

## **TP53 somatic mutations and LOH profile in colorectal cancer in Romania**

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### **Abstract**

*Somatic mutations in TP53 tumor suppressor gene represent common events in colorectal cancer, followed frequently by loss of heterozygosity in the course of disease progression. In order to study the profile of these genetic alterations in Romanian oncologic patients TP53 gene sequencing and loss of heterozygosity analysis were performed in 62 tumor samples and 60 paired normal colon tissue samples collected during curative surgical resection. The rate of TP53 somatic mutations (46.8%) was concordant with published data while the type of mutations and their association with tumor sites exhibited a different pattern. The most prevalent mutation type was the nucleotide transition GC>AT (62.1% of total mutations) that occurred exclusively at the CpG dinucleotide sites. Allelic imbalance at TP53 locus was detected in 50% of colorectal tumors. The geographical variation of CRC might be explained by large differences in lifestyle habits and diet across various regions and ethnical groups. However, larger studies are needed to identify and characterize the distinct features of the colorectal cancer mutational profile in respect to geographical location, combined also with nutrigenomics analysis of the particular dietary nutrients specific for the region.*

**Keywords:**colorectal cancer, TP53 somatic mutations and LOH profile, nutrigenomics.

### **1. Introduction**

Colorectal cancer (CRC) is the second most common cancer in women (614 000 cases per year) and is the third most common in men (an estimated 746 000 cases per year), representing almost 10% of the global cancer incidence. CRC incidence burden seems to increase in countries transitioning towards higher levels of development and tends to stabilize or decline in highly developed ones (WORLD CANCER REPORT 2014 [1]).

There is wide global geographical variation in incidence, with rates varying ten-fold in both sexes worldwide, the highest estimated rates being in Australia/New Zealand and the lowest in Western Africa. In adult male and female Romanian population, this type of cancer in second place as incidence and mortality (GLOBOCAN 2012, IARC -7.8.2016 [2]). The geographical variation of CRC might be explained by large differences in lifestyle habits and diet across various regions and ethnical groups. Several compounds that result from adherence to the Western diet are considered possible etiologic factors for CRC such as N-nitroso compounds (NOC) related to increased intake of animal fat, heme iron resulted from high consumption of red meat or heterocyclic amines and polycyclic aromatic hydrocarbons generated by processing meat at high temperature. On the other hand, dietary fiber might

exert a benefic role in protection against CRC cancer development (HAGGAR & BOUSHEY [3]).

In spite of the fact that the term “colorectal cancer” might suggest a homogeneous disease, it is known that is only appropriate as a topographical indication, CRC being a very heterogeneous disease, developing through a multi-pathway sequence of events guided by clonal selections (WORLD CANCER REPORT 2014 [1]). Pathways operating in the development of CRC may be roughly categorized into chromosomal and microsatellite instability, genomic mutations including suppression of tumor suppressor genes and activation of tumor oncogenes, microRNA, and epigenetic modifications. As cancer advances, invasion and metastases are facilitated by the epithelial-mesenchymal transition (EMT), with additional genetic adjustments (KANTHAN & al. [4]).

The *TP53* tumor suppressor gene, located on the short arm of the chromosome 17 (17p13.1), encodes a 53-kD nuclear phosphoprotein that regulates multiple genes in response to different cellular injuries thus having a key role in guarding genomic stability. P53 presents the common hallmarks of a transcription factor: an amino-terminal transactivation domain, a core DNA-binding domain (DBD) and carboxy-terminal tetramerization and regulatory domains. Activated p53 suppresses cellular transformation essentially by inducing growth arrest, DNA repair and differentiation in damaged cells or initiating apoptosis when DNA repair fails. Consequently, when p53 function is compromised, as is in most tumor cells, it is usually due to somatic mutations. The critical role of *TP53* in tumorigenesis has been further emphasized by the high frequency (36.1%) of its somatic mutations in cancer patients across 20 tissues, the most of any known gene (MARTINCORENA & CAMPBELL [5]). Even more, it was observed that in human cancers the somatic mutations of *TP53* occur in most conserved mammalian domains of the gene, emphasizing the importance of its wild-type (WT) activity [W.M. KAMP & al. [6]). Whereas somatic *TP53* mutations contribute to sporadic cancer, germline *TP53* mutations are present in a rare autosomal dominant syndrome known as Li-Fraumeni Syndrome (LFS), characterized by high incidence of early-onset cancers (BROSH & ROTTER [7]). Somatic mutations are distributed all over *TP53* coding regions, however most of them were observed in exons 4-9, which encode the DBD of the protein. In addition to the loss of function that a mutation in *TP53* may cause, many p53 mutants are able to support tumor development by additional mechanisms. It is well documented that in a heterozygous situation, mutant p53 antagonize WT p53 tumor suppressor activities in a dominant negative (DN) manner. The inactivation of the WT p53 by the mutant p53 in a DN mechanism relies on the fact that the transcriptional activity of WT p53 is based on tetramer formation and mutant p53 will alter their DNA binding function (MILNER & al. [8], MILNER & MEDCALF [9], SIGAL & ROTTER [10]). However, the heterozygous state is most of the times transient, as *TP53* mutations are often followed by loss of heterozygosity (LOH) during tumor progression. The LOH of the short arm of chromosome 17 indicates a selective advantage for inactivation of the remaining *TP53* WT allele, suggesting that the DN activity of mutant p53 is not sufficient to inactivate entirely WT p53 (RIVLIN & al. [11]). Furthermore, many mutant p53 are able to exert additional oncogenic influence by gain-of-function (GOF) activities acquired through different mechanisms. DN, LOH and GOF effects may have a key role in the positive selection of missense mutations in *TP53* during tumorigenesis.

Despite a remarkable amount of data accumulated on *TP53* gene mutations in CRC, disagreement still exists regarding the association of *TP53* mutations with survival and drug response. Nevertheless, accumulated sequencing data showed that different types of *TP53* mutation might play a fundamental role in defining the biologic behavior of CRC from

specific sites and consequently the prognosis of patients (RUSSO & al. [12]). On the other hand, genotyping of the *TP53* mutations in CRC might be useful for p53-targeted therapy that has emerged recently as a promising treatment tool. By small molecule drugs it becomes possible to target selectively the mutant p53, thus inducing apoptosis in tumor cells. (BYKOV & al. [13]).

The aim of our study was to assess the contribution of *TP53* somatic mutations profile on incidence and development of colorectal cancer in patients from Romania, as mutational heterogeneity may characterize distinct tumor phenotypes in different geographic areas and data from this region are only scarcely presented in the literature.

## 2. Materials and Methods

Samples examined in this study were collected from 62 patients with sporadic colorectal cancer who underwent curative surgical resection at the *Prof. Dr. Alex. Trestioreanu* Institute of Oncology, Bucharest. The patient group included 23 women and 39 men with ages ranging from 39 to 85 years (median 64 years) of Romanian descent examined by genealogical inquiry. The anatomical distribution of the tumors was as follows: proximal colon (caecum through the splenic flexure), distal colon (descending and sigmoid colon) and rectum (the rectosigmoid junction and rectum). Overall, 31 tumors were localized in the colon and 31 in the rectum. Tumors were staged according to the AJCC criteria and histologic grade was determined by reviewing complete medical records. Immediately after resection, 62 tumor and 60 paired normal colon tissue samples were collected, snap-frozen in liquid nitrogen and stored until DNA isolation. To confirm the original diagnosis and histopathological tumor cell content, an adjacent sample was fixed in 10% buffered formalin and embedded in paraffin. Routine hematoxylin- and eosin-stained sections were reviewed by a pathologist. The content of tumor cells in the section slides assigned as “tumor sections” was on average 90% (range 80–100%) while in the section slides assigned as “normal sections” tumor cells were not present.

The study was approved by the institutional review board. All subjects provided written informed consent according to national and international regulations.

*Genomic DNA* was isolated from fresh frozen tissue using the commercial QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s recommendations. All samples generated a good quality DNA that was stored at  $-20^{\circ}\text{C}$  until sequencing.

The entire coding sequence of the *TP53* gene (exons 2–11) was analyzed using *DNA direct sequencing*. Standard M13 tails were added to primers previously described (<http://p53.iarc.fr/>) in order to facilitate subsequent sequencing. The PCR was performed in an EppendorfMastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany) using a touchdown protocol (available on request). Purification of the PCR product was performed using USB® ExoSAP-IT® (Affymetrix Inc., Santa Clara, California, USA). DNA sequencing reaction used M13 primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Thermo Fisher Scientific Corp.) according to the manufacturer’s recommendations. Sequencing amplification products were purified using the BigDye® XTerminator™ Purification Kit (Applied Biosystems) and subjected to capillary electrophoresis using a 3500 Genetic Analyser (Applied Biosystems). Sequence traces were analyzed using KB™ Basecaller Sequencing Analysis Software, Version 1.4, and Variant Reporter™ Software, Version 1.0 (Applied Biosystems). For heterozygous insertions, a parallel analysis was performed using Mutation Surveyor software ([www.softgenetics.com](http://www.softgenetics.com)).

Two polymorphic microsatellite markers were used to detect *LOH and/or microsatellite instability (MSI)* at chromosome 17p: TP53(AAAAT)<sub>n</sub> (FUTREAL & al. [14]) located in the

first intron of the *TP53* gene, and TP53(CA)<sub>n</sub> (JONES & NAKAMURA [15]) situated in the first intron of the gene. Fluorescently labeled primers were used to yield PCR products of approximately 140-175 bp for the AAAAT 5 bp repeat, and 103-135 bp for the CA 2bp repeat (CAWKWELL & al. [16])

PCR amplifications were carried out using a Veriti 96 Well Thermal Cycler (conditions available on request). The samples were run on a Genetic Analyzer 3500 (Applied Biosystems). Data were processed by ABI software GeneMapper version 4.1.

The *allelic imbalance* (AI) value was calculated according to Applied Biosystems' GeneScan Reference Guide. The allelic ratio was calculated for each paired normal and tumor samples by dividing the peak ratio of tumour tissue to the peak ratio of normal tissue. AI was scored when the allele peak ratio was < 0.67 or > 1.35. Tumor samples with novel allele peaks compared to the corresponding normal samples were classified as having MSI. Cases showing homozygosity or MSI were defined as uninformative for allelic imbalance analysis. Correlation between *TP53* mutations and clinico-pathological parameters was assessed by two-tailed Fisher's exact test (GraphPad Prism 5, Version 5.04, GraphPad Software Inc.). Values of  $p < 0.05$  were considered statistically significant.

### 3. Results

Sixty-two colorectal tumor samples and 60 matched normal samples were sequenced in this study in order to analyze somatic modifications in the *TP53* gene. Table 1 shows the clinico-pathological features of the patients and includes patients tumor stage, grade, lymphatic invasion and distant metastasis.

Table 1. Clinico-pathological features of the patients included in the study.

Variables	Number	%
<b>Tumour localization</b>		
Proximal colon	14	22.6
Distal colon	17	27.4
Rect	31	50.0
<b>Type</b>		
Carcinoma	1	1.6
Adenocarcinoma	52	83.9
Mucoid	9	14.5
<b>Depth of tumor invasion</b>		
T2	23	37.1
T3	35	56.4
T4	4	6.5
<b>Nodal status</b>		
N0	42	67.7
N1	11	17.8
N2	7	11.3
Nx	2	3.2
<b>Differentiation</b>		
G2	49	79.0
G3	11	17.8
Gx	2	3.2
<b>Distant metastasis</b>		
M0	47	75.8
M1	14	22.6
Mx	1	1.6
<b>Duke's stage</b>		
A	16	25.8
B	21	33.9
C	11	17.7
D	14	22.6

**Somatic mutations in the *TP53* gene.** Using PCR-direct sequencing analysis a total of 20 somatic mutation types were identified in the CRC samples investigated in this study. Thirteen of these mutations (65.0%) were missense single base substitutions, whereas six (30.0%) were protein-truncating mutations (3 non-sense, 1 frameshift and 2 intron splice site mutations). One samples had a non frameshift insertion. Representative electropherograms of the *TP53* variants characterized in the colorectal tumors are shown in figure 1.

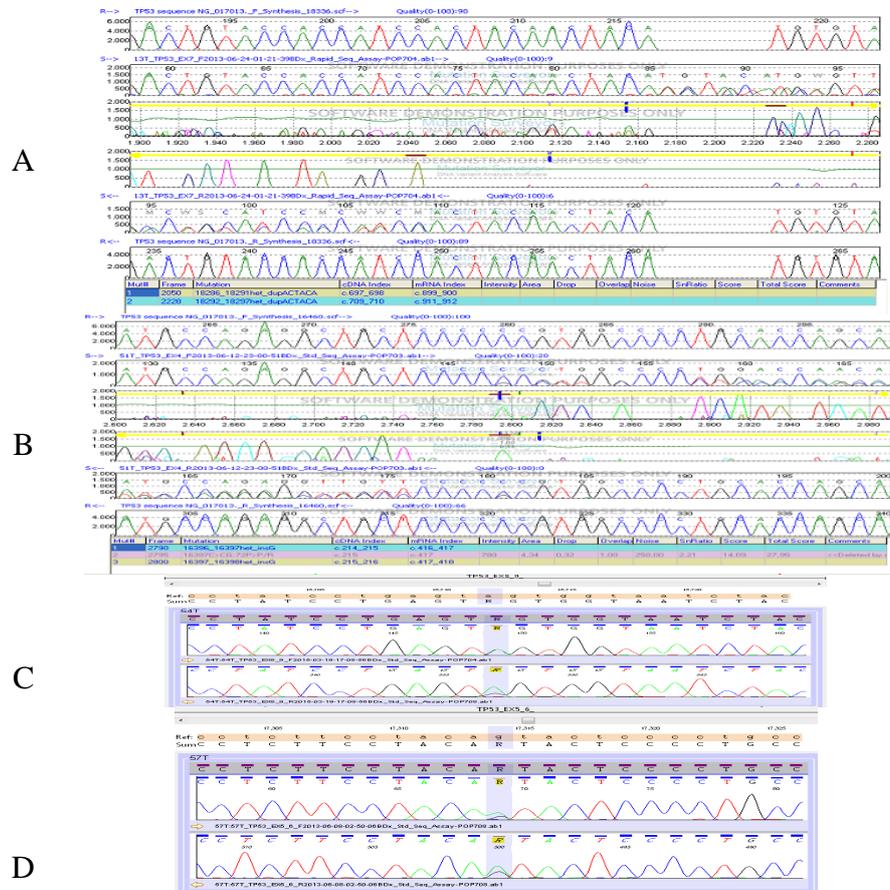


Figure 1. Representative electropherograms of heterozygous indels and splicing mutations detected in *TP53* gene.

The Mutation Report of the Mutation Surveyor software includes: the reference trace, the sample trace, the sample trace conserved sector, the sample trace mutation sector, the shifted mutation sector aligned to the reference and the mutation trace with the position of the indel event. The brown bar indicates a heterozygous duplication of ACTACA (A) and a heterozygous insertion of a G nucleotide (B). Electropherograms showing c.783-2 A>G (C) and c.376-1 G>A (D) splicing mutations.

All the missense single base substitutions were found in heterozygous state. Nonsense mutations were detected at the codons 196, 213 and 343 leading to premature termination of p53 protein. Two insertion events, c.214\_215insG and c.709-710 insACTACA, were detected in *TP53* exon 4 and exon 7, respectively. To the best of our knowledge, the latest is reported for the first time. Two cases showed intronic mutations that altered splice acceptor sites. One change was a transition G → A at the 3' end of intron 4 (c.376-1G>A). The second was an A→G transitions at the -2 position of the splice acceptor site for intron 7 (c.783-2A>G). A summary of the results is shown in table 2.

Taken together, *TP53* somatic mutations were detected in 29 (46.8%) of colorectal tumor samples, with a higher frequency observed in distal colon 9/17 (52.9%) and a lower frequency in rectal tumors 14/31 (45.2%) and proximal colon tumors 6/14 (42.9%).

When the mutation distribution pattern was analyzed, 25/29 (86.2%) of the *TP53* mutations found were located to DNA-binding domain, with a higher frequency in exon eight 12/29

(41.4%) (figure 2). Mutations in four hotspot codons (R175, G245, R273 and R282) accounted for 48.3% (14/29) of all *TP53* mutations. The most frequently mutated codon was 273, which comprised 31.0% (9/29) of the mutations found. Outside DNA binding domain, we detected a nonsense mutation p.Glu343\* (c.1027G>T) in the  $\alpha$ -helix of the tetramerization domain and an insertion event (p.V73fs\*76 (c.216\_217insC) in the proline-rich region.

Table 2. *TP53* somatic mutations in colorectal samples.

Samples	Gender	Age	Localization	Exon / Intron	Codon	Position	Nucleotide change	Amino acid change	Effect	Structural motif	CpG site	Substitution Type	TA class	LOH
11T	F	66	Rectum	4	105	c.313G>T	GGC-TGC	p.Gly105Cys	missense	NDBL/beta-sheets	no	GC>TA	NF	AI
35T	M	80	Distal colon	4	110	c.329G>T	CGT-CTT	p.Arg110Leu	missense	NDBL/beta-sheets	yes	GC>TA	NF	AI
51T	M	40	Distal colon	4	73	c.215_216	insG		frameshift	Pro-rich/SH3	yes		NA	ROH
57T	M	69	Rectum	intron 4		c.376-1G>A			splice		no	GC>AT	NA	AI
20T	M	59	Rectum	5	152	c.455C>T	CCG-CTG	p.Pro152Leu	missense	NDBL/beta-sheets	yes	GC>AT	NF	AI
52T	M	72	Distal colon	5	154	c.461G>T	GGC-GTC	p.Gly154Val	missense	NDBL/beta-sheets	no	GC>TA	NF	AI
65T	M	70	Rectum	5	175	c.524G>A	CGC-CAC	p.Arg175His	missense	L2IL3	yes	GC>AT	NF	AI
68T	F	59	Proximal colon	5	175	c.524G>A	CGC-CAC	p.Arg175His	missense	L2IL3	yes	GC>AT	NF	AI
7T	M	74	Rectum	6	197	c.589G>T	GTG-TTG	p.Val197Leu	missense	NDBL/beta-sheets	no	GC>TA	PF	MSI
8T	M	66	Rectum	6	196	c.586C>T	CGA-TGA	p.Arg196*	nonsense	NDBL/beta-sheets	yes	GC>AT	NA	AI
30T	M	49	Distal colon	6	213	c.637C>T	CGA/CGG-TGA	p.Arg213*	nonsense	NDBL/beta-sheets	yes	GC>AT	NA	AI
13T	M	53	Proximal colon	7	237	c.709-710	ins ACTACA		non-frameshift	L2IL3	no		NA	AI
53T	M	44	Proximal colon	7	242	c.724T>C	TGC-CGC	p.Cys242Arg	missense	L2IL3	no	AT>GC	NF	AI
55T	F	60	Distal colon	7	234	c.701A>G	TAC-TGC	p.Y234C	missense	NDBL/beta-sheets	no	AT>GC	NF	AI
69T	F	64	Rectum	7	245	c.733G>A	GGC-AGC	p.Gly245Ser	missense	L2IL3	yes	GC>AT	NF	AI
54T	M	61	Proximal colon	intron 7		c.783-2A>G			splice		no	GC>AT	NA	AI
5T	M	82	Proximal colon	8	282	c.844C>T	CGG-TGG	p.Arg282Trp	missense	L1S/H2	yes	GC>AT	NF	AI
19T	F	56	Distal colon	8	273	c.818G>A	CGT-CAT	p.Arg273His	missense	L1S/H2	yes	GC>AT	NF	AI
22T	F	73	Distal colon	8	273	c.818G>A	CGT-CAT	p.Arg273His	missense	L1S/H2	yes	GC>AT	NF	AI
24T	F	52	Rectum	8	273	c.817C>T	CGT-TGT	p.Arg273Cys	missense	L1S/H2	yes	GC>AT	NF	ND
25T	M	74	Rectum	8	273	c.817C>T	CGT-TGT	p.Arg273Cys	missense	L1S/H2	yes	GC>AT	NF	AI
27T	F	74	Rectum	8	278	c.832C>A	CCT-ACT	p.Pro278Thr	missense	L1S/H2	no	GC>TA	NF	AI
34T	M	62	Rectum	8	273	c.817C>T	CGT-TGT	p.Arg273Cys	missense	L1S/H2	yes	GC>AT	NF	AI
36T	M	83	Distal colon	8	273	c.818G>A	CGT-CAT	p.Arg273His	missense	L1S/H2	yes	GC>AT	NF	AI
40T	F	82	Distal colon	8	273	c.817C>T	CGT-TGT	p.Arg273Cys	missense	L1S/H2	yes	GC>AT	NF	ROH
46T	M	56	Rectum	8	273	c.818G>A	CGT-CAT	p.Arg273His	missense	L1S/H2	yes	GC>AT	NF	AI
61T	F	57	Rectum	8	282	c.844C>T	CGG-TGG	p.Arg282Trp	missense	L1S/H2	yes	GC>AT	NF	AI
62T	M	76	Proximal colon	8	273	c.818G>A	CGT-CAT	p.Arg273His	missense	L1S/H2	yes	GC>AT	NF	AI
31T	M	39	Rectum	10	343	c.1027G>T	GAG-TAG	p.Glu343*	nonsense	C-term/TAD	no	GC>TA	NA	ROH

Reference sequences used are: GenBank NC\_000017.10 (genomic), NM\_000546.4 (cDNA), UniProt P04637 (protein).

Among somatic mutations, transition showed the greatest incidence 72.4% (21/29), with GC>AT transition occurring predominantly at CpG islands. GC>TA transversion was detected in 20.7% (6/29) cases, with a predominance at non-CpG sites.

The study also showed a statistically significant increase in the incidence of *TP53* mutations in Dukes' Stage C and D when compared with A and B ( $p=0.0092$ ; OR=0.225; 95% CI=0,07621 to 0,6695). No difference in mutation rate of *TP53* was detected between well-differentiated and poorly differentiated tumors. Further, patient sex, age and tumor size were not correlated with *TP53* mutations in primary colorectal tumors.

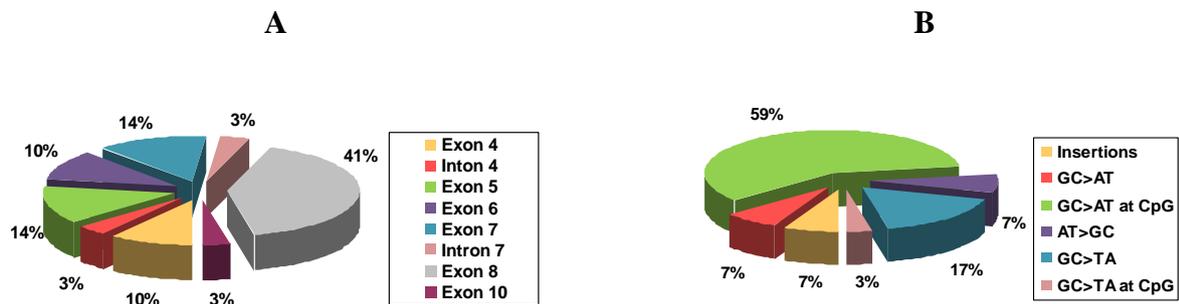


Figure 2. *TP53* somatic mutations in colorectal tumor samples. **A** – location of mutations; **B** – mutation events.

Tumor DNA used for the analyses derived from whole tumor homogenates thus, documenting LOH by microsatellite analysis did not allow for a distinction between LOH and allelic imbalance (AI). Since normal tissue DNA was not available in 2 cases, only 60 tumors were included in the LOH assay. 52 out of the 60 tumor DNA (86.7%) was informative at one or both *TP53* loci. The number of patients informative for each polymorphic region were 49 (94.2%) in *TP53*(CA)n and 41 (78.8%) in *TP53*(AAAAT)n. Seventeen cases (32.7%) had loss at both informative loci. In total, 26 of 52 (50%) patients showed AI in the analyzed p53 areas. The remaining 26 cases were determined to have retention of heterozygosity (ROH). One patient (1.7%) was homozygous for both markers. Seven out of 60 cases (11.7%) had MSI at *TP53*(CA)n locus, while only 2 incidences were found at *TP53*(AAAAT)n. Figure 3 provides representative results of our AI analysis.

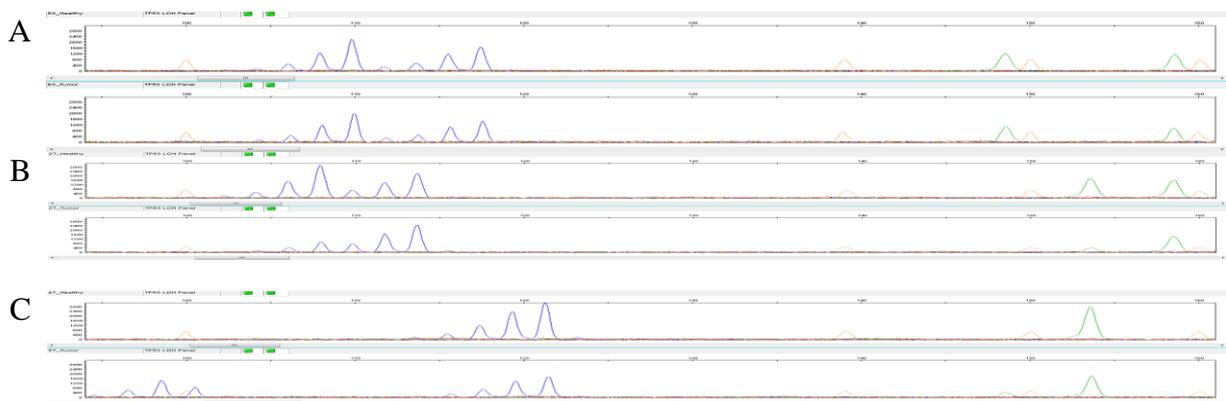


Figure 3. Loss of heterozygosity and microsatellite instability in *TP53* gene identified in colorectal samples.

A) a representative electropherogram of a retention of heterozygosity case; B) Loss of heterozygosity in *TP53*(AAAAT)n and *TP53*(CA)n loci is interpreted as reduced height of one allelic peak as compared to corresponding normal DNA. C) MSI positive case identified as additional peaks in tumor profiles.

Out of 52 informative tumor DNA, combined *TP53* AI and mutation of the gene was observed in 24 (46.2%) of the cases predicting a non-functional state of the gene. For 23 (44.2%) cases neither AI nor a gene mutation were demonstrated. Tumors with *TP53* mutation had a higher incidence of AI (88.8%) at *TP53* than tumors without a mutation

(8.0%) ( $p < 0.0001$ , OR 0,01087, 95% CI 0,001661 - 0,07115). Only in one out of seven tumors with MSI (14.3%) a *TP53* mutation was found.

Although the frequency of AI in distal colorectal cancer (8 of 17 cases, 47.1%) was higher than that of proximal (6 of 14 cases, 42.9%) and rectal cancer (12 of 31 cases, 38.7%), this difference was not statistically significant.

#### 4. Discussion and Conclusions

Here we report 20 different tumor suppressor gene *TP53* mutations in CRC samples obtained from a group of Romanian patients. In our study, the *TP53* mutation rate was 46.8%, which is in agreement with the published frequencies of *TP53* mutations in colorectal cancer (IACOPETTA [17]; LEROY & al. [18]; CIOBOTARU & al. [19]) and the IARC mutation database <http://p53.iarc.fr>). Distal colon tumors were found to have more mutations than proximal colon and rectal tumors (52.9%, 42.9%, 45.2%, respectively) in Romanian CRC cases investigated in this study. This higher frequency of mutations observed in distal colon tumors compared to tumors located at other sites was observed also in AI despite that the difference in the frequency of AI for tumors with different locations was not significant.

Analysis of the relative distribution of mutations within exons showed a lower proportion of mutations in exons 5 and 7 and a higher proportion in exon 8 comparing to those reported in the IARC database. Mutation analysis revealed a high percentage of missense mutations (22/29, 75.9%) compared with frameshift mutations (2/29, 6.9%). Most mutations identified in this work clustered in the DNA binding domain, and apart from the mutation in codon 197, that partially retains *TP53* function, all mutations reside in DBD were non-functional. About 57.7% of the mutations in this domain fell within 4 hotspot residues (R175, G245, R273, and R282). Unlike previous studies that have reported a high incidence of mutations at codon 248 (13.8% of total mutations) no mutations were found at this specific site in our samples (LASKY & SILBERGELD [20]).

The new mutation reported in this work involved an insertion of 6 base pairs within the sequence of exon 7 that does not alter the reading frame of the protein. The patient carrying the mutation was a 53-year-old man, with metastatic proximal colon cancer, who underwent chemotherapy before surgery.

The nonsense mutation p.Glu343\* and the frameshift mutation p.V73fs\*76 detected outside the DNA binding domain could both affect *TP53* functional activity.

The most prevalent mutation type was the nucleotide transition GC>AT (65.5% of total mutations). This result is consistent with previous studies reporting a higher prevalence of transition than transversion mutations of *TP53* however, the frequency of transitions appear to be lower in Romanian samples compared to samples from different geographic locations (VERGINELLI & al. [21]; PARK & al. [22]; SOUSSI & BÉROUD [23]).

It is worthwhile to mention that GC>AT transitions detected in the current study occurred mainly at the CpG dinucleotide sites. The preponderance of GC>AT transitions at CpG sites has been attributed to spontaneous deamination of 5-methylcytosine to thymine. (IVANOV & al. [24], BAELE & al. [25]). Such deamination processes yield T:G mismatches and, subsequently, GC>AT transitions at the next round of DNA replication (PARK & al. [22]). On the other hand, GC>AT transition mutations have been postulated to be inducible by alkylating agents and may be induced by NOC formed endogenously in the presence of haem. Haem or its oxidized form, haemin, that might be excessively present in red meat diet, promotes, on the other hand, apoptosis exerting a supplementary selective pressure (DIACONU & al. [26]). Nitric oxide (NO), a key signaling molecule involved in the regulation of peristalsis, gut vasomotor functions and mucosal inflammation, may contribute

to transition mutagenesis at CpG dinucleotide sites acting directly at 5-methylcytosines, by nitrosative deamination in oxidizing environments, and, indirectly, at guanines, by base alkylation after conversion to nitrate, bacterial reduction to nitrite and endogenous formation of NOC (GOODMAN& al. [27], SAWA & OSHIMA [28], LALA & CHAKRABORTY [29], HUGHES [30]). Mutagenesis at CpGs may be facilitated by NO-induced inhibition of DNA repair (SAWA & OSHIMA [28], LALA & CHAKRABORTY [29]). Furthermore, NO promotes apoptosis via *TP53* and therefore exerts a critical selective pressure for *TP53* mutation (FORRESTER& al. [31], MIHARA& al. [32]).

In the present study, a statistically significant increase of the incidence of *TP53* mutations was found in Dukes' Stage C and D when compared with stages A or B. No associations have been shown with other clinico-pathological features.

It is well known that ninety-nine percent of all exons are flanked by the intronic dinucleotides GT and AG at the 5' and 3' splice sites respectively. Mutations in tumor suppressor genes at the invariant intronic dinucleotide often cause exon-skipping events that truncate proteins, acting like classical nonsense mutations. The real incidence of splice site mutations on *TP53* gene has been underestimated in the past because intron–exon boundaries were rarely analyzed. Large-scale DNA sequencing studies have allowed identification of a consistent number of genetic lesions that affect alternative splicing. These mutations are likely to contribute considerably to the colorectal carcinogenesis. In the present study, two intronic splice-site mutations were found. The first mutation (c.376-1G>A) involved an invariant residue at the consensus splice acceptor site of intron 4 and was previously reported in colorectal tumors. The second mutation (c.783-2A>G), located at the splice acceptor site of intron 7, was detected in carcinoma of lung, prostate and upper aerodigestive track, but not in colorectal cancer. Mutations found in the primary tumors were absent in DNA from the matched non-malignant tissues indicating that these alterations were somatic events.

Missense mutations in *TP53* in human tumors are usually followed by LOH at the corresponding locus. Both genetic alterations occur frequently in later stages of the progression to colorectal carcinoma. (FEARON & JONES [33]) The loss of p53 in CRC not only allows the cells to divide at an uncontrolled rate, but this fast replication further supports the development of even more genetic mutations because the cell cycle is not interrupted when errors occur and no efficient repairing actions are taken (CHANG& al. [34]) In colorectal cancer 50% to 70% of cases were showed to exhibit LOH at the *TP53* locus (FREED-PASTOR& C. PRIVES [35]) *TP53* mutations and LOH have been linked, in many cases, with poor therapy response, high metastasis potential and worse outcome (CHANG& al. [34], PEREZ& al. [36], BARRATT& al. [37]).

In this work two polymorphic microsatellite markers at the *TP53* genetic locus were used to assess LOH in the samples that had normal DNA. The TP53(CA)<sub>n</sub> marker was more informative compared to TP53(AAAAT)<sub>n</sub> markers. Overall, 50% of colorectal tumors displayed AI. Investigating the correlation between the AI results with respect to tumor locations we found that AI showed a higher frequency in distal colorectal cancer (8 of 17 cases, 47.1%) than in proximal (6 of 14 cases, 42.9%) and in rectal cancer (12 of 31 cases, 38.7%), although this difference was not statistically significant. Similar findings have been reported in several other studies (PEREZ& al. [36], SUGAI& al. [38]). We found no association between AI at the *TP53* locus and clinico-pathological features. Consistent with reports by other investigators the frequencies of p53 mutations were significantly correlated with AI status (SUGAI& al. [38]).

Previous studies have shown that MSI occurs in 15% to 20% of sporadic colon cancers. In our study, 11.7% of cases showed MSI, most of them were located in proximal colon cancer. An inverse correlation was observed between MSI and *TP53* gene mutations.

Despite large-scale sequencing studies having identified numerous mutated genes in CRC, a complete view on the genomic modifications and their clinical significance for CRC tumorigenesis should be replicated in samples from different geographic locations analyzing *TP53* mutation and LOH profile for their role in defining the biologic behavior of CRC from specific sites and consequently disease progression and clinical outcomes taking into consideration also particular dietary nutrients characteristic to the region, in comprehensive nutrigenomics studies.

Although there are controversies regarding the prognostic value of *TP53* mutations, their characterization might be relevant in the context of the newly developed p53 targeted therapeutic strategies.

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