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Human papillomavirus type 18 in colorectal cancer

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Human papillomaviruses (HPVs) have been recognized as the etiological agent of warts, and they may also be associated with many cancers. HPV-18 is very common both in genital papillomas and in large bowel cancer. The relation between HPV-18 infection and natural course of colorectal cancer has not been fully defined. In this study, normal mucosa and colorectal cancer tissue were evaluated for the presence of HPV gene to determine whether or not HPV was involved in the development of colon neoplasm. The DNA extracted from colon tissue was screened for HPV by polymerase chain reaction (PCR) to amplify the viral gene fragment. These PCR products were digested with restriction enzyme, and Southern blotting was then performed to confirm the existence of HPV-18. The nucleotide sequence related to HPV-18 DNA was detected in 53% (10/19) of the normal mucosa specimens and in 84% (16/19) of the colorectal cancer specimens. The correlation between cancer samples and positive rate of HPV-PCR was statistically significant by chi-square test ($p < 0.01$). These data indicate that HPV-18 can infect the normal mucosa of the colon, and that this infection may be a risk factor for the development of colorectal cancer. The presence of HPV-18 DNA in patients with colorectal cancer suggests that the pathogenesis of colorectal cancer includes viral involvement.

Key words: Colorectal cancer, human papillomavirus (HPV), polymerase chain reaction (PCR), restriction enzyme digestion, Southern blot hybridization

Squamous cell carcinomas of the urogenital region and aerodigestive tract have previously been reported to be highly associated with human papillomavirus (HPV) infection in studies involving polymerase chain reaction (PCR) and DNA hybridization [1,2]. Many HPV types have been identified and among them, types 16 and 18 have been found in malignant lesions, especially in high-grade cervical intraepithelial neoplasia and uterine invasive squamous cell carcinomas [3,4]. The technique of PCR has recently been used to detect and type HPV in specimens obtained from genital lesions [5-11]. By using this technique, we also observed several types of cervical cancer in Taiwan that were highly associated with HPV-16 and HPV-18 [12].

Before the widespread use of PCR-based techniques [5-12], colon tissues were screened for HPV by using immunohistochemical staining and *in situ* DNA hybridization techniques [13,14]. Previous reports have found HPV antigen in 23% of normal colon specimens, 60% of benign tumors, and 97% of carcinomas [13]. The HPV viral genome was also demonstrated in 27% of benign tumors, and in nearly 43% of all carcinomas tested [13]. On the other hand, HPV was discovered in 43% of colon cancer cell lines [15] and 86% of cases of anal cancer [16,17]. Many investigations have shown that HPV has an etiologic role in cervical dysplasia [18]. Cervical dysplasia may occur in association with co-factors, such as tobacco use, infection with Epstein-Barr virus, cytomegalovirus, herpes simplex virus II, and human immunodeficiency virus. Anal carcinoma is rare, with a reported annual incidence in Switzerland of about 0.6 cases per 100 000 people [18], of which elderly women and homosexual men are more frequently affected [18]. Epidemiological data suggests that a sexually transmitted factor has an important role in the genesis of anal carcinoma [18]. The colorectal cancer tissue and the neighboring normal mucosa of the same patient were thus evaluated for the presence of HPV gene to determine whether HPV was involved in the development of colon neoplasm. In this study, HPV-18 was detected in 84% of patients with colorectal

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cancer when the E6/E7 gene region of the virus was used as primers for PCR in elderly men.

Materials and Methods

Clinical specimens

From the 19 patients with colorectal cancer, 38 tissue samples were derived from both the tumor lesions and their neighboring normal mucosa for investigation. The samples of normal mucosa were surgically taken 6 cm away from the tumor border. All clinical samples were collected during primary surgery. Immediately following resection, both cancer tissue and normal mucosa tissue were stored at -70°C before experiments.

Tissue was homogenized with a homogenizer and centrifuged for 10 min at 2500 x g (IEC DPR-6000, Needham Heights, MA, USA). The cellular pellets were suspended in 1 mL of the extraction buffer [19]. Half of the pellet was used for HPV-DNA detection by direct PCR using early primers, and the other half was stored at -70°C.

Preparation of DNA

The method used for DNA preparation was as described previously [19]. Briefly, the homogenates were first digested overnight at 37°C with proteinase K at a final concentration of 100 mg/mL in extraction buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 7.9; 0.5% sodium dodecyl sulfate). Proteins were removed by extraction twice with buffered phenol and once with chloroform:isoamylalcohol (24:1). The DNA was precipitated at -70°C for 30 min with two volumes of ethanol in the presence of 0.1 M NaCl, and resuspended in 50 µL H_2O after centrifugation. The concentration of DNA was determined by using a fluorometer (Hoefer, San Francisco, CA, USA).

Primers

The HPV-18 DNA-PCR primers were derived from the E6/E7 region of the HPV genome. The sequences of the primers (sense: 5'-CGACAGGAACGACTCCAACGA-3', antisense: 3'-TCAATTAGTAGTTGTAAATGGTCG-5', and probes: 5'-TAAGGCAACATTGCAAGACA-3'), the genomic locations (sense: 540-560 and antisense: 718-741), and the sizes of the amplified products (202 base pair [bp]) are listed in Fig. 1.

Polymerase chain reaction

The amplification was performed with a DNA Thermal Cycler (MJ Research, Inc., Waltham, MA, USA). One microgram of DNA purified from HeLa (human cervical cancer) cells was used as positive control, and 1 µg of DNA extracted from each specimen was used for PCR

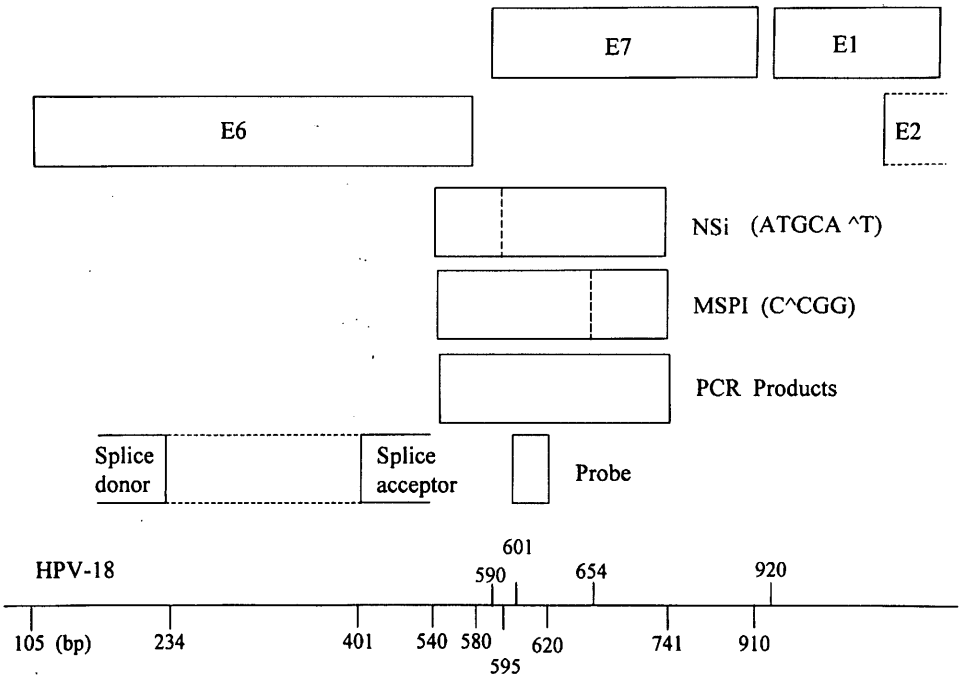

Fig 1. The E6/E7 region of the HPV-18 genome. The graph showed the position of the oligonuclotide primers used for PCR, the sites for restriction endonuclease digestion, and probe for hybridization. Note that the splice donor site and splice acceptor site are absent in the primer region, and that the amplification generates a fragment of 202 bp.

in a reaction mixture containing 50 mM KCl, 10 mM Tris HCl (pH8.3), 1.5 mM MgCl, 0.001% gelatin, 100 μM of each deoxynucleotide (dNTP), 0.3 μM of each of the two primers, and 2.5 U of *Taq* polymerase (Promega, Madison, WI, USA) to a total volume of 100 μL. The samples were overlaid with a drop of mineral oil to prevent evaporation. The samples were heated for 2 min at 95°C to separate double-stranded DNA and subjected to 35 cycles of PCR amplification. Each cycle included primer annealing for 1 min at 55°C, primer extension for 1 min at 72°C, and DNA denaturation for 1 min at 95°C. After the last cycle, the samples were incubated for 7 min at 72°C to complete the extension of the primers.

Restriction enzyme analysis

Anticontamination procedures were followed during all manipulations. Analysis of PCR products by re-striction endonuclease digestion was carried out using 100 μL of the reaction mixture. The amplified DNA in each sample was extracted with phenol-chloroform, precipitated with absolute ethanol, and finally resuspended in a minimal volume of H_2O. Restriction digestion with two units of *Msp*I or *Nsi*I (GIBCO BRL, Gaithersburg, MD, USA) was allowed to proceed for 2 h at 37°C [21]. Digestion patterns were analyzed on a 4% agarose gel and visualized as previously described [22]. After electrophoresis, the DNA bands were denatured in the denaturing buffer (1M NaCl, 0.5N NaOH) and transferred onto a "gene screen" Zetaprobe membrane (Bio-Rad, Hercules, CA, USA) for overnight. After neutralization (1.5M NaCl, 0.5M Tris-HCl, pH 7.4) and air-drying, the filter was illuminated with ultraviolet light (UV) to fix the DNA.

Slot blotting

For 20 μL of each PCR-amplified DNA specimen, 80 μL of 20x SSC (1x SSC: 0.15 M sodium chloride; 0.015 M sodium citrate, pH 7) were added. The samples were denatured by the addition of 10 μL of 1 M sodium hydroxide and heating at 95°C for 5 min. The mixtures were chilled on ice, and each sample was spotted into a nylon membrane filter (Bio-Rad). After air-drying, the filter was wrapped in plastic and placed DNA-side down on a UV crosslinker at 312 nm 0.3 J/cm^2 (Biolink, New Haven, CT, USA) to fix the DNA.

Probe labeling

Ten picomoles of oligonucleotide probes were 3'-end labeled with a γ-^{32} dATP (3000 Ci/mmole) using a DNA 3'-end labeling kit (Boehringer Mannheim, Mannheim, Germany).

Slot blot or Southern blot hybridization

For hybridization, the filters were prehybridized at 34°C for 4 h in a solution containing 6x SSC, 2x Denhardt solution (1x Denhardt solution: 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl-pyrolidone) [20], 0.25% sodium dodecyl sulfate, and 0.1 mg/mL denatured and sonicated salmon sperm DNA. Hybridization was performed at 37°C overnight in a similar solution with a labeled oligonucleotide probe (3×10^8 cpm/mL). The filter was washed twice in 2x SSC, 0.05% sodium dodecyl sulfate at room temperature for 30 min. The last wash was for 10 min at 45°C. The filter was exposed to Kodak X-O mat XAR film (Eastman Kodak, Rochester, NY, USA) at -70°C for 10 to 24 h.

Results

Using primers of HPV from E6/E7, the early region of HPV-18 gene, the PCR product was identified by restriction endonuclease digestion. The results of gel electrophoresis are shown in Fig. 2. The HPV-18 positive control from HeLa cells showed a fragment of 202 bp of DNA amplified by the PCR (Fig. 2, Lane 4). This fragment was digested into 88 bp and 114 bp fragments with *Msp*I endonuclease (Fig. 2, Lane 5) or into 147 bp and 55 bp fragments with *Nsi*I digestion (Fig. 2, Lane 6). These fragments were further used as

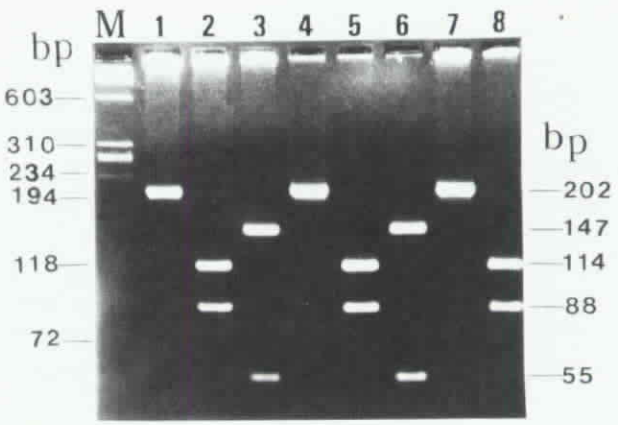

Fig. 2. Electrophoresis patterns of PCR products from amplified E6/E7 gene fragments for HPV-18 and their restriction endonuclease (*Msp*I, *Nsi*I) digestions. Markers: *Hae* III-digested ϕx174 DNA markers (1353, 1078, 872, 603, 310,281,271, 234, 194, 118, 72 bp). Lane 1: a fragment of 202 bp from a colorectal sample showing HPV; Lane 2: *Msp*I digestion of Lane 1 (114 and 88 bp); Lane 3: *Nsi*I digestion of Lane 1 (147 and 55 bp); Lane 4: the fragment from HeLa cells as HPV-18 control; Lane 5: *Msp*I digestion of Lane 4; Lane 6: *Nsi*I digestion of Lane 4; Lane 7: PCR product of a normal mucosa sample showing HPV; Lane 8: *Msp*I digestion of Lane 7.

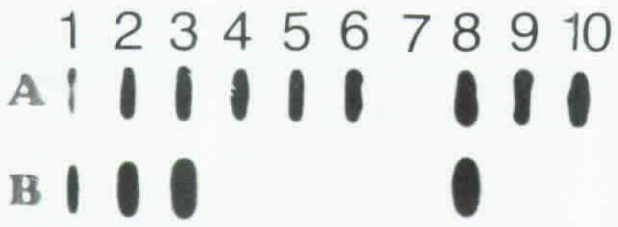

Fig. 3. Slot blot hybridization of HPV-18 from PCR-amplified sample. Row A: colorectal cancer: 1-6 and 9-10; clinical samples, 7; distilled water (negative control), 8; HeLa (positive control). Row B: normal mucosa: 1-6 and 9-10; clinical samples, 7; distilled water (negative control), 8; HeLa (positive control).

Table 1. Correlation between colorectal cancer and HPV-18

HPV-PCR	No. of cases	Clinical sample	
		Normal mucosa	Colorectal cancer
Positive	26	10	16
Negative	12	9	3
Total	38	19	19

Note: chi-square = 10.3, df = 1, $p < 0.01$.

positive references for DNA detected from clinical samples (Fig. 2, Lanes 1-3 and 7-8). The PCR products were also confirmed by slot blot hybridization in general (Fig. 3). Fig. 3 shows the results of slot blot hybridization with a HPV-18 probe when the samples of colorectal cancer lesion (Line A) and those of normal mucosa (Line B) were detected.

Using the above criteria, the incidence of HPV-18 in the normal mucosa specimens was found to be 53% (10/19). The frequency of HPV-18 found in colorectal cancer specimens was 84% (16/19). There were 10 patients with a positive result for HPV-18 by PCR in both colorectal cancer lesion and the normal mucosa specimens.

The histologic classification of cancer and its correlation to HPV-18 by PCR is summarized in Table 1. The incidence of HPV-18 involvement in patients with colorectal cancer lesion was significantly greater than that in patients with normal mucosa ($p < 0.01$) (Table 1).

Discussion

HPVs, especially HPV-16 and HPV-18, have been extensively studied in various invasive cancers [21], which include colorectal tumors. By using Southern blot hybridization, Cheng *et al* [16] reported that all three colorectal cancer cell lines contained HPV-16 and HPV-18 DNA sequences. Kirgan *et al* [13] examined benign and malignant colonic tissue and showed that HPV could be detected in tissue sections.

In this study, when the E6/E7 region of the viral oligonucleotide primer was used for PCR, 16 of 19 (84%) tumor specimens and 10 of 19 (53%) normal mucosa specimens obtained 6 cm away from the tumor lesion of the colorectal cancer patients contained HPV-18 DNA. The technique of DNA amplification by PCR has shown to be an extremely powerful tool in gene analysis [22,23]. Our results indicate a significant difference between the normal mucosa and the cancer tissue in HPV-18 infection, which suggests that HPV-18 infection was correlated with colorectal cancer. Restriction endonuclease digestion of the amplified products reassures accurate typing of HPVs.

When using general primers of HPVs selected from the L1 region for PCR, the positivity varied. The reason could be that L1 consensus primers are significantly less sensitive than E6/E7 derived type specific primers for HPV-18 in detecting HPV DNA from colorectal cancer tissue, most probably owing to the disruption of the L1 gene (but not the E6/E7 genes) during integration of the viral genome, which is thought to be a prerequisite for malignant transformation [24-26]. HPV E6/E7 gene is probably involved at least in the early steps of the pathogenesis and progression of colorectal cancer, whereas few mature virus particles exist in the late stage [11,15,24]. Further investigations are needed to clarify the involvement of HPV-18 DNA, E6/E7.

The E6/E7 oncogenes are constantly expressed in cancer cells [14]. The regulatory mechanisms of E6/E7 expression are not known in cancer cells although E6/E7 are capable of immortalizing cells, inducing cell growth, and promoting chromosomal instability in the host cell. Blocking the function of E6/E7 gene leads to reversion of malignant phenotype of the cancer cells [14]. E6/E7 in high-risk HPVs, however, can act as independent oncogenes because of their even separation and capability of immortalizing cells. The mammalian phosphoprotein P53 has been shown to have an essential role in regulating cell division, as it is required for the transition from phase G_0 to G_1 of the cell cycle [28]. The *P53* gene has now been clearly shown to be the most commonly mutated oncogene in a wide variety of human cancers, which include the most frequent, adenocarcinomas [29]. This information provides a useful marker for early detection of HPV in colon cancers.

HPV-18 has a high incidence of positivity in patients with colorectal cancer, cervical cancer, and squamous cell carcinomas of the anal and colorectal canal [1,3-4,11-16,19,26]. The results of this study support that genital HPV infection predisposes patients

not only to cervical cancer, but also to colorectal cancer, possibly by means of contiguous contamination.

Acknowledgments

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References

1. Das BC, Sharma JK, Gopalkrishna V, Das DK, Singh V, Gissmann L, Zur Hausen H Luthra UK. A high frequency of human papillomavirus DNA sequences in cervical carcinomas of Indian women as revealed by Southern blot hybridization and polymerase chain reaction. J Med Virol 1992;36:239-45.
2. Gjoen K, Siebke JC, Flikke M, Hager R, Ertzeid G, Halsos A, Ekgren J, Norling B, Grinde B, Orstavik I. Genital human papillomavirus infection in Oslo studied by dot blot DNA hybridization and the polymerase chain reaction. J Med Virol 1991;34:159-64.
3. Vandenvelde C, Scheen R, Van Pachterbeke C, Loriaux C, Decelle J, Hubert T, Delhaye C, Cattoor JP, Duys M, Van Beers D. Prevalence of high risk genital papillomaviruses in the Belgian female population determined by fast multiplex polymerase chain reaction. J Med Virol 1992;36:279-82.
4. Lambropoulos AF, Agorastos T, Frangolides E, Karahaliou R, Bontis J, Dozi-Vassiliades I. Detection of human papillomavirus using the polymerase chain reaction and typing for HPV 16 and 18 in the cervical smears of Greek women. J Med Virol 1994;43:228-30.
5. Hallam N, Green J, Gibson P, Powis J, Bibby J. Prevalence of HPV cervical infection a family planning clinic determined by polymerase chain reaction and dot blot hybridization. J Med Virol 1991;34:154-8.
6. Falcinelli C, Glaas E, Kleter B, Quint WG. Detection of the human papillomavirus type 16 mRNA-transcripts in cytological abnormal scrapings. J Med Virol 1992;37:93-8.
7. Higgins GD, Vzelin DM, Phillips GE, Mcevoy P, Marin R, Burrell CJ. Transcription patterns of human papillomavirus type 16 in genital intraepithelial neoplasia: evidence for promoter usage within the E7 open reading frame during epithelial differentiation. J Gen Virol 1992;73:2047-57.
8. Vormwald-Dogan V, Fischer B, Bludau H, Freese UK, Gissmann L, Glitz D, Schwarz E, Durst M. Sense and antisense transcripts of human papilloma virus type 16 in cervical cancer. J Gen Virol 1992;73:1833-8.
9. Rose BR, Jiang XM, Thompson CH, Tatternsall MH, Cossart YE. Detection of human papillomavirus type 16 E6/E7 transcripts in fixed paraffin-embedded cervical cancers by the polymerase chain reaction. J Virol Methods 1991;35:305-13.
10. McNicol P, Paraskevas M, Guijon F. Variability of polymerase chain reaction-based detection of human papillomavirus DNA is associated with the composition of vaginal microbial flora. J Med Virol 1994;43:194-200.
11. Cheng JY, Sheu LF, Meng CL, Lee WH, Lin JC. Detection of human papillomavirus DNA in colorectal carcinomas by polymerase chain reaction. Gut 1995;37:87-90.
12. Hsu WP, Ho ES, Yang CH, Lee YM, Chang CM, Liu WT. Prognostic significance of human papillomavirus detected with polymerase chain reaction in cervical cancer. Chin Med J (Taipei) 1993;51:97-102.
13. Kirgan D, Manalo P, Hall M, Mcgregor B. Association of human papillomavirus and colon neoplasms. Arch Surg 1990;125:862-5.
14. Miyake Y, Funjiwara Y, Ohue M, Yamamoto H, Sugita Y, Tomita N, Sekimoto M, Shiozaki H, Monden M. Quantification of micrometastases in lymph nodes of colorectal cancer using real-time fluorescence polymerase chain reaction. Int J Oncol 2000;16:289-93.
15. Cheng JY, Meng CL, Chao CF, Gau SD, Lin JC. Human papillomavirus 16 DNA in NIH3T3 cells transformed by colonic cancer cellular DNA. Gut 1993;34:1710-3.
16. Cheng JY, Meng CL, Chao CF, Gau SD, Lin JC. Human papillomavirus type-related DNA and c-myc oncogene alterations in colon cancer cell lines. Dis Colon Rectum 1991; 34:469-74.
17. Frisch M, Fenger C, Van den Brule AJ, Sorensen P, Meijer CJ, Walboomers JM, Adami HO, Melbye M, Glimelius B. Variants of squamous cell carcinoma of the anal canal and perianal skin and their relation to human papillomaviruses. Cancer Res 1999; 59:753-7.
18. Poletti PA, Halfon A, Marti MC. Papillomavirus and anal carcinoma. Int J Colorectal Dis 1998;13:108-11.
19. Soler C, Allibert P, Chardonnet Y, Cros P, Mandrand B, Thivolet J. Detection of human papilloma virus types 6,11,16, and 18 in mucosal and cutaneous lesions by the multiplex polymerase chain reaction. J Virol Methods 1991;35:143-57.
20. Contorni M, Leoncini P. Typing of human papillomavirus DNAs by restriction endonuclease mapping of the PCR products. J Virol Methods 1993;41:29-36.
21. Cone RW, Minson AC, Smith MR, McDougall JK. Conservation of HPV-16 E6/E7 ORF sequences in a cervical carcinoma. J Med Virol 1992;37:99-107.
22. Snijders PJ, Van den Brule AJ, Schrijnemakers HF, Snow G, Meijer CJ, Walboomers JM. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J Gen Virol 1990; 71:173-81.
23. Morrison EA, Gold Berg GL, Kadish AS, Burk RD. Polymerase chain reaction detection of human papillomavirus: quantitation may improve clinical utility. J Clin Microbiol 1992;30:2539-43.
24. Cheng JY, Sheu LF, Lin JC, Meng CL. Detection of human papillomavirus DNA in colorectal adenomas. Arch Surg 1995; 130:73-6.
25. Leppert M, Burt R, Hughes JP, Samowitz W, Nakamura Y, Woodward S, Gardner E, Lalouel JM, White R. Genetic analysis of an inherited predisposition to colon cancer in a family with a variable number of adenomatous polyps. N Engl J Med 1990; 322:904-8.
26. Tilston P. Anal human papillomavirus and anal cancer. J Clin Pathol 1997;50:625-34.
27. Kessis TD, Slerbos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L, Cho KR. Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc Natl Acad Sci USA 1993;90:3988-92.
28. Buchman VL, Chumakov PM, Ninkina NN, Samarina OP, Georiev GP. A variation in the structure of the protein-coding region of the human *P53* gene. Gene 1988;70:245-52.
29. Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV, Lane DP. *P53* mutations in colorectal cancer. Proc Natl Acad Sci USA 1990;87:7555-9.