








Down Expression of Zyxin is Associated with Down Expression of p53 in Colorectal Cancer

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Original Article

Colorectal cancer (CRC), which is among the most prevalent cancers worldwide, is caused by environmental and genetic factors. It has been shown that the *p53* gene is associated with CRC pathogenesis; moreover, Zyxin (ZYX) may play a role in *p53* level and activity. Therefore, the present research aimed to investigate the levels of P53 and ZYX genes and proteins in CRC tumor samples. Cancerous tissues (n=31) and matched non-cancerous tissues (n=31) were randomly obtained from 31 patients with CRC. Total RNA was extracted using RNXplus, and gene expressions were assessed by Real-time PCR. Furthermore, the Western blot technique was used to investigate the expression of ZYX and P53 proteins. The obtained results revealed that the expression of ZYX and *p53* genes in cancerous tissues showed no significant difference compared to matched non-cancerous tissues. On the other hand, measuring protein expression using the Western blotting technique indicated that the ZYX (P=0.0081) and *p53* (P=0.0065) expression in tumor tissues significantly decreased compared to those in matched non-cancerous tissues. Correlation analysis indicated a significant correlation between ZYX and P53 proteins (r=0.746, P=0.013). Based on our findings, ZYX might have a suppressive function in CRC and is associated with P53.

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Introduction

The colorectal cancer (CRC) is considered one of the most prevalent cancers in the world (1), and it is the third leading cause of cancer-related mortality in both genders (2). Although it is well-established that environmental and genetic factors are crucial in the development of CRC (3, 4), its etiology remains unclear (5). The *p53* gene mutation plays a key role in CRC pathogenesis (6). Several proteins are important in controlling the P53 level and function, one of which is Homeodomain Interacting Protein Kinase 2 (HIPK2). The HIPK2 increases the stability of P53 through phosphorylation, amplifying its pro-apoptotic effect. Thus, the proteins that may be involved in regulating HIPK2 function may also be important in regulation of P53 level and function; one of these proteins is Zyxin (ZYX) (7). The ZYX, an 82-kD phosphoprotein (8), consists of 572 amino acids and has two major sections: an N-terminal domain and a C-terminal domain, which are important for its interaction with various proteins (9). The ZYX is present in both the nucleus and the cytosol and can translocate between these regions (10). The ZYX is also essential in various process types, including skeletal organization, transcriptional regulation, differentiation, and oncogenesis (10). It is proposed that ZYX might have a regulatory role in HIPK2-P53 signaling pathway (7).

There are conflicting findings regarding the ZYX function in cancer pathology. Some studies point to the fact that ZYX functions as a tumor suppressor in gastric cancer, Ewing sarcoma, and prostate cancer, while others suggest that ZYX has an oncogenic role in ovarian, glioma, and breast cancer (11-14). Due to the potentially significant role of ZYX in the regulation of P53, we aimed to investigate the expression of the ZYX gene and protein and its possible association with *p53* expression in CRC.

Materials and methods

Sample specimens

A total of 31 fresh frozen CRC samples (case) and 31 matched non-cancerous samples (control) were obtained from the Iran National Tumor Bank, founded by the Cancer Institute of Tehran University of Medical Sciences, for Cancer Research, Iran. The samples size was calculated according to the following formula (based on the mean and standard deviation of previous studies in this field):

$$n = \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2} = \frac{(1.96 + 1.84)^2 (0.48^2 + 3.64^2)}{(0.69 - 3.64)^2} = 23$$

The Research Ethics Committee of Kermanshah University of Medical Sciences (Kermanshah, Iran, Ethics Approval number: IR.KUMS.REC.1399.626) approved this study. Moreover, all the procedures were in accordance with the 1964 Declaration of Helsinki (version 2013). The samples were selected based on inclusion and exclusion criteria. The inclusion criterion consisted of patients with CRC. The exclusion criteria included patients who had received chemotherapy, radiotherapy, or anti-inflammatory drugs, as well as patients with other cancers that had metastasized to the colon or rectum. The CRC was diagnosed based on histopathological examination, and the histopathological information was obtained from the Iran National Tumor Bank. Furthermore, the adjacent non-cancerous tissues were assessed through histopathological examination and reported as non-cancerous tissues.

Determination of ZYX and p53 gene expressions

In the first step, up to 50 mg of fresh frozen tissue was homogenized using liquid nitrogen, and total

RNA from the cancerous and matched non-cancerous tissues was extracted using RNX plus solution (Sinaclon, Iran). The quality of the total RNA was examined by 1% Tris-acetate EDTA-agarose gel electrophoresis. In the next step, using 2 µg of total RNA, complimentary DNA (cDNA) was synthesized via reverse transcriptase enzyme using cDNA synthesis kit (Yekta Tajhiz Azma, Iran), based on the manufacturer's instructions. The expression of ZYX and p53 genes in cancerous and matched non-cancerous tissues was determined using quantities RT-PCR with the Real Q plus 2x maser, mix Green (Ampliqon) on Applied Biosystems, Step One Plus, USA. Additionally, the products of Real-Time PCR were evaluated by agarose gel electrophoresis. The forward and reverse sequences of the primers used (15-17) are listed in Table 1. The expression (fold change) of the studied genes was calculated using $2^{-\Delta\Delta C_t}$ formula and β -actin considered as a reference housekeeping gene (18).

Table 1. Primer sequences used in Real-Time PCR

Genes		Primer sequence	Reference
β actin	F	CATGTACGTTGCTATCCAGGC	(15)
	R	CTCCTTAATGTCACGCACGAT	
ZYX	F	ATCCTCAGAGGCAGAATGTGG	(16)
	R	AAGCAGGCGATGTGGAAC	
p53	F	CAGCACATGACGGAGGTTGT	(17)
	R	TCATCCAAATACTCCACACGC	

Preparation of tissue homogenates and total protein determination

For preparing tissue homogenate, the tissues were rinsed by cold saline and crushed in liquid nitrogen. Subsequently, the tissue homogenates were suspended in ice-cold Radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail (KIAZIST, IR), and incubated on ice for 20 min. Then, the homogenates were centrifuged for 15 min at 20000g, -20°C and the supernatants were collected and aliquoted in three micro-tubes and stored at -20°C. The BCA method was used to assess the total protein concentration in tissues homogenates using BCA reagent (KIAZIST: KBCA-96. IR).

Western Blot Analysis

The expressions of ZYX and P53 proteins in cancerous and matched non-cancerous tissues were studied using the Western Blot method. In the first step, 40µg of total protein from tissue homogenates were separated on a 10% SDS-PAGE gel. Next, the protein bands from the gel were transferred to nitrocellulose membrane. The membranes were then incubated for 2 h in in PBST (PBS and 0.1% Tween-20) containing 5% nonfat skim milk (Sigma-Aldrich, USA) at room temperature. After the blocking step, the nitrocellulose membranes were washed twice with PBST and once with PBS (20 mM PBS, pH 7.4). In the next step, the nitrocellulose membrane was incubated with mouse monoclonal antibody against ZYX (1:1000 dilution, Sigma-Aldrich, USA, Z0377), mouse monoclonal antibody against P53 (1:2000 dilution, STJ, USA, STJ96954) and mouse monoclonal antibody against beta-actin (1:1000 dilution, Santa Cruz sc-47778) overnight at 4°C. Next, the membranes were washed twice with PBST (each time for 10 min) and once with PBS for 5 min. After washing, the membranes were incubated with HRP-Goat anti-mouse secondary antibody (1:3000 dilution, Biolegend) for 2 h at room temperature. Finally, after three washing steps (twice with PBST and once with PBS, each step lasting 10 min), the protein bands were visualized using BioRad ECL Select Western Blotting

Detection Reagent (BioRad, USA). ImageJ software (version 1.47, National Institute of health, USA) was used for densitometric analysis of the bands.

Statistical analysis

The SPSS 16 software was used to analyze the collected data. The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. Statistical differences between the studied groups were examined using Student's *t*-tests (paired *t*-test), and Pearson's correlation coefficient was applied to test possible correlation. The data were presented as mean \pm SD, and a *P*-value < 0.05 was considered statistically significant.

Results

Demographic and Clinicopathological characteristics of the participants

As shown in Table 2, most patients were over 50 years old, did not consume alcohol, were non-smokers, and had no family history of CRC. In most cases, the tumors were of adenocarcinoma type with differentiation grade II and were larger than 3 cm. The characteristics of other pathological information regarding the patients, including tumor size, histology, vascular and lymphatic invasion, tumor staging (e.g., primary tumor, lymph node involvement, and metastasis) and TNM stage are presented in Table 3.

ZYX and p53 gene expressions in cancerous and matched non-cancerous tissues

As shown in Fig 1(A), no statistical difference was observed between the ZYX mRNA fold change in the participants' cancerous tissues and the matched non-cancerous tissues ($P=0.37$). Additionally, as indicated in Fig 1(B), no significant difference was found between *p53* gene expression in cancerous tissues and that in the matched non-cancerous tissues ($P=0.213$).

ZYX and P53 protein expression in cancerous and matched non-cancerous tissues

The protein expression of ZYX and P53 in cancerous tissues was significantly lower compared to that in the matched non-cancerous tissues (Fig 2, A). Furthermore, our assessments indicated that ZYX and P53 protein expression showed significant reductions in cancerous tissues compared to that in the matched non-cancerous tissues ($P=0.0081$ and $P=0.0065$, respectively; Fig.2.B and Fig.2.C).

Table 2. Demographic characteristics of the participants

Characteristics	Categorization	N(%)
Age	<50 year	6(19.4)
	≥ 50 year	25(80.6)
Alcohol	Drinker	0(0)
	Non-Drinker	31(100)
Smoking status	Non smoker	22(71)
	DX-Smoker at Diagnosis	5(16.1)
	but Discontinued	4(12.9)
	Smoker	
Family history	Yes	13(41.9)
	No	18(58.1)

Table 3. Frequency of clinical (pathological) information of the patients

Characteristics	Categorizations	N(%)
Tumor Size	<3 cm	5(16.1)
	≥3 cm	26(83.9)
Histology	Adenocarcinoma	25(80.6)
	Mucinous (Colloid) adenocarcinoma	5(16.1)
	Other	1(3.2)
Histology grade	Grade I (Well Differentiated)	6(19.4)
	Grade II (Moderately Differentiated)	19(61.3)
	Grade III (Poorly Differentiated)	3(9.7)
	Grade IV (Undifferentiated)	2(6.5)
	Grade X (Unknown)	1(3.2)
Vascular invasion	Yes	30(96.8)
	No	1(3.2)
Lymphatic invasion	Yes, Nos	29(93.5)
	No	1(3.2)
	Unknown	1(3.2)
Pathological T	T2	4(12.9%)
	T3	23(74.2)
	T4	4(12.9)
Pathological N	Nx	1(3.2)
	N0	12(38.7)
	N1	11(35.5)
	N2	7(22.6)
Clinical Metastasis	M0 (the primary tumor)	27(87.1)
	M1 (manifest metastasis)	3(9.7)
	N/A	1(3.2)
TNM staging	Stage I	3(9.7)
	Stage IIA	8(25.8)
	Stage IIB	1(3.2)
	Stage IIIB	10(32.3)
	Stage IIIC	6(19.4)
	Stage IV	3(9.7)

Correlation analysis

The correlation between ZYX and P53 proteins was examined using correlation analysis. Our assessment showed a direct and significant correlation between ZYX and P53 proteins ($r=0.746$, $P=0.013$) in colorectal tumors.

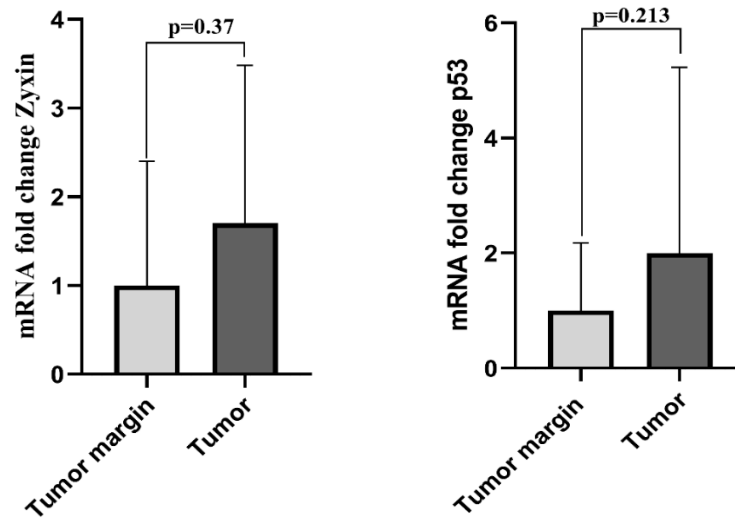


Fig. 1. ZYX and *p53* gene expression levels were determined by qRT-PCR. The relative mRNA expression of (A) ZYX and (B) *p53*, fold changes in gene expression were expressed as mean \pm SD.

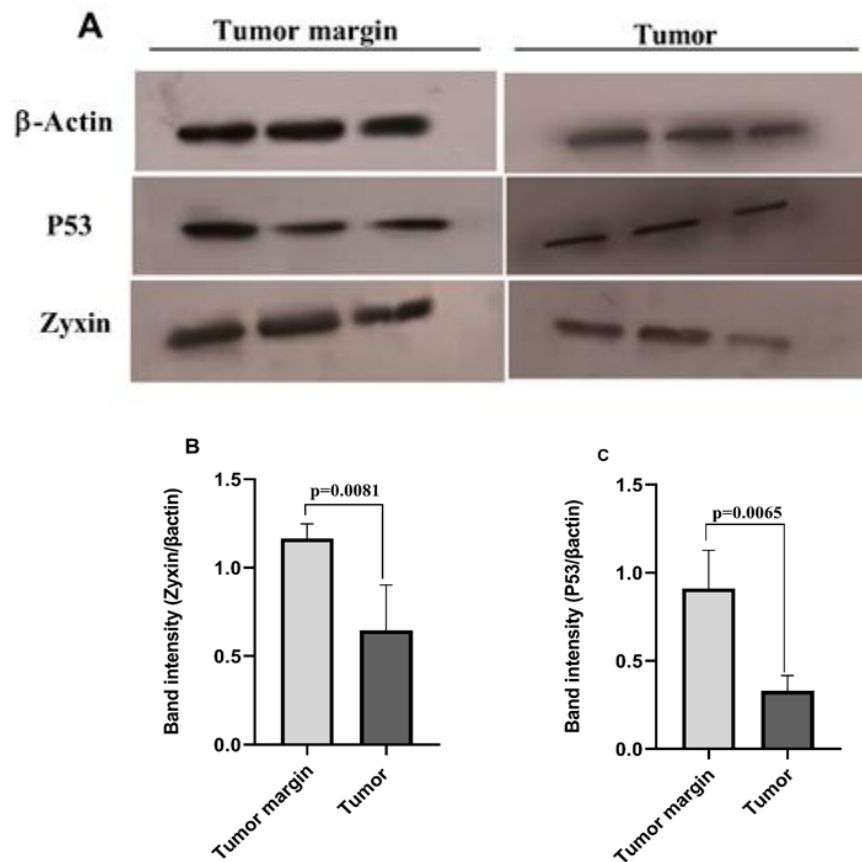


Fig. 2. The protein levels of ZYX and P53 in tumor tissues (n=5) and non-cancerous tissues (n=5) as determined by Western blotting. The Band intensity of the studied proteins was assessed using ImageJ software (A) and expressed as mean \pm SD for (B) ZYX and p53. Columns with **** or ** represent significant difference ($P < 0.05$) compared with tumor margin.

Discussion

In the present research, we aimed to investigate the possible role of ZYX in CRC pathogenesis, as well as its correlation with P53 at both gene and protein levels. Based on our results, although no significant difference was observed between tumors and matched non-cancerous tissue regarding the expression of *p53* and *zyx* genes, the expressions of ZYX and P53 proteins significantly decreased in tumor tissue. Additionally, our findings revealed a direct correlation between ZYX and P53 proteins.

Demographic data of CRC patients showed that 80.6% of cases were over 50 years old, 71% were non-smokers, and 100% were non-alcoholic. It has been shown that the incidence of CRC increases after the fifth decade of life (19), as 50% of cancers and 70% of all cancer deaths occur in older age (20). According to pathological information, tumor size was larger than 3 cm in 83.9% of patients. Tumor growth is an important variable indicated by T classification. However, tumor size in univariate and multivariate analyses in the colon is significantly associated with cancer progression and patient survival (21). Moreover, pathologically, 80.6% of tumors were adenocarcinomas, and 16.1% were mucosal adenocarcinomas. The most common tissue subtype of CRC is adenocarcinoma; mucosal adenocarcinoma is a distinct subtype characterized by abundant mucosal components comprising at least 50% of tumor volume (22). In terms of histological grading, 61.3% of the tumors were moderately differentiated, and 19.4% were well differentiated. Most colorectal tumors exhibit vascular and lymphatic invasion. The presence of a lymphatic attack in CRC could indicate the ability of cancerous cells to metastasize to lymphatic nodes. On the other hand, the invasion of tumor cells into blood vessels and lymphatic vessels is a crucial step in the spread and metastasis of tumor cells (23). However, when discussing demographic data, the small sample size of the present study is one of its limitations.

The *p53* gene, which is mutated in most tumors, plays a crucial role in the pathogenesis of CRC (24). One study showed that HIPK2 can control the level and function of P53 by phosphorylation in Ser46, which leads to P53 stability (25). The ZYX (the LIM domain of ZYX protein) may indirectly regulate P53 by controlling HIPK2 (7), and it has been shown that ZYX has different functions in different tumors. Given the possible role of ZYX in the regulation of P53, our aim was to investigate the expression of the *zyx* gene and protein and its possible association with P53 in CRC.

The findings of gene expression analysis revealed that the expression of the *zyx* and *p53* genes in cancerous tissues was not significantly different from that in matched non-cancerous tissues. However, the results of gene expression analysis indicated a trend toward an increase. On the other hand, the expression of ZYX and P53 proteins in cancerous tissues was significantly decreased compared to matched non-cancerous tissues. Although, a direct relationship between gene expression and protein level is expected, there are instances where the gene expression results differ from those of protein expression. Only about 40% of changes in protein concentration can be explained by mRNA abundance, which can be explained by other factors such as post-transcriptional regulation, translation, varying half-life of mRNA and protein, and protein cleavage and degradation (26). Our observations were consistent with the previous studies that noted the correlation between gene and protein expression during dynamic conditions, such as stress responses, may differ from ideal correlations. They demonstrated that a mismatch between gene and protein expression can occur (27-30).

In the current research, we found that the expression of ZYX and P53 proteins in cancerous tissues significantly decreased compared to matched non-cancerous tissues. A direct and significant correlation was found between ZYX and P53 proteins expression; consequently, a tumor suppressor role for ZYX in CRC was proposed. Similar to our findings, several studies have suggested a tumor suppressor role for ZYX. In a study conducted by Jing lou et al., the expression of ZYX in tumor tissues from 73 gastric cancer patients was investigated. In a study carried out by Lou et al., they proposed a tumor suppressor role for ZYX in gastric cancer and found that overexpression of ZYX decreases epithelial–mesenchymal transition (EMT) processes in a gastric cancer cell line (12). Moreover, Amsellem V et al. found that in human Ewing tumor-derived cells, ZYX is decreased and transferred to EWS-FLI1-transformed fibroblasts, and SK-N-MC cells significantly decreased cell motility and anchorage-independent growth, suggesting a tumor suppressor role for ZYX in Ewing tumor cells (31). Additionally, Lin et al. reported a tumor suppressor function for ZYX in prostate cancer (32). Furthermore, Wei et al. examined the expression of ZYX osteosarcoma cells and observed that ZYX prevents cell migration and proliferation, and invasion, and reported a tumor suppressor role for ZYX (13).

Although some studies suggest a tumor suppressor role for ZYX, several others indicate an oncogenic function for ZYX. Chenhan Zhong et al. in 2019, contended that the expression level of ZYX increased in human CRC tissues, and its reduction interferes with the proliferation and metastasis of CRC cells; however, our results were inconsistent with those of Chenhan Zhong et al. (33). Another study revealed that ZYX is associated with tumor grade and stage (34). The ZYX's oncogenic and tumor suppressor activities are probably associated to other factors, such as Siah-1 ubiquitin ligase, test condition, and organ involved. In addition, ZYX modulates Siah-1 dimerization and possibly its activity. Therefore, an increase in the level of ZYX during DNA damage helps to separate Siah-1 ubiquitin ligase from its substrates. Since ZYX regulates HIPK2 stability through Siah-1, it is suggested that ZYX may control the stability of P53 and HIPK2 through regulation of Siah-1 availability (7). In the study by CAI et al., it was reported that the expression of ZYX in tumor tissues of patients with hepatocellular carcinoma significantly decreased compared to the liver tissues of healthy individuals. In addition, they stated that overexpression of ZYX was correlated with alterations in cell cycle proteins and increased cell migration and invasion (14).

The findings of our investigation demonstrated that the levels of ZYX and *p53* gene expression in cancerous tissues were not significantly different compared to matched non-cancerous tissues; however, the level of ZYX and P53 proteins in cancerous tissues showed a significant decrease compared to matched non-cancerous tissues. We conclude that ZYX may play a major role in the regulation of P53 and may have a tumor suppressive function in CRC.

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