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Human Papillomavirus Infection, p53 Overexpression and Histopathologic Characteristics in Colorectal Cancer

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ABSTRACT

Background

There is evidence of a possible etiological role of human papillomaviruses (HPVs) in the development of colorectal cancer. Loss of p53 tumor suppressor gene function has been found in many malignancies and it can occur in a variety of ways, including gene mutation and interaction with the E6 protein of oncogenic HPVs. The objective of this study was to determine the prevalence of HPV infection and p53 overexprssion in colorectal cancer (CRC) tissue samples and its association with tumor histopathologic characteristics.

Materials and Methods

60 tissue sections from patients with CRC were investigated by immunocytochemistry techniques for aberrant expression of p53 using the streptavidin-biotin-peroxidase method with monoclonal antibodies. The HPV status was also analyzed using type-specific primers for HPV16/18 by polymerase chain reaction (PCR).

Results

Overall, 21 (35%) of 60 patients were found positive for HPV DNA; HPV 18 was detected in 19 (32%) and HPV16 in 11 (18%) of 60 samples. An abnormal expression of tumor-suppressor protein p53 was observed in 29 (48%) samples. p53 overexpression was observed in 15 (71%) of 21 HPV-positive and in 14 (36%) of 39 HPV-negative patients (p=0.009). Similar significant difference was found in p53 overexpression in HPV18-positive patients (p=0.007) but not in those positive for HPV 16 (p=0.261). HPV DNA presentation was not significantly associated with histopathologic characteristics including tumor stage (p=0.509), grade (p=0.668), peri-neural invasion (p=0.265) and lympho-vascular invasion (p=0.275).

Conclusions

p53 inactivation caused by HPV infection may play a role in the pathogenesis of colorectal cancer. There is no association between HPV infection and histopathologic characteristics.

Keywords: HPV, p53, Colorectal cancer, Histopathologic characteristics

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INTRODUCTION

Colorectal adenocarcinoma, with more than 800,000 new cases diagnosed per year, is the third

leading cause of cancer deaths worldwide.(1), According to report of Iranian Ministry of Health, Cure and Medical Education, colorectal cancer (CRC) is the third most common cancer in Iran. Although the pathogenesis of CRC is being well-understood at molecular level, there are still confusing concepts regarding its etiology.(2)

More than 130 different human papilloma virus (HPV) types have been associated with benign and malignant tumors of cutaneous and mucosal origin.(3), HPVs are associated with a broad spectrum of human diseases ranging from subclinical disease conditions to benign and malignant tumors.(4, 5), HPV 6 and 11 (low risk types) are usually associated with subclinical or benign diseases.(4, 5), HPV 16 and 18 and to some extent HPV 33 and 31 are often associated with malignant disease (high-risk HPV).(4, 5), There is association between HPV and various cancers such as skin, cervical, vulval, vaginal, anal, penile, oral, laryngeal, and esophageal carcinoma.(4-6)

Several studies have evaluated the role of various types of HPV in CRC and found it in 32%-97% (7, 8) of tumoral tissue in comparison to 0%-53% (9, 10) of normal tissues. However, in colorectal adenocarcinoma, the presence of HPV DNA remains controversial.(2), Identification of HPV as a predisposing factor for colorectal carcinogenesis would have significant implications in human health, allowing the opportunity to identify high risk groups or develop new therapeutic modalities, besides the general contribution to better understanding the biology of this disease.(2)

On the other hand, the most frequently mutated gene in human cancer is the p53 tumor suppressor gene.(11), Loss of p53 tumor suppressor gene function has been found in many malignancies and can occur through either gene mutation or by interaction with the E6 protein of oncogenic HPV.(12), In contrast to other cancers, there is only few data on HPV infection and p53 mutation or inactivation in CRC.(13-15), Buyru, et al, reported that there is an inverse correlation between p53 mutation rate and prevalence of HPV infection and suggested that p53 inactivation caused by HPV infection may play a role in the pathogenesis of colon cancer.(14)

The objectives of this study were 1) to determine the prevalence of HPV DNA in colorectal tumor tissue samples using a polymerase chain reaction (PCR)-based approach to detect HPV 16 and HPV 18 types; 2) to assess the prevalence of p53 overexprssion in tumors by immunohistochemistry (IHC); 3) to determine if there is a correlation between HPV infection of colorectal tumor tissue and p53 overexprssion; and 4) to determine the association between HPV infection and p53 overexprssion with tumor histopathologic characteristics.

MATERIALS AND METHODS

Study group

In the present study tissue samples of 60 colorectal adenocarcinomas taken from patients who underwent surgery were analyzed. Samples were identified and obtained from a series of paraffin-embedded tissues collected between 2004 and 2005 in the pathology service of a Health Institute in Tehran, Iran.

Immunohistochemistry for p53

Paraffin-embedded tissue sections of 4-µm thick were placed on a poly-lysine-coated glass slide. One section from each of the 60 samples was stained with hematoxylin and eosin for re-examination to confirm the diagnosis. New paraffin sections were used for immunohistochemical staining for p53.

Paraffin was removed from tissue sections by incubating the sample at 60°C for two hours and washed three times in xylene for complete dewaxing. The sections were gradually rehydrated using alcohol and distilled water. Slides were then incubated for 10 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Antigen retrieval was achieved by boiling

sections in 0.01μM citrate buffer (pH=6.0) in microwave oven (four cycles, 10 min each, 300 W). Sections were then incubated with the primary monoclonal antibodies anti-p53 (clone DO-7, DAKO A/S, Denmark; dilution 1:50) which detected both the wild and mutant types. The reaction products were visualized using a streptavidin biotin immunoperoxidase complex (DAKO, Denmark) as secondary antibody complex with diaminobenzidine (DAB). Sections were then counterstained with hematoxylin and mounted with permanent mountant DPX.

Evaluation of p53 expression was semi-quantitative; and reported as negative (0); few isolated cells, less than 10% (+); moderate number of positive cells, 10%-50% (++); and large number of positive cells, 50%-100% (+++).

Determination of HPV infection by PCR

DNA was isolated from paraffin-embedded samples using the DNA extraction kit (DIATom DNA prep 100 Genefanavaran). The amount of DNA was quantified by Nanodrop spectrophotometer. PCR was performed using type-specific primers for high-risk HPV subtypes 16 and 18 for all the 60 tissue samples using Eppendorf thermocycler (Table 1).

PCR was carried out using a PCR amplification kit (Genefanavaran). A reaction mixture of 20 μL was prepared standard buffer with 2.5 mM $MgCl_2$, 1 units of Taq DNA polymerase enzyme, 200 μM of each dNTP, 1.5 μM of each primer (forward and reverse) and 2 μg of genomic DNA per 20μL reaction mixture. Reaction mixture without DNA template was used as a negative control and that with known DNA template was used as a positive control which yielded PCR products of expected results. Forty cycles of amplification were carried out for HPV 16 and 18. PCR products were stored at 4°C for further analysis.

The PCR products were analyzed by electrophoresis on 1.5% agarose gel along with ethidium bromide. A molecular weight marker of 100 bp range (Fermentas) was also run simultaneously to identify the molecular size of the PCR products. The DNA bands were visualized by UV trans-illumination and analyzed using a gel-documentation system.

Statistical analysis

Possible associations between HPV DNA presentation, p53 overexpression and clinical/ histopathologic characteristics were determined using the χ^2 test. Data analyses were performed by SPSS (Chicago, IL). The significance level (α) was set to 0.05 for all statistical tests used.

Table 1. Details of the primers used for HPV analysis.

Type	Viral region	strand	Sequence(5'-3') product size
HPV 16	L1	forward	GCCTGTGTAGGTGTTGAG 246 bp
HPV 16	L1	reverse	TGGATTTACTGCAACATTGG
HPV 18	E1	forward	GTGGACCAGCAAATACAG 162 bp
HPV 18	E1	reverse	TCCAACACGTGGTCGTTGCA

RESULTS

Overall, 21(35%) of 60 patients were found positive for HPV DNA; HPV 18 was detected in 19 (32%) and HPV16 in 11 (18%) of 60 samples. An abnormal expression of tumor-suppressor protein p53 were observed in 29 (48%) samples (Table 2). Overexpression of p53 was observed in 15 (71%) of 21 HPV-positive and 14 (36%) of 39 HPV-negative patients (p=0.009). Similar significant difference was found in p53 overexpression in HPV18-positive patients (p=0.007) but not in those positive for HPV16 (p=0.261) (Table 3).

HPV DNA presentation was not significantly associated with histopathologic characteristics including tumor stage (p=0.509), grade (p=0.668), peri-neural invasion (PNI) (p=0.260) and lympho-vascular invasion (LVI)

Table 2. Tumor and patient's characteristics according to HPV status.

			Total	HPV-	HPV+	p–value
			No. (%)	No. (%)	No. (%)	
Total			60 (100)	39 (65)	21 (35)	
Gender	Male		36 (60)	23 (59)	13 (62)	0.829
	Female		24 (40)	16 (41)	8 (38)	
Age	<60 years		28 (47)	20 (51)	8 (38)	.091
	≥60 years		32 (53)	19 (49)	13 (62)	
p53	Negative		31 (52)	25 (64)	6 (29)	0.009
	Positive		29 (48)	14 (36)	15 (71)	
	Positive	<10%	6 (21)	2 (14)	4 (27)	0.053
		10-50%	10 (34)	5 (36)	5 (33)	
		>50%	13 (45)	7 (50)	6 (40)	
Tumor stage	T1		1 (2)	1 (2)	0 (0)	.509
	T2		10 (16)	5 (13)	5 (24)	
	T3		40 (67)	28 (72)	12 (57)	
	T4		9 (15)	5 (13)	4 (19)	
Differentiation grade	Grade 1		45 (75)	28 (72)	17 (82)	.668
	Grade 2		9 (15)	7 (18)	2 (9)	
	Grade 3		6 (10)	4 (10)	2 (9)	
PNI (N=41)	negative		28 (68)	19 (63)	9 (82)	.260
	positive		13 (32)	11 (37)	2 (18)	
LVI (N=41)	negative		26 (63)	18 (60)	8 (73)	.453
	positive		15 (37)	12 (40)	3 (27)	

α= 0.05

(p=0.453). In grade 1 cancer, 17 (38%) of 45 patients showed HPV DNA; in grade 2 the frequency was two of nine (22%) and in grade 3, it was two of six (33%). Correlation analysis did not show any significant correlation (p=0.485; Spearman's r=0.092) between HPV DNA presentation and different histopathologic grade of CRC. No correlation was also observed between HPV DNA presentation and different T stage of CRC (p=0.912; Spearman's r=0.015).

No significant association was also found between p53 overexpression and histopathologic characteristics including tumor stage (p=0.434), grade (p=0.233), PNI (p=0.265) and LVI (p=0.275). p53 overexpressed in 24 (53%) of 45 patients with grade 1 disease, in four of nine (44%) patients with grade 2 disease and in one of six (17%) with grade 3 disease (Table 4).

Althought grade 1 cancer showed a higher p53 overexpression, the correlation between p53 overexpression and disease grade was not statistically significant (p=0.145; Spearman's

Table 3. Association between p53 overexpression and HPV16 and 18 infection.

		Total	p53-	p53+	p-value
		No. (%)	No. (%)	No. (%)	
	Total	60 (100)	31 (52)	29 (48)	
HPV16	Negative	49 (82)	27 (55)	22 (45)	.261
	Positive	11 (18)	4 (36)	7 (64)	
HPV18	Negative	41 (68)	26 (63)	15 (37)	0.007
	Positive	19 (32)	5 (26)	14 (74)	

Table 4. Histopathologic characteristics according to p53 status.

		Total	p53-	p53+	p-value
		No. (%)	No. (%)	No. (%)	
Total		60 (100)	31 (52)	29 (48)	
Tumor stage	T1	1 (2)	1 (3)	0 (0)	.434
	T2	10 (16)	6 (19)	4 (14)	
	T3	40 (67)	18 (58)	22 (76)	
	T4	9 (15)	6 (20)	3 (10)	
Differentiation grade	Grade 1	45 (75)	21 (68)	24 (83)	.233
	Grade 2	9 (15)	5 (16)	4 (14)	
	Grade 3	6 (10)	5 (16)	1 (3)	
PNI	negative	28 (68)	16 (76)	12 (60)	.265
	positive	13 (32)	5 (24)	8 (40)	
LVI	negative	26 (63)	15 (71)	11 (55)	.275
	positive	15 (37)	6 (29)	9 (45)	

r=0.191) (Table 4). Similar lack of correlation was also onserved between p53 overexpression and T stage of CRC (p=0.965; Spearman's r=0.006).

DISCUSSION

Oncogenic papillomaviruses have been shown to be involved in development of benign and malignant lesions of the cervix and other anogenital sites such as penis, vulva, vagina and anus.(2), Infection with high-risk subtypes of HPV is now considered as one of the possible etiologies along with tobacco and alcohol consumption in the multifactorial etiology of head and neck tumors.(11), The evidence regarding a link between HPV infection and esophageal cancer is also gradually accumulating.(17, 18, 22), Recently, a few reports have claimed the presence of HPV DNA in colorectal tumors. Based on its well-known role in

cervical and anogenital carcinogenesis, some authors proposed an association between HPV DNA and CRC.(2), However, in colorectal adenocarcinoma, the presence of HPV DNA remains controversial. The role for HPV has not been established in two large studies conducted by Shroyer et al. (19) and Shah, et al. (20) using PCR. But there have been two smaller studies which have shown that HPV in colorectal adenocarcinoma. In a study from Taiwan, HPV DNA was detected in 11 of 37 adenomas and 37 of 70 carcinomas using PCR and southern blot hybridization.(21), Another recent report has also found evidence of HPV 18 in adenocarcinoma of the colon (no squamous features). HPV 18 DNA was found in 10 of 19 cases in the normal mucosa and in 16 of 19 cases in the colorectal adenocarcinoma specimens, using PCR.(10)

In our study, 21 of 60 patients (35%) were found positive for HPV DNA; HPV 18 was detected in 19 (32%) and HPV 16 in 11 (18%) of 60 patients. In other studies, the prevalence of HPV in CRC ranged from 32%-97%.(7, 8), This wide range of HPV infection incidences may be attributed to various geographic area regarding to HPV prevalence, different detection methods used, varying number of tissue samples from various locations studied, and different composition of groups of patients regarding various host-tumor parameters.

The role of HPV in malignant diseases is well explored but it is still not fully described. What is known is that three HPV genes, E5, 6 and 7, can alter proliferation, a characteristic common to many oncogenic viruses.(5, 23), E5 encodes proteins that appear to activate epidermal growth factor receptors. E6 and E7 of HPV 16 and 18 can control transformation and express their oncogenic potential, particularly in the cervix.(5, 23), Two tumor suppressor gene products, p53 and Rb, are inactivated by binding E6 and E7, respectively.(24), Therefore HPV oncoproteins may induce tumor development not only through p53-independent pathways but also via p53 inactivation.(14), The normal p53 is known to induce apoptosis by activating the death gene bax and down regulating survival genes like bcl-2.(25), Loss of p53 tumor suppressor gene function has been found in many malignancies and it can occur in a variety of ways, including gene mutation and interaction with the E6 protein of oncogenic HPVs.(12), The loss of function of the p53 check point regulation due to its interaction with high-risk HPV E6 may thus impair the apoptotic response to virally infected cells.(26), Since the normal p53 is non-immuno-detectable, due to its shorter half-life, p53 overexpression is often considered to indicate the mutant form that is unable to regulate the cell cycle and apoptosis.(27), Several studies have evaluated the correlation between HPV infection and p53 gene mutations and p53 overexpression in various tumor types including head and neck (28, 12), esophageal (29, 30) and cervical cancer.(27-29), The majority of studies showed an inverse correlation between HPV infection and the presence of p53 mutations and suggested that the p53 protein inactivation by complex formation with high-risk HPV 16 and 18 subtypes may be responsible for the overexpression of p53 observed in these cancers, especially in cervical cancer in which unlike most of the solid tumors, the mutation in p53 gene is found to be infrequent.(14, 27, 28, 30, 31, 33, 34), Nevertheless, other studies could not find any significant correlation between p53 expression and the presence of high-risk HPV types.(35, 36, 32, 38-43)

In contrast to other cancers, there is only few data on HPV infection and p53 gene mutation or inactivation in CRC.(13-15), Buyru, et al, (14) showed that although the prevalence of p53 mutation in CRC is usually higher than 50%, (14) in HPV-positive tumors the p53 mutations are rare and concluded that there is an inverse correlation between p53 mutations and HPV infection and suggested that p53 inactivation caused by HPV infection may play a role in the pathogenesis of colon cancer.(14)

In our study, like other reports, the abnormal expression of tumor-suppressor protein p53 were observed in 29 (48%) of 60 samples. Higher numbers of p53 positive cases were found in HPV 16 and 18-positive than in HPV 16 and 18-negative patients (71.4% vs. 35.8%). Although Buyru, et al, (14) only evaluated p53 mutation and did not report p53 expression in tumor samples, our results in accordance with their conclusion also suggested that the p53 protein inactivation by complex formation with high-risk HPV 16 and 18 subtypes may be responsible for the overexpression of p53 observed in CRC.

There is only few data on any probable association between HPV infection and histopathologic characteristics in various tumor types. Recently, in CRC, Weinberger, et al. (44) demonstrated significant association between the presence of viral antigens and tumor staging in a subset of 447 adenocarcinomas of colon but Perez, et al, (2). showed that the prevalence of HPV decreased with increasing severity of the disease. Other studies also reported that HPV E6/E7 gene is probably involved at least in the early steps of the pathogenesis and progression of CRC, whereas few mature virus particles exist in the last stage.(10)

In our study, HPV DNA presentation was not significantly associated with histopathologic characteristics including tumor stage (p=0.428), grade (p=0.668), PNI (p=0.265) and LVI (p=0.275). Furthermore, no significant correlation was also found between HPV DNA presentation and different histopathologic grade and different T stage of CRC.

Few studies have analyzed the p53 status according to hisopathologic characteristics. p53 immunodetection in the early stages of esophageal carcinogenesis has been reported in the literature, mainly in the basal layer of noninfiltrative lesions.(16, 35), The implications of these findings are not yet clear, and it has been suggested that mutations take place primarily in the basal epithelial layer, spreading later throughout the mucosa, as the degree of dysplasia rises.(37), The association between p53 expression and cervical cancer progression is not well-understood. Recently, some studies have reported that the p53 expression is a late event and was seen in advanced cervical intraepithelial lesion (CIN III) and invasive cancer (27, 46) and only in invasive cancer.(27, 45), Some other studies have shown an increased p53 expression in early stages (CIN I and CIN II) (35) and early grade (32, 36) of cervical lesions. Also a highly significant correlation was observed between the intensity of p53 expression and different stages of cervical lesions during the development of cervical cancer from mild dysplasia to invasive cancer.(27)

There is no data on association between p53 expression and histopathologic characteristics or CRC progression. In our study, there was no significant association between p53 overexpression and histopathologic characteristics. Although no correlation was found between p53 overexpression and different histopathologic grades of CRC (p=0.145; Spearman's r=0.191), grade 1 cancer showed higher p53 overexpression and we concluded that p53 expression is probably an early event in colorectal cancer development.

CONCLUSION

p53 inactivation caused by HPV infection may play a role in the pathogenesis of CRC. There is no association between HPV infection and histopathologic characteristics.

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