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ARTICLE

p53 Protein Accumulation and p53 Gene Mutation in Colorectal Cancer

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Comparison of immunohistochemical methods for detection of protein p53 accumulation and molecular techniques for analysis p53 gene mutation in colorectal cancer is presented. Thirty eight patients were included: all underwent surgery without preoperative treatment. Sex of patients, tumor localisation, macro and microscopic type of cancer and staging according to Astler-Coller and Jass classifications were evaluated. Protein p53 accumulation was detected by the streptavidin-biotin method using DO-7 (Dako) antibody. The number of cells stained were classified semiquanititatively according to a scoring system: (–) no positive cells, (+) : 10-30% positive cells, (++) : 40-70% positive cells, (+++) : >70% positive cells. For all cancer samples, exons 5 to 9 of p53 gene were amplified from isolated genomic DNA. PCR products were subjected to single standed conformational polymorphism analysis. All product were also directly sequenced on ABI PRISM 377 apparatus using fluorescent dideoxyterminators chemistry. The protein p53 accumulation was detected in 53% (20/38), whereas p53 gene mutation was seen in 55% (21/38). Among them, 15 patients (39%) with overexpression showed mutation in exon 5-8 gene p53. Discrepancies between results were noted in 29%. In conclusion, the necessity of both methods – immunohistochemical and molecular – is indicated for the objective evaluation of functional and structural status of p53 gene and protein. (Pathology Oncology Research Vol 6, No 4, 275–279, 2000)

Keywords: p53 protein, p53 gene, molecular biology, immunohistochemical studies, colorectal cancer

Introduction

The p53 tumor-suppressor gene is the most commonly altered gene in human neoplasia. The abnormality of this gene plays a fundamental role in tumor development and progression.[20,23,29] It has clinical application to identify the early step of colorectal carcinogenesis. Sequential genetic mutations, including chromosome delection and loss of suppressor genes have been described by Vogelstein as a one model of carcinogenesis of colorectal cancer in the adenoma-carcinoma sequence.[14,21,28]

The p53 tumor-suppressor gene located on the short arm of chromosome 17 encodes a 53-kD nuclear phosphoprotein.The structure of p53 protein is composed of five identifable regions serving different functions.[1,9,19] Each of these domain has distinct, but inter-dependent regulation and functions. The binding domain is the main region of the p53 protein. It plays fundamental role in the interaction with DNA in a sequence-specific manner. The majority of missense mutations in colorectal cancer occurs in this region. The consequence of such an event is the loss of the ability of p53 to specifically bind DNA in a sequence-specific manner. p53 is a transcription factor which regulates cell growth and prevents cells from entering S phase. This suppressor gene is a negative regulator of the cell cycle and promotes apoptosis after DNA damage.

Missense mutations in colorectal cancer are more frequent than complete or partial deletions or other

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rearrangements of the gene.[10,11] The missense single mutation results in the replacement of the amino acid by another in the p53 protein. Mutational analyses are principally confined to exons 5-8, because the majority of p53 mutations are located in these regions.

p53 accumulation and mutation is a new biological factor with clinical signisficance. According to several studies alterations of specific oncogenes and tumor suppressor genes correlate with tumor aggressiveness and poorer survival.[4,13,25] Advances in chemo or/and radiotherapy of colorectal cancer require to select the patients according to prediction of the response to treatment.[27]

The aim of this study is to clarify the relationship between protein p53 accumulation and gene p53 mutation in a group of Polish patients.

Materials and Methods

Between June 1996 and March 1997 at the Maria Sk³odowska-Curie Memorial Cancer Centre and Institute of Oncology, Warsaw, Poland, 38 patients with colorectal cancer underwent surgery without preoperative treatment.

Tumor tissues were formalin fixed, paraffin embedded and stained with haematoxylin and eosin. The operative material was analysed macro- and microscopically. The site of localization and macroscopic type of tumor were evaluated (1 – polypoid, 2 – ulcerative and localised, 3 – ulcerative and nonlocalised, 4 – infiltrating). The microscopic examination included tumor type and grade according to World Health Organization grading system,[17] clinico-pathological staging classification according to Astler-Coller and Jass.[16]

Immunostaining Procedures

The 5 µm sections from tumor tissue with surrounding colorectal mucosa were evaluated using a immunohistochemical method. The rehydrated slides were immersed in the solution (buffer Citrate pH 6,0, molarity 0,01) and heated for 12 min in microwave (model Siemens, wattage 600W). Following rinsing in TBS pH 7,6 (Tris-Buffered Saline) endogenous peroxidases were quenched by incubation in 3% H2O2 for 5 min. Incubation with primary antibody followed rinsing with TBS. Incubation at room temperature with a 1:50 dilution of DO-7, DAKO was carried for 60 min. This antibody recognizes wild-type as well as mutated p53 protein. Following three rinses with TBS the slides were incubated with linking antibody for 30 min, followed by 30 min with steptavidin-horseradish-peroxidase (LSAB, +p/x kit) diluted as recommended by the manufacturer and then incubated for 2-5 min with the chromagen 3,3'-diaminobenzidine tetrachloride (DAB, DAKO). After each incubation samples were rinsed three times in TBS. Samples were then counterstained with haematoxylin for 1 min and nuclei blued under running water. Slides were then dehydrated and mounted. Control sections were used to replace the primary antibody with a nonrelated monoclonal antibody. As a positive control, a section of colorectal cancer with high p53 expression was used. All slides were scored according to number of cells stained in 1000 cells; none (–), weak (+, 10-30%), moderate (++, 40-70%) and intense (+++, >70%).

Molecular Analysis

SSCP of p53 gene fragments – Genomic DNA was isolated with Proteinase K/phenol-chloroform extraction. Each of exons 5 to 9 of the p53 gene was amplified (PCR) in a mixture containing 1× PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1U of Taq Polymerase, and 0.6M of each of amplification primers in final vol. of 30 µl. Forty cycles of denaturation at 94°C for 10 s, annealing for 10 s and extension at 72°C for 30 s were performed. The primers and annealing temperatures were as follows:

exon 5 5'-TGTTCACTTGTGCCCTGACT-3' and 5'-CAGCCCTGTCGTCTCTCCAG-3' (57°C),
exon 6 5'-ACAGGGCTGGTTGCCCAGGGT-3' and 5'-CTCCCAGAGACCCCAGTTGC-3' (57°C),
exon 7 5'-GGTCTCCCCAAGGCGCACTGG-3' and 5'-AGGCTGGGGCACAGCAGGCC-3' (61°C),
exon 8 5'-ATTTCCTTACTGCCTCTTGC-3' and 5'-AAGTGAATCTGAGGCATAAC-3' (52°C),
exon 9 5'-GCAGTTATGCCTCAGATTCAC-3' and 5'-AAGAAGAAAACGGCATTTTGA-3' (52°C).

For SSCP, 10 µl of PCR product was mixed with 10 µl of 0.1 M NaOH/10 mM EDTA, incubated at 50°C for 10 min, combined with 6 µl of 0.1% bromophenol blue/0.1% xylene cyanole in formamide and immediately loaded onto 10% acrylamide gel (acrylamide:bis-acrylamide 40:1) with 10% glycerol, prepared in 0.5× TBE buffer. Electrophoresis was carried out at 300 V (23 W of power) for 8 h at: 4°C and 30°C for exon 5, 18°C for exon 6, 22°C for exons 7 and 8, 4°C and 24°C for exon 9. DNA bands were visualized by silver staining.

Automated dye terminator sequencing was performed on PCR products representing blocks of exons 5-6 and 7-9 of p53 gene. The PCR reactions were carried out as above with the following primers:

block 5-6; 5'-GTTTCTTTGCTGCCGTGTTCC-3' and 5'-ATTAAGCCTCACTAAAGGGATTGCA-CATCTCATGGGGTTAT-3' (60°C),
block 7-9; 5'-ATTAACCCTCACTAAAGGGAATCTT-GGGCCTGTGTT-3' and 5'-ACGGCATTTTGAGTGTTAGACTGGA-3' (60°C).

The sequencing was performed according to manufacturer's protocol using Dye Terminator sequencing kit (Perkin Elmer) and following sequencing primers:

for block 5-6: 5'-TGTTCACTTGTGCCCTGACT-3' and
5'-CTCCCAGAGACCCCAGTTGC-3';
and amplification primers
for block 7-9: 5'-ATTTCCTTACTGCCTCTTGC-3' and
5'-AGGCTGGGGCACAGCAGGCC-3'.

Ethanol-precipitated reactions were loaded onto 5% LongRange/8 M urea 36cm-long gel and analyzed on an ABI377 automated sequencing apparatus (Perkin Elmer).

Results

Clinico-pathological Data

The colorectal cancers were obtained from 21 (55%) men and in 17 (45%) female (age range 28 to 79). Twenty seven (71%) cases were localized in the rectum and 11 (29%) in the colon. Polypoid and localized macroscopic tumor type dominated among our cases, type 1 and 2 were observed in 28 (74%) cases, type 3 and 4 in 10 (26%) cases. Cancer infiltration was limited to the bowel wall in only 6 patients: B1 – 4/38 (11%), C1 – 2/38 (5%). In the remaining 84% of tumors cancer infiltration was also seen in the adjacent adiposal tissue: B2 – 16/38 (42%), C2 – 15/38 (39%), D1 – 1/38 (3%). According to the Jass classification 2 (5%) cases were prognostic group I, 13 (34%) group II, 10 (27%) group III and 13 (34%) group IV. All 38 tumors were diagnosed microscopically as adenocarcinoma. The majority of them were moderatelly differentiated (28/38, 74%).

Table 1. **Clinico-pathological features and protein p53 accumulation in colorectal cancer**

Clinico-pathological factors	*Number of patients*	*p53 (+)*	*p53(–)*
Sex			
men	21	7	14
female	17	12	5
Localization			
colon	27	15	12
rectum	11	4	7
Macroscopic type of tumor			
1	11	4	7
2	17	11	6
3	8	3	5
4	2	1	1
Astler-Coller			
B1	4	3	1
B2	16	10	6
C1	2	2	0
C2	15	3	12
D	1	1	
Jass classification			
I	2	0	2
II	13	1	12
III	10	5	5
IV	13	7	6
Grade			
G1	7	2	5
G2	28	16	12
G3	3	1	2

Protein p53 accumulation in colorectal cancer was assessed with monoclonal antibody DO7. Nuclear staining in at least 10% of the tumor cells was categorized as a positive reaction. Immunoreactivity in normal colorectal mucosa was seen only in one case. p53 overexpression was found in 19/38 (50%) cancer cases. The majority of these were female patients (12/17, 70%) compared to male patients (7/21, 33%).

The relationships between p53 expression and clinico-pathological staging were evaluated. Protein p53 accumulation was seen only in one patient from group II according to Jass classification compared to 5 of 10 (50%) from the third group and 7 of 13 (54%) from the fourth group. We noted also that p53 overexpression dominated colorectal cancer without metastases (13/20, 66%) – B1 and B2 stage according to Astler-Coller classification comparing to cases with metastases (6/16, 34%) – C1, C2 and D stage.

The relationships between protein p53 accumulation and clinico-pathological data are summarized in *Table 1*.

Molecular Analysis

SSCP analysis revealed the presence of mutations in 21 of 38 cases. All mutations were confirmed by direct sequencing.

The protein p53 accumulation was detected in 53% (20/38), whereas p53 gene mutation was seen in 55% (21/38). Among them, 15 patients (39%) with p53 overexpression showed mutation in exon 5-8 gene p53. Discrepancies between these results were noted in 29% (11/38). *Table 2* presents data of p53 accumulation and mutation of this gene.

Discussion

Alterations of the p53 gene can be evaluated by DNA sequence analysis, immunohistochemistry to assess protein p53 accumulation and by allelic loss at 17p. Although each of these methods gives the information of gene abnormality, they all have limitations.

Molecular analysis is a cumbersome, time-consuming and difficult method on archival material. In contrast,

Table 2. **Correlation between protein p53 accumulation and p53 mutation**

	p53 accumulation + 20/38 (53%)	*p53 accumulation – 18/38 (47%)*
p53 mutation + 21/38 (55%)	15/38 (39%)	6/38 (16%)
p53 mutation – 17/38 (45%)	5/38 (13%)	12/38 (32%)

immunohistochemical methods are cheaper, less labor intensive and more familiar to pathologist as a standard procedure in routine diagnosis. The major problem with evaluation of protein p53 accumulation arises from fixation of material, choice of methods, sensitivity and specificity for different antibodies as well as interpretation of the results.[12,18] The immunohistochemical detection of p53 protein varies considerably, depending on the methods and antibodies used.

In the present study we examined the expression of p53 protein in tissues fixed in formalin and embedded in paraffin wax. For immunohistochemistry the DO-7 commercial monoclonal antibody was used following microwave irradiation of the tissues in an antigen retrieval system. This method has been applied in several other studies.[6,24,25]

Wild-type p53 (non mutated) has a short half-life of about 15 min[22] and is turned over rapidly by an ATP-ubiquitin degradation pathway. Mutant p53 protein has a greater stability with half-life prolonged up to 20 h in some cases and accumulates in the nuclei of cells. It can be detected immunohistochemically using monoclonal or polyclonal antibodies.

Immunohistochemical detection is determined by the level of the antigen, affinity and dilution of the antibody and sensitivity of the detection system. In this study, immunoreactive p53 was detected by the labelled Streptavidin-biotin method and the DO-7 (Dako) antibody described previously.[25]

Several immunohistochemical studies have evaluated p53 overexpression in colorectal cancer and other tumors.[15,26] They applied similar methods, although different antibodies for p53 were used. There are only a few studies, which correlate the frequency of p53 mutation detected by SSCP with p53 overexpression analysed using immunohistochemical methods on the same series of colorectal carcinoma specimens.[6,8,25] Protein p53 accumulation has been found in 24-72% colorectal cancer and mutation of p53 gene occurs in approximately 50% colorectal cancers. Our results are similar, p53 overexpression was found in 53% (20/38) and point mutation in 55% (21/38) cases. The relationship between mutation of p53 gene and p53 overexpression is not direct. In the present study in 71% cases (27/38) we observed concordance between immunohistochemistry and SSCP; among them 39% cases (15/38) were immunopositive tumors that had mutation in exons 5-9. Other studies showed comparable results, Smith found concordance of 61% and 71% for monoclonal antibodies Pab1801 and Pab240 respectively,[25] Dix reported corcordance 69% with monoclonal antibody DO7.[8]

Discordance in our study was observed in 29%. Accumulation of p53 protein without mutation was observed in 20% in other papers (2,3, 6,7). Mutation of p53 gene without immunopositivity was seen in 10% in other (2,6) and 16% (6/38) in the present study.

The major discrepancies between p53 gene mutation and protein p53 accumulation concern cases with high protein overexpression and without mutation of p53 gene. The immunohistochemical reaction dose not always correlate with mutation of p53 gene. The accumulation of p53 protein could be induced by a number of causes, such as interaction with other proteins or inhibition of degradation.[25,29] In immunopositive and SSCP- negative tumors p53 protein may accumulate in the absence of gene mutation by forming complexes with other molecules, such as viral oncoproteins; human papilloma virus E6 protein in cervical carcinomas, adenovirus E1B protein, the simian virus 40 large T antigen. SSCP positive and immunonegative tumors, often exhibit nonsense mutations that destabilize p53 protein.

We have previously demonstrated an accumulation of p53 protein in 75% of early colorectal cancer (21). In the present study p53 overexpression was found in 66% of cases without metastases (B1, B2) and in 34% of cases with metastases (C1, C2, D). We agree[21] with Valentinni,[28] that immunohistochemistry is a suitable method for routine clinical applications only for the detection of mutation in the early step of colorectal carcinogenesis.

In conclusion, p53 overexpression and mutation of p53 gene show concordance in 75% of cases. The parallel usage of both methods – immunohistochemical and molecular – is required for the proper evaluation of functional and structural status of p53 protein and gene.

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