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New Mutations in the Human *p53* Gene – a Regulator of the Cell Cycle and Carcinogenesis

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Abstract—Mutations in the tumor suppressor gene *p53* often lead to disarrangement of the cell cycle and of genetic integrity control of cells that may contribute to tumor development. We studied *p53* gene mutations in 26 primary tumors of colorectal cancer patients. Mutations in *p53* were found in 17 tumors (65.4%). All point mutations affected the DNA binding domain of p53 and were localized in exons 4-8 of the gene. Mutant p53 isoforms with altered domain structure and/or with alternative C-terminus arising from frameshift mutations or abnormal splicing were found in six tumors. Mutations Leu111Gln and Ser127Phe were shown in colorectal cancer for the first time. Isoforms p53-305 with C_4 insertion in codons 300/301 and p53i9* including an additional 44 nucleotides of the 3′-end of intron 9 were discovered for the first time. Mutations of *p53* were associated with lymph node metastases and III/IV stage of tumors that are signs of unfavorable prognosis in colorectal cancer.

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Key words: *p53*, tumor suppressor gene, mutations, isoforms, alternative C-terminus, polymorphism, colorectal cancer

The *p53* gene is among the most intensively studied genes in molecular oncology because its product plays a key role in regulation of cell cycle, apoptosis, control of genome integrity, and many important processes that run in cells (see reviews [1-4]). Mutations in the *p53* gene lead to disarrangements of these processes and assist cells with defective control of division to survive. This may contribute to development of tumors, particularly human colorectal cancer (CRC) [5, 6]. Thirty to sixty percent of CRC were reported to contain mutations in the *p53* gene [5]. All data on p53 mutation are accumulated in the IARC TP53 database (http://www-p53.iarc.fr/) and UMD p53 Mutations Database (http://www.umd.be:2072/).

Most p53 mutations are concentrated in evolutionarily conserved regions (ECR) [7-9] and mutational hotspots that reside in the DNA binding domain of p53. However, one may find mutations also in other p53 domains, which are responsible for transactivation, tetramerization, nuclear export, or other important functions of the protein [10]. Normal p53 can exist as several isoforms that arise because of alternative splicing [11-13] or transcription from internal promoter in intron 4 [12]. The p53 isoforms may lack some functions of the major protein, i.e. DNA binding activity [12, 14]. At the same time, some p53 isoforms regulate their own transcriptional activity [12] and cooperate with isoforms of relative proteins p63 and p73 in delicate regulation of cell differentiation and response to stress [13]. New p53 isoforms can also arise due to mutations [15, 16]. In particular, mutations can result in loss of the C-terminus of the p53 molecule [17]. The C-terminus is important for autoregulation of p53 and for cooperation between p53, p63, and p73 isoforms [18-20], as well as for modifying of effects of p53 point mutations [21].

Correlations of p53 mutations with advanced tumor stage, metastases, distal or rectal localization of tumors, and with unfavorable prognosis in CRC have been demon-

Abbreviations: CRC) human colorectal cancer; ECR) evolutionary conserved region; n.) nucleotide; Ti) transition; Tv) transversion; TNM) classification of malignant tumors by International Union Against Cancer, 2002.

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strated in a number of studies [6, 22]. Tumors without p53 mutations were reported to respond to radio- and chemotherapy better than tumors with p53 mutations [5, 22], apparently because of more resistance of the latter to DNA damage. In spite of extensive research, the clinical significance of p53 mutation analysis remains uncertain.

In present work, we addressed again the problem of p53 mutations and polymorphism in CRC. To ensure that mutations are expressed at the RNA level, we sequenced cDNA of *p53* through exons 2-10, and introns from genomic DNA in some cases to reveal the cause of a mutation.

MATERIALS AND METHODS

Tumors. Tumor samples were collected during resection from colorectal cancer patients and were provided by 24th Municipal Hospital (Moscow, Russia) and Blokhin Cancer Research Center (Moscow, Russia). Samples were immediately dipped into RNAlater (Qiagen, USA), kept at 4°C overnight, and then stored at −20°C. Tumors were staged and classified according to clinical signs and histological analysis.

RNA isolation and p53 sequencing. RNAs were isolated from tumor tissues using TriReagent (Sigma, USA) with quality control by spectrometry and agarose electrophoresis, and then precipitated and stored in 80% ethanol with 0.3 M sodium acetate at −80°C. cDNA was prepared from 1 μg of RNA by Reverse Transcription system with random primers (Promega, USA) using the Promega manual. *p53* cDNA was amplified in four overlapping fragments that covered exons 2-10 (Fig. 1). The first fragment included exons 2 and 3 and was amplified with primers (all written from 5′ to 3′ ends) F1 (TGC TTT CCA CGA CGG TGA CAC) and R1 (CAG TTG GCA AAA CAT CTT GTT GAG G). The second fragment included exons 5 and 6 and was amplified with primers F2 (TAC CAG GGC AGC TAC GGT TTC) and R2 (GCC GCC CAT GCA GGA ACT GT). The third fragment covered exons 7, 8, and 9 and was amplified with primers F3 (TGG CCC CTC CTC AGC ATC TTA) and R3 (CAA GGC CTC ATT CAG CTC TCG). The fourth fragment included exons 9 and 10 and was amplified with primers F4 (CGG CGC ACA GAG GAA GAG AAT C) and R4 (CTG ACG CAC ACC TAT TGC AAG C). All PCRs were performed in 50-μl volumes using DNA amplification kit with Taq-polymerase (Sileks, Russia) and amplifier Mastercycler personal (Eppendorf, Germany) with a standard protocol: 95°C preheating, 2 min; 30 cycles of amplification (95°C, 30 sec; 64°C, 1 min; 72°C; 1 min), and final elongation at 72°C, 5 min. PCR products were purified by agarose gel electrophoresis and DNA purification columns (Helicon, Russia).

Purified DNA fragments were sequenced from nested primers in both directions. The first fragments were sequenced with primers SF1 (GAC GGT GAC ACG CTT CCC TGG AT) and SR1 (TCT TGT TGA GGG CAG GGG AGT AC). The second fragments were sequenced with primers SF2 (GGT TTC CGT CTG GGC TTC TTG) and SR2 (CAC ATG TAG TTG TAG TGG ATG GTG GT). Sequencing primers for the third fragments were as follows: SF3 (ATC CGA GTG GAA GGA AAT TTG CGT) and SR3 (CTC ATT CAG CTC TCG GAA CAT CTC). The fourth fragments were sequenced with primers SF4 (ACA GAG GAA GAG AAT CTC CGC AAG) and SR4 (CCT ATT GCA AGC AAG GGT TCA AAG A).

Intron 3 of the *p53* gene was amplified with primers i3F (GAA GCG AAA ATT CAT GGG ACT) i3R (GAA CCA TTG TTC AAT ATC GTC) and sequenced with primers i3SF (GGG ACT GAC TTT CTG CTC TTG TCT) and i3SR (CGG GGA CAG CAT CAA ATC ATC CAT T). Intron 4 was amplified as a single 957-bp fragment with primers F2 (see above) and i4R (TCA TGT GCT GTG ACT GCT TGT AGA TG). Sequencing of intron 4 amplicon was carried out with four primers: two forward, SF2 (see above) and i4SF (AAG ACC AGC CTG GGT AAC ATG ATG); and two reverse, i4SR1 (TAG ATG GCC ATG GCG CGG A) and i4SR2 (ATC ATG TTA CCC AGG CTG GTC TTG A).

Sequencing was performed using ABI PRISM® BigDye™ Terminator v.3.1 reagents kit (Applied Biosystems, USA) with subsequent product analysis by an automated ABI PRISM 3100-Avant DNA sequencer (Applied Biosystems). Analysis of chromatograms, assembly and alignment of sequences were performed using Vector NTI v.10 program (Invitrogen, USA). Experimental sequences were aligned to canonical ones of *p53* mRNA (ac. No. NM_000546) and *p53* gene (ac. No. X54156) from NCBI GenBank.

Statistics. The tumors were grouped by presence and type of *p53* mutations and the groups were collated by the following clinical signs: tumor stage (I-IV), TNM classification, size, growth pattern, depth of invasion, level of histological differentiation, and by sex and age of the patients. Statistical analysis was performed using Statistica 5.5 program (StatSoft Inc., USA). χ^2 criterion and one-sided Fisher exact test (P_1 for groups including less than three patients) were used for group comparison.

RESULTS

We analyzed tumor tissue samples from 26 Caucasian colorectal cancer patients older than 35 years of age. We amplified and sequenced p53 cDNA (exons 2-10) from these tumors. Amplification and sequencing tactics are summarized in Fig. 1a. Sequencing results and some clinical signs of the tumors are shown in Table 1. Total percentage of tumors with p53 mutations that altered coding sequence was 65.4% (17/26). The *p53* from tumor CC222

Table 1. Mutations of *p53* in colorectal tumors

Patient No.	Age, years	Sex	TNM	Stage	Mutation[1]	Type of mutation	Codon	ECR	Exon	Domain affected	Alternative C-terminus, aa	Codon 72
CC222	63	m	T4N0M0	II	CCT (Pro)→CCC (Pro) (WT)	Ti (C→T)	295	–	8	–	–	Arg/Arg
CC351	55	m	T4N0M1	IV	WT	–	–	–	–	–	–	Pro/Pro
CC474	n.d.	n.d.	n.d.	n.d.	WT	–	–	–	–	–	–	Arg/Arg
CC684	73	m	T4N0M0	II	WT	–	–	–	–	–	–	Arg/Pro
CC712	63	m	Villiferous	—	WT	–	–	–	–	–	–	Arg/Pro
CC741	60	m	T4N0M0	II	WT	–	–	–	–	–	–	Arg/Arg
CC931	73	f	T4N0M0	II	WT	–	–	–	–	–	–	Arg/Arg
CC965	86	f	T3NxMx	II	WT	–	–	–	–	–	–	Arg/Pro
CC1105	67	f	T4N0M0	II	WT	–	–	–	–	–	–	Arg/Arg
CC197	65	f	T3N0M0	II	CGG→CAG (Arg→Gln)	Ti (G→A)	248	IV	7	DBD	–	Arg/Arg
CC220	72	m	T3N0M0	II	CGG→CAG (Arg→Gln)	Ti (G→A)	248	IV	7	DBD	–	Arg/Arg
CC223	n.d.	n.d.	n.d.	n.d.	TGC→TAC (Cys→Tyr)	Ti (G→A)	176	III	5	DBD	–	Pro/Pro
CC299	74	f	T4N2M1	IV	del(C)[2]; frameshift	del	176-178	III	5	see Fig. 2b	68	Arg/Arg
CC692	73	m	T2N0M0	I	ins(C_4)[3]; frameshift	ins	300/301	after V	8	see Fig. 2c	4	Arg/Arg
CC694	76	f	T4N2M1	IV	del(nn. 14081-14089)	del	del (cc. 252-254)	IV	7	DBD; see Fig. 2d	–	Arg/Arg
CC697	52	m	T4N0M0	II	del(C)[4]; frameshift	del	300/301	after V	8	see Fig. 2e	43	Arg/Pro
CC702	50	f	T4NxMx	III	CGT→TGT (Arg→Cys)	Ti (C→T)	273	V	8	DBD	–	(Arg)/Pro[5]
CC747	74	f	T3N1M0	III	GGC→AGC (Gly→Ser)	Ti (G→A)	245	IV	7	DBD	–	Arg/Arg
CC773	57	f	T4N2M0	III	CTG→CAG (Leu→Gln)	Tv (T→A)	111	before II	4	DBD	–	Arg/Arg
CC780	35	f	n.d.	n.d.	TGC→TTC (Cys→Phe)	Tv (G→T)	135	II	5	DBD	–	Arg/Arg
CC862	61	f	T4N1M1	IV	del(nn. 11982-12032) (Δe4)	del	del (cc. 33-125)	II	intron 3	see Fig. 2f	–	0/0
CC950	66	f	T2N0M0	II	TCC→TTC (Ser→Phe)	Ti (C→T)	127	II	5	DBD	–	Arg/Arg
CC1011	70	m	T4N1M0	III	CGC→CAC (Arg→His)	Ti (G→A)	175	III	5	DBD	–	Arg/Arg
CC1103	72	m	T4N1M0	III	CGC→CAC (Arg→His)	Ti (G→A)	175	III	5	DBD	–	Arg/Arg
CC1114	64	m	T4N3M0	III	A→G (n. 17570); frameshift	Ti (A→G)	–	–	intron 9	see Fig. 2g	27	Arg/Arg
CC1149	76	f	n.d.	n.d.	CGT→CAT (Arg→His)	Ti (G→A)	273	V	8	DBD	–	Pro/Pro

Note: aa, amino acid residues; WT, wild type protein; n.d., no data; DBD, DNA binding domain; Ti, transition; Tv, transversion.

[1] Nucleotide numbers correspond to NCBI GenBank Ac. No. X54156.

[2] Deletion of C in C_5 (nn. 13207-13211).

[3] Insertion of C_4 in C_5 (nn. 14567-14571).

[4] Deletion of C in C_5 (nn. 14567-14571).

[5] Partial loss of heterozygosity (Arg allele).

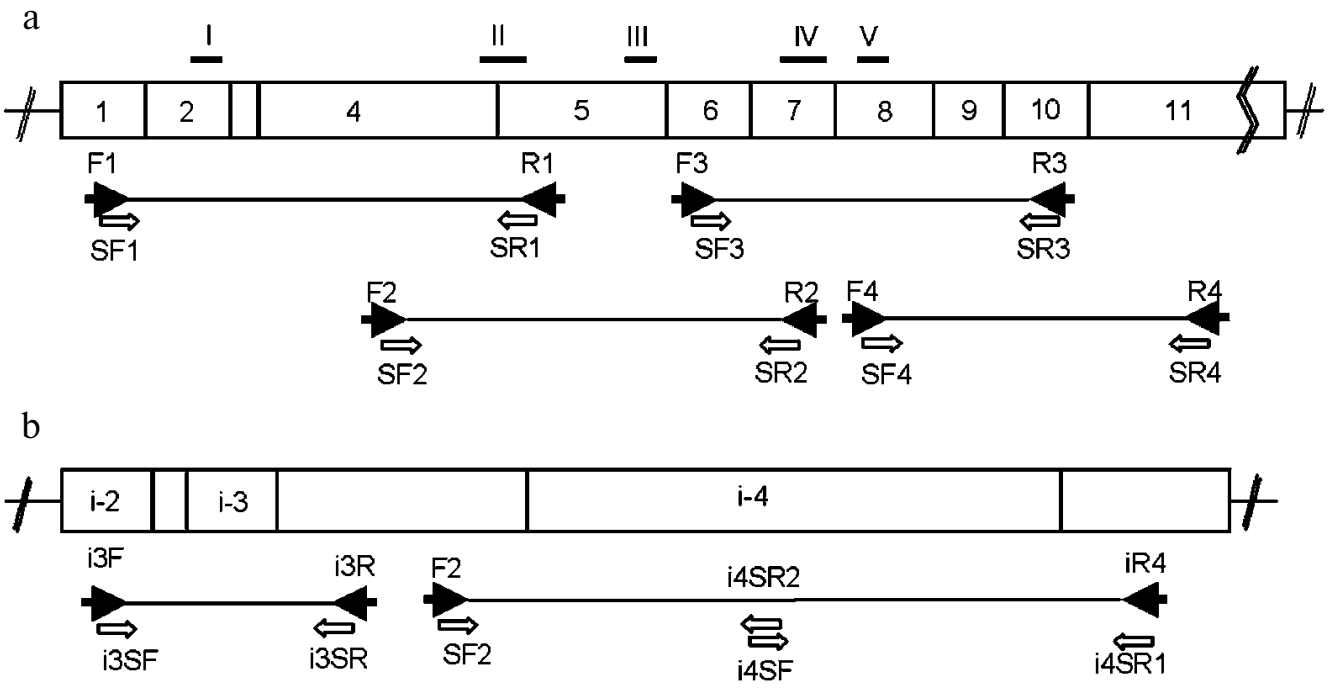


Fig. 1. Tactics of amplification (primers are shown as solid arrows) and sequencing (white arrows; all not in scale). a) Sequencing tactics of *p53* cDNA. Exons are designated as Arabic numerals and conserved regions as Roman ones. b) Sequencing tactics of introns 3 and 4 (i-3, i-4) of *p53* gene.

with silent point mutation in codon 295 was considered as wild type when calculating correlations with clinical signs but was taken into account when calculating number of mutations. Sixty-one percent of all mutations were transitions (seven G→A, three C→T, and one A→G), 11% transversions (one T→A and one G→T), and 22% (in four tumors) were deletions. One case was insertion. Transition/transversion ratio (Ti/Tv) was 5.5; this value lies between the 2.3 and 9.5 limits reported for colorectal cancer and is not far from averaged Ti/Tv index [23]. Mutations that altered functional domains of p53 were found in six tumors. Putative structures of mutant p53 from these tumors are depicted in Fig. 2; ECRs I-V (amino acids 13-23, 117-142, 171-181, 234-258, and 270-286, correspondingly) [7-9] and functional domains of p53 [10] are marked according to published data. In four of these tumors (CC299, CC692, CC697, CC1114), p53 had aberrant C-terminal domains resulting from frameshift (Table 2 and Fig. 2), which theoretically could result in loss or gain of function [24].

In tumor CC1114 mutant isoform p53i9* was found by both sequencing and agarose gel electrophoresis of RT-PCR product (Fig. 3). This isoform arose from mutation A→G (n. 17570) in the acceptor splicing site of intron 9 of the *p53* gene (Fig. 3, nucleotide numbers are denoted according to canonical *p53* gene sequence No. X54156 from NCBI GenBank). Due to this mutation, the normal acceptor splicing site was damaged and alternative

Table 2. Sequences of alternative C-termini of four mutant p53 isoforms

Patient No.	p53 isoform[1]	Amino acid number[2]	C-Terminus sequence
CC299	p53-254	178-245	TMSAAQIAMVWPLLSILSEWKEICVWSIWMTETLFDIVWWCPMSRLR-LALTVPPSTTTTCVTVPAWAA
CC692	p53-305	302-305	PREH
CC697	p53-342	301-342	QGALSEHCPTTPAPLPSQRRNHWMENISPFRSVGVSASRCSES
CC1114	p53i9*	332-358	VLCIYLLLPLLCCCGSVGVSASRCSES

[1] Structures of mutant isoforms are shown at Fig. 2.
[2] Additional amino acid numbers are denoted.

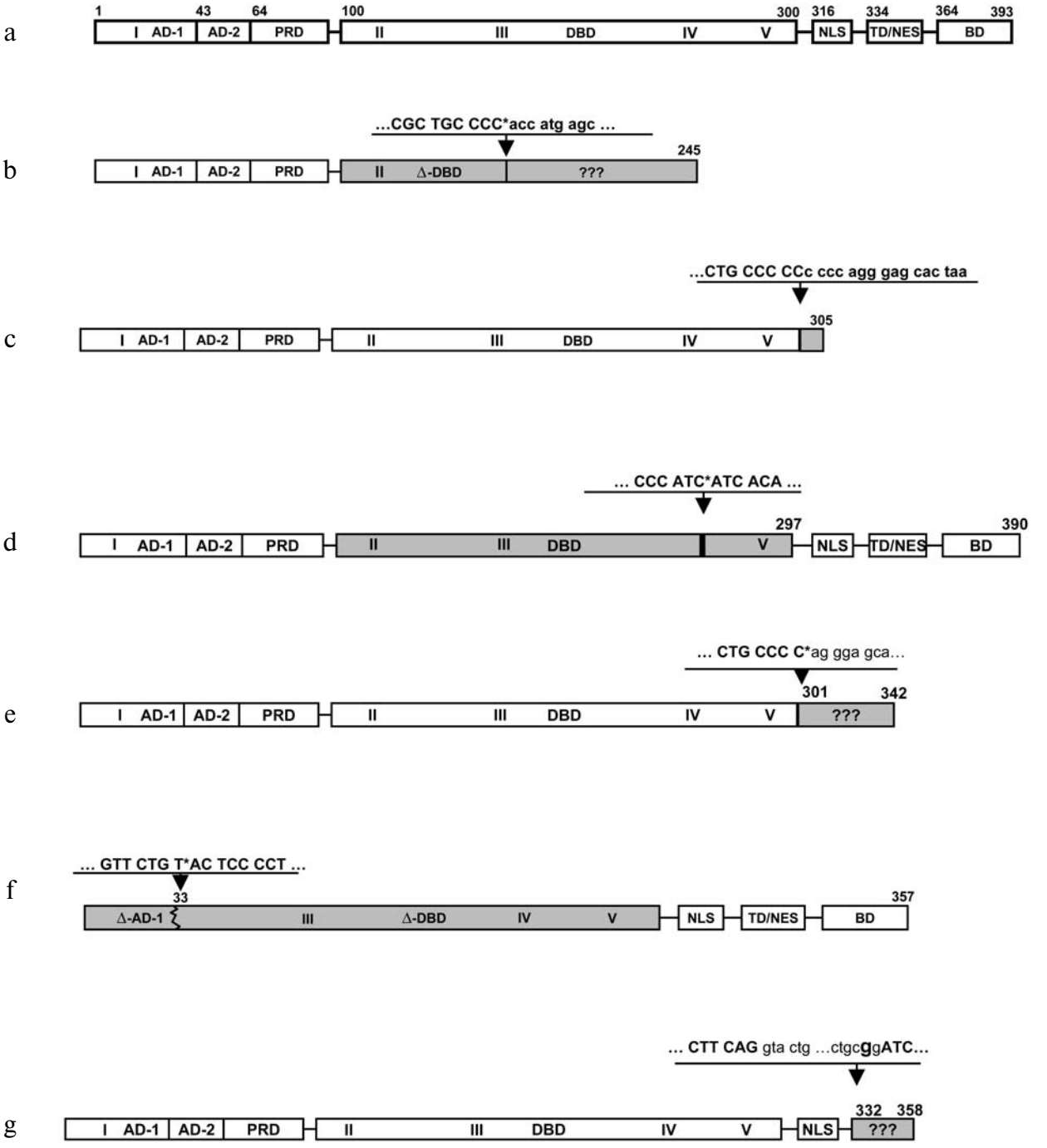
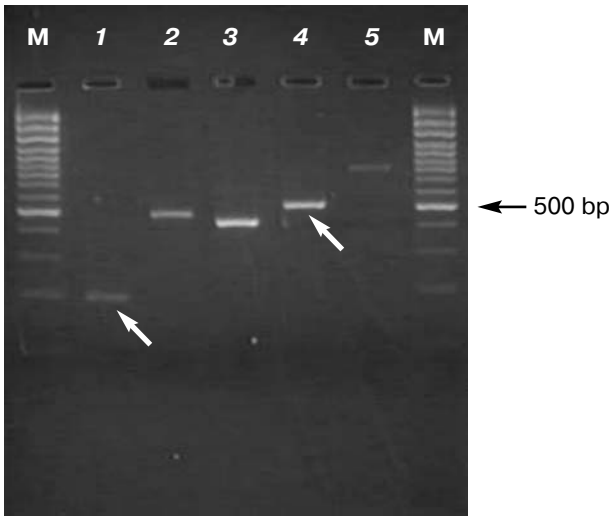
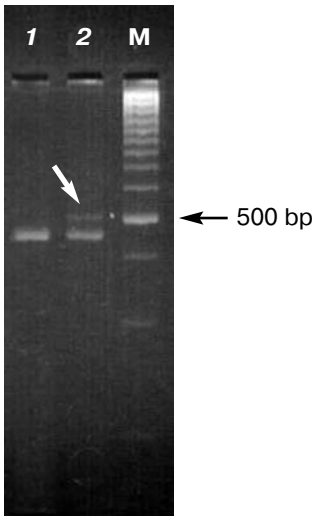


Fig. 2. Wild type p53 and putative mutant p53 isoforms. Altered domains are shown in gray. a) Wild type p53 monomer (393 amino acids). Functional domains are designated as in [10]. Evolutionarily conserved regions (ECRs) are designated as in [7-9]. b) Mutant p53 isoform from tumor CC299. Deletion of C in codons 176-178 (shown by asterisk) resulted in frameshift and arising of an 68 amino acid length aberrant C-terminus beginning from 178 codon (small letters). c) Mutant p53 isoform from tumor CC692 has abnormal four amino acids at the C-terminus resulting from C_4 insertion in C_5 track of codons 300 or 301. Domains NLS, TD/NES, and BD have been lost. d) Mutant p53 isoform from tumor CC694 lost codons 252-254 (ECR IV) due to in frame deletion of nine bases and then has defective DNA-binding domain. Asterisk shows a deletion point. e) Mutant p53 isoform from tumor CC697. Deletion of C in codon 300 or 301 (shown by asterisk) resulted in frameshift and formation of aberrant C-terminus of the protein. f) Mutant p53 isoform from tumor CC862 has aberrant AD-1 and DBD domains resulting from deletion of exon 4 (detailed in text and Fig. 3). AD-2 and most of ECR II have been lost, but other ECRs have been retained. Asterisk shows a position of deleted exon 4. g) Mutant p53 isoform from tumor CC1114. Point mutation A→G in splice acceptor site of intron 9 (lower case bold letters) led to including of part of intron 9 (lower case letters) in mRNA and formation of aberrant C-terminus of the protein.

a



b



c

Contig_862	150	…ACAACGTTCTGT----------	162	----------ACTCCCCTGCCC…
NM_000546	337	…ACAACGTTCTGTCCCCCTTGCCGTCCCAA…	615	…GTGACTTGCACGTACTCCCCTGCCC…

d

862_i3	52	…CTGGGGGGCTG---------- ----------GTCCCAAGCAAT…
X54156	11970	…CTGGGGGGCTGAGGACCTGGTCCTCTGACTGCTCTTTTCACCCATCTACAG*TCCCCCTTGCCGTCCCAAGCAAT…

e

Contig1114_i9*	1046	…TTCACCCTTCAGGTACTGTGTATATACTTACTTCTCCCCCTCCTCTGTTGCTGCGGATCCGTGGGCGTG…
NM_000546	1233	…TTCACCCTTCAG----------ATCCGTGGGCGTG…

f

```
********************** NetGene2 v. 2.4 ************************

Acceptor splice sites, direct strand
------------------------------------
               pos 5'→3'  phase strand  confidence  5'    intron exon      3'

                 15601       0     +        0.16       GCCTCCCCAG^TAGCTAGGAC
                 15604       0     +        0.25       TCCCCAGTAG^CTAGGACCAC
                 17527       1     +        0.43       TTTAACTCAG^GTACTGTGAA
                 17571       0     +        1.00       GTTGCTGCAG^ATCCGTGGGC
```

Fig. 3. Splicing p53 isoforms in tumors CC862 and CC1114. a) Identification of p53Δe4. *1*, *4*) RT-PCR of RNA from tumor CC862 with primers F1/R1 (*1*) and F1/R2 (*4*); *2*, *3*, *5*) RT-PCR of RNA from tumor CC1105 (wild type *p53*) with primers F1/R1 (*2*), F2/R2 (*3*), and F1/R2 (*5*). M, molecular weight marker 100 bp ladder plus (Fermentas, Lithuania). Electrophoresis of RT-PCR products in agarose gel. Products of RT-PCR originated from mutant p53 isoforms are shown by arrows. b) Identification of p53i9*. RT-PCR or RNA from tumors CC1105 (*1*) (wild type *p53*) and CC1114 (*2*) with primers SF3/SR3. c) Alignment of *p53* cDNA sequence from tumor CC862 with canonical *p53* mRNA sequence NM_000546. Deleted sequence (CC862) corresponds to exon 4 of *p53* gene. d) Alignment of intron 3 sequence of *p53* from tumor CC862 with canonical sequence X54156. Asterisk shows splicing point at the border of intron 3 and exon 4 in X54156 sequence. e) Alignment of cDNA sequence of *p53* from tumor CC1114 and normal *p53* mRNA NM_000546; 44 nucleotides of alternative exon 9 of mutant p53i9* isoform correspond to 3′-end part of intron 9. Mutation in n. 17570 (by X54156) is designated. f) Analysis of intron 9 sequence of wild type *p53* gene by means of NetGene2 program. Nucleotides 17536 and 17570 are shown by bold font. A→T change (n. 17536, rs2856754 polymorphism) does not affect splicing site prediction. A→G change (n. 17570, as in *p53* isoform from tumor CC1114) results in loss of normal splicing site (n. 17571) and in use of alternative site (n. 17527).

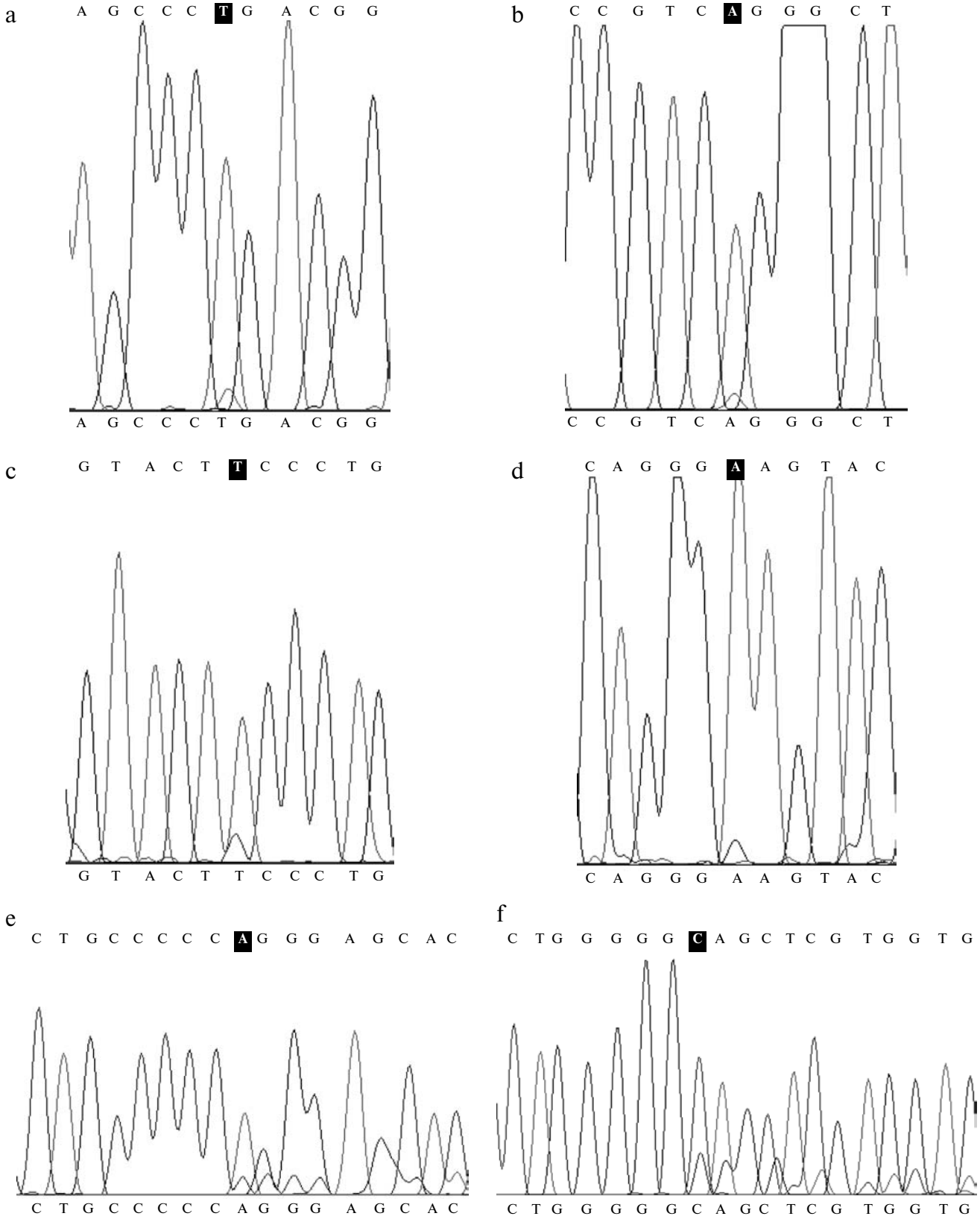


Fig. 4. Identification of new p53 mutations on sequencing chromatograms: a, b) Leu111Gln (CTG→CAG) mutation, tumor CC773. Only a small part of the tumor tissue sample contains this mutation; c, d) Ser127Phe (TCC→TTC) mutation, tumor CC950. Most of the tumor tissue sample contains this mutation; e, f) C_4 insertion in C_5-stretch of codons 300 and 301 (CC692); a, c, e) sense (coding) strands; b, d, f) antisense strands.

Table 3. Association of *p53* mutations with tumor stage and with lymph node metastases (*N*)

p53 type	Stages 0, I, II	Stages III, IV	Portion of tumors with stage ≥III	$N = 0$	$N \geq 1$	Portion of tumors with $N \geq 1$
WT	7	1	12.5%	7	0	0%
Mutant	5	9	64.3%	5	8	61.5%
P_1			0.026			0.015

Note: $N = 0$ means the absence of metastases; $N \geq 1$ means one or more metastases in lymph nodes.

splicing site began to work; so new *p53* isoform mRNA included 44 nucleotides of the 3′-end part of intron 9. Loss of acceptor splicing site because of mutation A→G (n. 17570) can be displayed by analysis of *p53* gene sequence by the program NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/) [25, 26] (Fig. 3). Known p53 polymorphism rs2856754 in nucleotide 17536 (homozygous T, see NCBI SNP) was found in CC1114 also; this nucleotide was included in p53i9* mRNA.

A mixture of normal and mutant *p53* transcripts was detected in most tumors. However, tumor CC862 contained a single shortened transcript that lost exon 4 (and primer F2 annealing site) with reading frame preserved (Figs. 2 and 3). Therefore, we amplified p53 cDNA corresponding to the N-terminus of the protein with F1/R2 primers and sequenced with SF1/SR2 primers. To reveal the cause of exon 4 loss, we sequenced introns 3 and 4 of the *p53* gene from tumor CC862 (Figs. 1b and 2d). Deletion of nucleotides 11982-12032 covering 40 bases of intron 3 including the acceptor splicing site along with 11 nucleotides of exon 4 was detected in this sample. This deletion apparently resulted in exon 4 loss from the final transcript. Several insignificant single nucleotide variations inside introns 3 and 4 of the *p53* gene from CC862 were found also, but they must not be realized at the protein level (data not shown).

We found mutations in p53 that were discovered in tumors of other tissues but shown first in CRC in two tumors. We made such conclusion based on the UMD p53 Mutations Database and on our own analysis of the literature. These mutations are Leu111Gln (CTG→CAG) in tumor CC773 and Ser127Phe (TCC→TTC) in tumor CC950. One other mutation, namely C_4 insertion in codons 300/301, was found in *p53* mRNA from tumor CC692. This p53 mutation seems to be discovered for the first time in human tumors (Figs. 2c and 4e). The presence of mutations in p53 was associated with lymph node metastases ($N \geq 1$ by TNM classification; $P_1 = 0.010$) and III/IV tumor stage ($P_1 = 0.026$; Table 3). Results of statistical tests by individual p53 mutations and by types of mutations were insignificant. The p53 allele loss of heterozygosity (LOH) was found in a single tumor, CC702, which was heterozygous by codon 72. All other samples were homozygous by codon 72 or had equal proportion of alleles. Unlike polymorphism, mutations were uninformative for LOH determination due to different content of normal tissue in tumor samples.

The frequency of codon 72 Pro allele (11/50, or 22%) in our sample was very close to the frequency that was published for healthy Caucasians (220/1080, or 20%; $\chi^2 = 0.01$; $p = 0.92$ [27]). We did not find any association between codon 72 polymorphism and presence of mutations in the *p53* gene.

DISCUSSION

The tactics of sequencing used in our work were similar to those used in [28], but the primers were redesigned completely. Unlike [28], we sequenced every RT-PCR product from nested primers in both directions. New p53 mutations found in tumor samples CC773, CC950, CC692, and CC1114 as well as all ambiguous sequencing data were checked by sequencing of products of independent PCRs. The total fraction of p53 mutations that must change protein sequence was 65.4%. This value somewhat exceeds results of other authors who used only sequencing of *p53* cDNA, but is close to data of researchers that used immunohistochemical detection of p53 mutations [5]. This may be because of our tumor sample peculiarities (modest number of samples; invasion through all bowel tissue layers and distal localization of most tumors) and of sequencing tactics that allowed us to ensure a mutation presence in questionable cases.

All mutations except mutations in introns were localized in exons 4-8. The most frequent type of mutation was transition, and G→A was the most frequent of these. Almost all mutations affected the DNA binding domain of p53 (Table 1), but several mutations must result in gross rearrangements of p53 domains and in appearance of abnormal p53 isoforms (Fig. 2). Totally, 11 missense point mutations were found. Two of them are known for different types of cancers and are included in IARC (R10) and UMD (2005) p53 mutation databases, but were found first in CRC by us. These are Leu111Gln and Ser127Phe. Both these mutations are localized in the

DNA-binding domain of p53 but do not contact with DNA directly (see 3D-analysis on IARC p53 Mutation Database Web site). The two mutations are known to reduce the ability of p53 to transactivate p53 target genes dramatically [29], so it may be supposed that amino acids 111 and 127 are critical for the structure of p53 DNA binding domain formation. Mutations that must alter domain structure of p53 were found in six tumors (Fig. 2 and Table 1). Two of these were in frame deletions that must result in the loss of functional sites inside of p53 protein (CC862 and CC694), and the four others were frameshift mutations (CC299, CC692, CC697, CC1114).

Interestingly, *p53* mRNA with complete loss of exon 4 sequence corresponded to a single transcript in tumor CC862 and was similar to p53 mutation in Li–Fraumeni syndrome that has been described [16]. The cause of exon 4 loss in *p53* from CC862 was deletion of 51 nucleotides including the splicing acceptor site of intron 3 (Fig. 3). We were not able to determine whether this mutation occurred in germline because pairwise normal tissue from the patient was not available. All other tumor samples contained admixture of wild type *p53* transcripts.

Mutant p53 mRNA from tumor CC1114 included a 44 nucleotide length fragment of intron 9, and this must lead to formation of p53i9* isoform with alternative C-terminus. Intron 9 and some other parts of *p53* transcript are known to be spliced alternatively in human lymphocytes and some other tissues [12, 14, 30]. Mature *p53* mRNA may include different parts of intron 9 of the gene, so several minor p53 isoforms may be translated. Existence of alternative splicing sites in intron 9 of the *p53* gene can be predicted by means of the NetGene2 program (Fig. 3f). A→G (n. 17570) nucleotide substitution, which was found in the *p53* gene from CC1114, must result in loss of the normal acceptor splicing site and in use of alternative splicing site, and we observed this experimentally. Since p53i9* arose due to mutation we may presume it to be constitutive (transcribing constantly) in tumor cells in contrast to normal cells, where the spectrum of p53 isoforms varies depending on tissues and conditions [12, 14].

A C_4 insertion in the C_5 track of codons 300/301 was found in the *p53* gene from tumor CC692. This frameshift mutation is found for the first time in human tumors. Because of the mutation, p53 must lose tetramerization and basic C-terminal domains as well as signals of nuclear localization and nuclear export, but acquire four additional amino acids (p53-305; Fig. 2c and Table 2).

In all, we found four frameshift p53 mutations in tumors CC299, CC692, CC697, and CC1114 (23.5% of the total number of mutations). These frameshifts must result in loss of normal C-terminal domains of the protein and in formation of p53 isoforms with alternative C-terminus.

The C-terminus of the p53 molecule is known to be responsible for the protein tetramerization and nuclear localization, and for such important functions as cell growth arrest, apoptosis, and autoregulation by means of MDM-2 [10, 18-21]. Loss of the C-terminus must result in impairment of these functions of p53. As an example, p53i9 [14] from human lymphocytes is defective in transcription and devoid of DNA binding activity. While normal p53 is a nuclear protein, p53β and p53γ [12] can be localized in cytoplasm also; p53β and mutant p53 isoform from neuroblastoma cells IGR-NB8 lacking 67 C-terminal amino acids were defective in activation of p21/WAF1 promoter [12, 17]. At the same time, the alternative C-terminus of p53 may be long enough to possibly acquire new functions ("gain of function" [24]). Thus, normal splicing isoforms p53β and p53γ have 10 and 15 abnormal C-terminal amino acids, correspondingly [12]. A mutant p53 isoform with an additional 107 amino acids at the C-terminus originated from duplication of exons 7, 8, and 9 was found in neuroblastoma cells IGR-N-91 [17]. This protein was as long as 500 amino acids, retained ability to bind to DNA, and acquired an additional oligomerization domain. In the present work, we found four mutant p53 isoforms that must have 4, 27, 43, and 68 abnormal amino acids at the C-terminus (Table 2). We could not establish any homology of these peptides with any known protein functional domains. It is possible, however, that these tails do not serve as a simple ballast but affect noticeably p53 function because they may change the spatial structure of the protein by means of amino acid interaction or by formation of abnormal S–S bonds. The question, what functions mutant p53 isoforms found in our work lose or gain, remains open. As mentioned above, interaction of normal p53 isoforms that arose from alternative splicing and/or transcription from internal promoter [11, 13] is one of the means of delicate and complicated regulation of p53 and of all the ensemble of relative proteins [5, 6, 22]. New abnormal p53 isoforms arising because of mutations apparently disturb native isoform balance and contribute to the process of carcinogenesis and to diversity of routes of tumor progression.

Statistical analysis of mutations found in our work revealed significant association of p53 mutations with lymph node metastasizing and with III/IV tumor stage, which are two important signs of unfavorable prognosis (Table 3). In this conclusion, we agree with many other researchers who have demonstrated prognostic significance of p53 mutations in CRC [6, 22].

Pro/Arg allelic variants of codon 72 of p53 differ in their biochemical and biological features, in particular, in their ability to induce apoptosis [31]. Many researchers have shown association of the relatively rare 72-Pro allele with higher incidence of different types of cancer including colorectal cancer [32-37]. This polymorphism was also reported to influence the effectiveness of chemotherapy in some cancer types [38, 39]. But there are a number of data arguing against such associations [40-46]. Codon

72 allele frequencies differ in human populations and races [27, 47], and vary from 0.21 to 0.33 in Caucasians (see rs1042522 on Ensembl Human SNPView and NCBI SNP Web sites). In our sample, the frequency of the Pro allele (22%) was very close to that in healthy Caucasians published in one of the most comprehensive studies (20%) [10]. Therefore, codon 72 polymorphism of p53 does not seem to be associated with incidence of colorectal cancer.

Thus, new p53 mutations and two new mutant isoforms p53i9* and p53-305 have been found in our work. Our results demonstrate association of p53 mutations with signs of unfavorable prognosis and lack of association of codon 72 polymorphism with incidence of colorectal cancer.

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