

p53 Intronic G13964C Variant in Colon Cancer and its Association with HPV

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Abstract. *Background:* Inactivation of p53 is the most common change identified in human cancer. Nucleotide alterations in p53 intron 6 have been reported to be associated with the dysregulation of p53 function and tumor development. The aim of our study was to assess whether the intron 6 G13964C variant of the p53 gene is associated with the presence of human papillomavirus (HPV) as a risk factor in colon cancer. *Materials and Methods:* To determine whether the intronic G13964C alteration is involved in colon carcinogenesis, surgical specimens from 55 patients with colon cancer and HPV-positive tumors were examined by PCR-RFLP. Blood samples from 77 healthy subjects were used as the control group. *Results:* The 13964C variant was found in 14.5% of colon cancer patients and 12.9% of the control subjects. One patient and two controls were homozygous for this base change. *Conclusion:* No evidence of a significant association between the p53 G13964C allele and colon carcinoma was found.

Colorectal cancer (CRC) is one of the leading malignancies worldwide and is the second most frequent cause of cancer death in developed countries. A series of genetic alterations have been associated with CRC and a multistep model of tumorigenesis involving activation of proto-oncogenes (ras gene family) and inactivation of tumor suppressor genes (*APC*, *SMAD4* and *p53*) have been proposed (1-3). The *p53* gene is one of the most frequently mutated genes in human cancers, although *p53* mutations occur late in the adenoma-carcinoma sequence of colorectal cancer (4).

P53 acts as a checkpoint control factor at the G₁/S-phase of the cell cycle. Mutations in the *p53* gene or a multifunctioning

p53 protein are observed in patients with most types of malignancies, including colon cancer (5, 6). The most commonly identified alterations of the *p53* gene are single base pair substitutions (6). These point mutations occur most frequently within five regions of exons 5-8 that have been highly conserved during evolution (7). An additional route to *p53* inactivation is the human papillomavirus (HPV) E6 protein which is known to functionally inactivate *p53* by binding to cause its rapid degradation (8-10). Infection by an oncogenic HPV may result in an E6-mediated loss of *p53* function, or mutation of the *p53* gene could result in its inactivation in the absence of HPV infection. Either event would be sufficient to release a cell from *p53* control. In addition to the mutations in the conserved region of *p53*, several polymorphisms and non-coding region mutations have recently been reported for the *p53* gene. There is accumulating evidence that novel mechanisms of gene regulation, including mutations in splice, donor and promoter elements, may be important in regulating gene expression (11).

Recently, Buller *et al.* have reported an intronic C to G base change at nucleotide 13964 of *p53* (12). The p53 G13964C substitution is not within the consensus splice site and there is so far no direct evidence that it affects the *p53* expression. Nonetheless, there is some indirect evidence that this variant has a functional role (13). An association of this polymorphism with human cancer susceptibility has been investigated in hereditary breast/ovarian cancer (12-14), Li Fraumeni syndrome (15) and leukemia (16), but the results are inconsistent. Its association with colon cancer with reference to HPV-associated tumors has not been studied yet. In a previous study, we showed that infection with oncogenic HPV types may play a distinct role in colon carcinogenesis (17). The frequency of *p53* gene mutations are significantly lower in human papillomavirus-positive colon tumors (18). The present study is based on evaluation of the *p53* intron 6 polymorphism as a putative modulator of HPV-associated colon tumor development.

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Table I. *p53* intron 6 genotype and allele frequencies in colon cancer patients and normal controls.

	GG (%)	GC (%)	CC (%)	n
Healthy controls	67(87)	8(10.4)	2(2.6)	77
Colon cancer patients	47(85.5)	7(12.7)	1(1.8)	55
HPV (+) colon cancer patients	36(81.8)	7(15.9)	1(2.2)	44

	Alleles	
	G (%)	C (%)
Patient	101 (91.8)	9 (8.2)
Control	142 (92.2)	12 (7.8)

Materials and Methods

To identify the intron 6 G13964C mutation of the *p53* gene, DNA was isolated from surgical specimens of 55 patients with colon cancer by phenol/chloroform extraction and analyzed using PCR-RFLP. Blood samples from 77 healthy subjects were used as the control group. Genomic DNA was prepared by proteinase K digestion and phenol/chloroform extraction followed by ethanol precipitation, as described previously.

A 131 bp sequence, spanning nucleotide 13964 of *p53*, was amplified by PCR using the following primers: Forward; 5'-GCCTCCCCTGCTTGCC-3', Reverse; 5'-CCGCCCATGCAGGA ACT-3'.

The PCR reaction was carried out in a total volume of 25 μ L containing 500 ng genomic DNA, 50 mM KCl, 2 mM MgCl₂, 10 pmol each of the primers, 1U Taq polymerase (MBI, Fermentas, Vilnius, Lithuania), 200 μ M dNTP mix and 20 mM Tris-HCl, pH 8.3.

Cycling conditions were 94°C for 4 min; followed by four cycles of 94°C for 20 sec, 60°C for 20 sec and 72°C for 20 sec; and 30 cycles of 94°C for 20 sec, 58°C for 20 sec and 72°C for 20 sec with a final extension of 72°C for 7 min. The PCR products were digested with the HhaI isoschizomere Hin6I.

Digestion was performed in a total volume of 25 μ L containing 10 μ L PCR product, 2.5 μ L 10 x digestion buffer (500 mM Tris-HCl, pH 8.2, 50 mM MgCl₂), 5 μ g BSA and 4U Hin6I (Fermentas) by overnight incubation at 37°C. The digested products were separated by 12% non-denaturing polyacrylamide gel electrophoresis at 100 V for 3 h in 1X TBE. The gel was stained with ethidium bromide and the genotypes were determined using a video gel documentation system (Vilber Lourmat, Torcy, France).

The resulting genotypes and allele distributions in the patient and control groups and association with colon cancer were analyzed using the Chi-square test.

Results

The G13964C variant of the *p53* gene was investigated by PCR-based RFLP analysis. The C allele was identified by the

presence of a single fragment of 131 bp and the G allele by two fragments of 98 and 33 bp, respectively. Heterozygous samples display all three fragments.

Among the 55 colon samples studied, 8 (14.5%) exhibited the G13964C base change in intron 6 of *p53*. One patient was homozygous and 7 patients were heterozygous for this base change. The G13964C transversion was observed in 10 (12.9%) control subjects, and two of the subjects were homozygous.

No difference was found with reference to the genotype distribution ($p=0.88$, $\chi^2=0.25$). The allele frequencies also displayed a similar incidence in the control and patient groups ($p=0.9$, $\chi^2=0.013$).

The allele frequencies and genotype distribution of the *p53* intron 6 G13964C variant in the cases and controls are summarized in Table I.

When the frequencies of the two variants in HPV-positive samples were compared with those in the control group, no difference between the two groups ($p=0.67$, $\chi^2=0.78$) was detected.

No difference was found in the subgroups, even when carcinomas positive for only HPV-16 and -18 were selected.

Discussion

Loss of *p53* tumor-suppressor gene function is observed in different human malignancies and can occur as a result of a variety of causes, including mutations and interaction with the E6 protein of oncogenic human papillomaviruses.

Allelic analysis of patients with HPV-associated tumors has shown that a common polymorphism in the amino acid sequence of the p53 protein may be implicated in the development of some cancers. Lehman *et al.* (13) have proposed that a mutation in intron 6 of *p53* may be critical for familial tumors in patients who do not have detectable germline *p53*-coding sequence or splice site mutations.

The association of this polymorphism with familial breast cancers has been extensively investigated (13, 14). The epidemiology of colon and breast cancer has several features in common; however, *p53* mutation patterns in these cancers are dissimilar (19).

It appears that intron 6 of the *p53* gene is potentially a hot-spot for mutation and represents a novel mechanism of gene regulation that appears to be important for tumor formation. Studies of the association between certain types of cancers and polymorphisms in intron 6 of *p53* have yielded conflicting results (13-16, 20). Initial studies reporting an association between intron 6 polymorphisms and lung (21) or familial breast cancer (13) were followed by contradictory studies reporting lack of association for both (14, 22) cancer types. A recent study investigating the incidence of the G13964C polymorphism in Polish cancer families, previously screened for germline *p53* mutations

and shown not to carry any known *p53* coding sequence mutations, also failed to find an association between the G13964C variation and cancer risk (16). Furthermore, Varley *et al.* analyzed the G13964C variant with respect to its association with cancer risk in LFS/LFL patients and have shown that this base change reflects a polymorphism rather than a mutation (15).

Our study was designed to investigate whether *p53* intron 6 genotypes are associated with susceptibility to colon cancer and whether this correlated with the presence and type of HPV infection in colon tumors. The genotype frequencies were similar in the patients and the control group. Our results do not support the hypothesis that the intron 6 polymorphic variation might influence individual susceptibility or viral *p53* inactivation in colon cancer. These data are in line with studies reporting a lack of association between *p53* intron 6 polymorphisms and lung and breast cancers (14) and with a more recent report on brain (23) tumors.

To our knowledge, this is the first report investigating an association of HPV infection and G13964C genotype in colon cancer patients. In our study group, HPV 18 and HPV 33 were the most prevalent HPV types. This is not surprising since HPV 18 is the predominant type found in adenocarcinomas, while HPV 16 is mainly associated with squamous carcinomas. Our data provide no evidence to support the suggestion by Lehman *et al.* (13) that the G13964C variant functions as a germline mutation associated with high risk of cancer.

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