing the samples twice with TBS, the peroxidase activity was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB), in sterile H_2O_2 solution for five minutes. Sections were then washed with tap water, counterstained with hematoxylin, dehydrated in different percentages of ethanol, and mounted in DPX [A mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene]. Sections of normal colon tissue samples were used as positive controls for p33^{ING1b} expression. Sections incubated with PBS instead of the corresponding primary antibody were used as negative controls.

Scoring System

The proportions of cellular staining for p33 ^{ING1b} were estimated under light microscopy at 100× and 400× magnifications by using Motic Image Advanced Plus Software, version 3.2. At least 1,000 cells per slide from 10 different fields were counted. The staining intensity was assessed on a four-point scale as follows: 0= negative, 1= weak, 2=intermediate, and 3=strong. The staining intensities were also verified by an independent pathologist. The percentages of stained cells were assessed on a three-point scale as follows: 1=0–15%, 2=16–50%, and 3=51–100%.

Statistical analysis

The SPSS software version 20 (SPSS Inc., Chicago, IL) was used for all statistical analyses. The paired t test was used to test the relationship between p33^{ING1b} expression in normal and tumor samples, as well as the relationship between p33^{ING1b} expression and clinicopathological variables. Data were represented as mean ± SD and P values less than 0.05 were considered as statistically significant.

RESULTS

General

Positive p33^{ING1b} staining was observed as a nuclear pattern with various staining intensities (figures 1,2). Overall, a significant reduction in p33^{ING1b} protein expression was noted in the tumor samples in comparison with the normal tissues

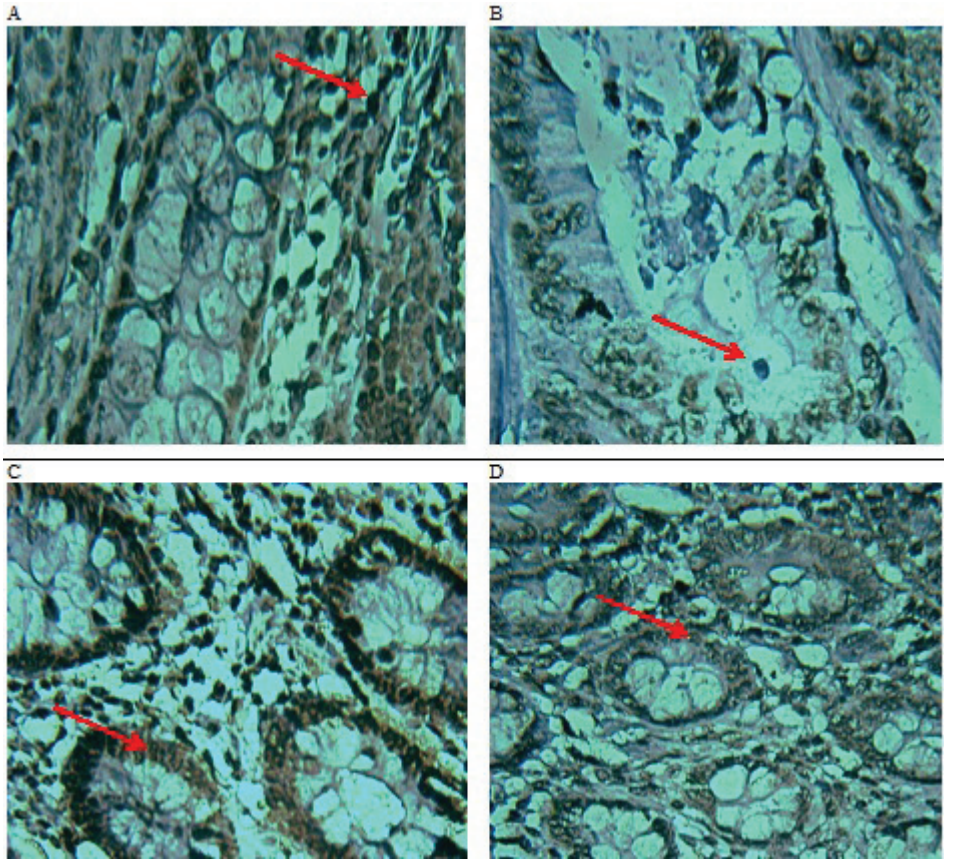
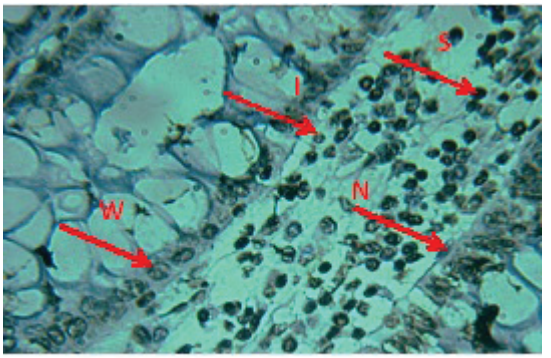


Fig. 1: Positive p33^{ING1b} staining



S: Strong, N: Negative, I:Intermediate, W: Weak

Fig. 2: Positive p33^{ING1b} staining was observed as a nuclear pattern with various staining intensities

($p< 0.000$) Table 1 shows the results of the paired t test comparing the mean tumor suppressor protein expression in tumor specimens and adjacent normal tissue samples. There was a significant difference ($p< 0.000$) between the first group (T.exp3-C.exp3) and group IV (T.exp0-C.exp0). But in the second group (T.exp.2-C.exp.2) and group III (T.exp.1-C.exp.1) this difference was not significant.

The overall correlation between the mean percentages of p33^{ING1b} expressing cells with demographic and clinical characteristics of patients with CRC

Out of the 70 samples analyzed, 41 (58.57%) came from the male patients and 52 (74.3%) were from patients above 50 years old. Among these patients whose ages ranged from 30 to 87 years (with an average age of 61.95 ± 13 years). Most of the patients with CRC (67.15%) were in stage B. The tumors were mainly non-mucinous (85.71%) and well differentiated (88.34%). And about two third of them (68.57%) were in the colon.

As table 2 shows the comparison of the mean tumor suppressor protein expression in tumor specimens with sex in the four-point scale, the second group (T.exp.2) and group III (T.exp.1) showed a significant difference (p=0.037 and p=0.029, respectively).

To compare the mean tumor suppressor protein

Table 1: Comparing the mean tumor suppressor protein expression in tumor specimens and adjacent normal tissue samples(*significant)

Groups		Mean	N	S. D	*p* value
Pair 1	T.exp3	23.65	70	12.48	.000*
	C.exp.3	35.71		16.50	
Pair 2	T.exp.2	38.70	70	14.88	.251
	C.exp.2	41.44		15.92	
Pair 3	T.exp.1	13.82	70	11.83	.246
	C.exp.1	11.98		11.44	
Pair 4	T.exp.0	23.85	70	11.46	.000*
	C.exp.0	10.87		6.17	

T: Tumor, C: Control (normal), exp.0: Negative; exp.1: Weak, exp.2: Intermediate; and exp3: Strong, S. D: Std. Deviation

Table 2: Comparing the mean tumor suppressor protein expression in tumor specimens with gender in four point scale (*significant)

Groups		Mean	N	S. D	*p* value
T.exp3	female	29	25.48	12.66	.307
	male	41	22.36	12.34	
T.exp.2	female	29	34.31	11.29	.037*
	male	41	41.80	16.39	
T.exp.1	female	29	17.48	11.76	.029*
	male	41	11.24	11.31	
T.exp.0	female	29	22.75	12.54	.504
	male	41	24.63	10.72	

Table 3: Comparing the mean tumor suppressor protein expression in tumor specimens with stage of tumor in four point scale

	Stage	df	Mean	F	*p* value
T.exp3	A,B	2	47.68	.300	.742
	C	67	159.08		
T.exp.2	A,B	2	674.10	3.242	.045*
	C	67	207.94		
T.exp.1	A,B	2	236.71	1.726	.186
	C	67	137.11		
T.exp.0	A,B	2	127.01	.966	.386
	C	67	131.50		

expression in tumor specimens with different levels of tumor stage, we used ANOVA test (Table 3). Results showed that different levels vary with each other. In fact T.exp.2 between different levels of tumor stage was significant (p=0.045). Also in this scale, stage A was significantly different from stage B and C (p=0.014).

Comparing the mean tumor suppressor protein expression in tumor specimens with the other variables showed no statistically significant difference (Tables 4-7).

Table 4: Comparing the mean tumor suppressor protein expression in tumor specimens with age in four point scale

	Age	N	Mean	S. D	*p* value
T.exp3	<50	18	24.83	15.17	.646
	≥50	52	23.25	11.55	
T.exp.2	<50	18	36.05	15.50	.386
	≥50	52	39.61	14.70	
T.exp.1	<50	18	15.38	13.34	.520
	≥50	52	13.28	11.35	
T.exp.0	<50	18	23.77	13.90	.973
	≥50	52	23.88	10.64	

Table 5: Comparing the mean tumor suppressor protein expression in tumor specimens with grade of tumor in four point scale

	Grade	N	Mean	S. D	*p* value
T.exp3	Moder diff	60	24.05	12.32	.523
	Well diff	10	21.30	13.82	
T.exp.2	Moder diff	60	38.45	15.05	.733
	Well diff	10	40.20	14.43	
T.exp.1	Moder diff	60	14.26	12.24	.452
	Well diff	10	11.20	9.01	
T.exp.0	Moder diff	60	23.30	11.47	.323
	Well diff	10	27.20	11.39	

Moder diff: Moderately differentiated, Well diff: Well differentiated

Table 6: Comparing the mean tumor suppressor protein expression in tumor specimens with type of tumor in four point scale

	Type	N	Mean	S. D	*p* value
T.exp3	Non-mucinou	7	23.28	13.71	.935
	Mucinous	63	23.69	12.45	
T.exp.2	Non-mucinou	7	37.28	24.17	.793
	Mucinous	63	38.85	13.77	
T.exp.1	Non-mucinou	7	15.28	10.70	.734
	Mucinous	63	13.66	12.01	
T.exp.0	Non-mucinou	7	24.28	11.14	.918
	Mucinous	63	23.80	11.58	

DISCUSSION

Several studies have shown that the levels of p33ING1 expression are reduced in some cancers including head and neck, squamous cell, stomach, liv-

Table 7: Comparing the mean tumor suppressor protein expression in tumor specimens with place of tumor in four point scale

	Place	N	Mean	S. D	*p* value
T.exp3	R.E	22	26.77	11.92	.159
	Colon	48	22.22	12.59	
T.exp.2	R.E	22	36.81	14.99	.478
	Colon	48	39.56	14.90	
T.exp.1	R.E	22	13.68	8.92	.945
	Colon	48	13.89	13.03	
T.exp.0	R.E	22	22.72	12.18	.580
	Colon	48	24.37	11.21	

R.E:Rectosigmoid

er, esophageal, lung, bladder, ovary, kidney, breast, and liver carcinomas.[15] However, the low levels of p33^{ING1b} expression have been reported with variable frequencies.[16-22] For instance, in breast cancer, the low levels of p33^{ING1b} expression were observed in 58–80% of cases.[16,20-21] This study was done on Iranian people and the expression intensity of p33^{ING1b} protein in the paraffin-embedded histological sections was determined using immunohistochemical analysis. Also the correlation between p33 ^{ING1b} expression in CRC and clinicopathological factors was examined.

Here, we showed that the mean intensity of nuclear p33^{ING1b} expression as well as the number of positive p33 ^{ING1b} expressing cells were low in all CRC cases. This is in support of the findings by Jian-guang and colleagues who reported that the p33^{ING1b} expression in CRC was reduced.[23] Also a study by Chen and colleagues on mRNA expression in sporadic CRC tissue samples by Real-Time PCR, showed that p33^{ING1b} mRNA levels were significantly lower in cancerous tissue. The authors further suggested that low levels expression of p33^{ING1b} might play an important role in CRC carcinogenesis and progression.[12]

The study by Jian-Guang and co-workers did not show a meaningful correlation between low levels of p33^{ING1b} expression and patients' sex, age, and tumor differentiation grades and site.[23] Jian-Guang and colleagues have also reported that the low levels of p33^{ING1b} expression markedly related with the Dukes classification. This is in agreement with an earlier report by Liang and colleagues indicating that the rates of p33^{ING1b} expression in cancerous tissues at Dukes' A and B stages were significantly higher than that at stages C and D.[24]

In conclusion, low expression of p33^{ING1b} may be an important molecular event in the pathogenesis of CRC. Our data suggest that reduced expression of p33^{ING1b} may be contribute to tumorigenesis and accompanied by the loss of cellular growth control. In fact cell growth is out of control in lower expression of p33 and dysfunctional program cell death. P33 expression might explain the etiology of CRC for reducing the expression of tumor suppressor proteins.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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