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ORIGINAL PAPER

The detection of mutations in the APC gene of Romanian patients with colorectal cancer through two independent techniques

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Abstract

Large mutations in the APC (adenomatous polyposis coli) gene have been detected especially on familial adenomatous polyposis (FAP). The mutations may appear not only inside the gene, but also at the microsatellite loci level. Therefore, we have developed two independent methods for the detection of mutations encompassing one or more exons, on patients with sporadic colorectal cancer, on APC or at the microsatellite loci of the same gene. Multiplex ligation-dependent probe amplification (MLPA), as a second method, is performed in one reaction for the initial quantification of all APC exon copy numbers.

In the current study, genomic DNA from 27 patients diagnosed with colorectal cancer was examined for mutations in the APC gene using microsatellite analysis and MLPA techniques (Multiplex Ligation – dependent Probe Amplification).

At the four microsatellites loci designated on chromosome 5 we found LOH/ MSI in 12/ 27 cases (44.44%) and 6/ 12 cases have AI on D5S421 loci. These results are correlated with our previous immunohistochemical studies (data not show).

In the case of the MLPA analysis only half of them (18.5%) have alterations such as deletion. The difference between the AI and MLPA analyses, which underlines the mutational mechanism of the APC gene, is still difficult to explain. These results underline the sporadic character of colorectal cancer in Romania and the clinical data also sustains them.

Keywords: colorectal cancer, APC, LOH, MSI, MLPA

Introduction

The large mutation in the *APC* (*adenomatous polyposis coli*) gene located on chromosome 5q21 has been detected especially in Familial Adenomatous Polyposis syndrome (FAP) [6, 16]. The tumor suppressor gene – *APC* has 15 exons and its 8.5 kb cDNA encodes 2843 amino acids. Exon 15, which comprises more than 75% of the coding sequence of the gene, is the most common target for both *germline* and somatic mutations [28]. Most of the mutations are small deletions or insertions, and about 95% of these lead to truncation of the *APC* protein with abnormal function [8]. Deletions at the codons 1061 (c.3183_3187delACAAA) and 1309 (c.3927_3931delAAAGA) are found to be the most frequent *germline* mutations, the latter being responsible for a severe clinical phenotype [3, 15].

Mutations may appear not only inside the *APC* gene, but also at the microsatellite loci. At this level, mutations may be of different types: deletions which can be termed *loss of heterozygosity* (LOH) or *microsatellite instability* (MSI). MSI are caused by a defective mismatch repair (MMR) system as a consequence of errors that occur during DNA replication [21, 24, 27]. The loss of heterozygosity (LOH) on tumor suppressor genes is considered one

of the key steps towards carcinogenesis of colorectal cancer [9]. The loss of one allele at a specific locus is caused by a deletion mutation or loss of a chromosome from a chromosome pair [13]. When this occurs on a tumor suppressor gene with an abnormal allele, neoplastic transformation occurs. LOH analysis has become an effective way to find informative loci candidate tumor suppressor genes [1, 5, 10].

Therefore, we have developed two independent methods for the detection of mutations on patients with sporadic colorectal cancer compared with familial adenomatous polyposis. The first was the microsatellite analysis of the *APC* gene. Multiplex ligation-dependent probe amplification (MLPA), as a second method, is performed in one reaction for the initial quantification of all APC exon copy numbers.

Materials and methods

Patients

Patients confirmed by oncologist with colorectal cancer were provided by Clinical Hospital “Sfantul Ioan” in Bucharest. In the current study, based on molecular analysis of the *APC* gene, we have examined 27 unrelated patients who include both familial and sporadic cases. All patients gave their consent before the blood and tissues were taken.

Of the 27 patients, 26 are sporadic cases, whereas the remaining individual, while having no family history, had undergone colectomy due to the presence of more than 100 intestinal polyps.

DNA isolation

Genomic DNA was isolated from venous blood (as a control) harvested on EDTA anticoagulant and tumor tissue from the same patient. DNA preparation was performed using the *Wizard® Genomic DNA Purification kit* (Promega) according to the manufacturer’s recommendations.

Microsatellite study

Four fluorescence labelled primers with FAM, HEX, TET, for polymorphic microsatellite markers on chromosome 5 were used to amplify DNA from normal and tumor tissues for LOH and MSI analysis. These microsatellite markers are found in the neighbourhood of the *APC* gene: D5S82, D5S489, and D5S656; while D5S421 is localized on the coding region of the gene. In order to analyze the polymorphic microsatellite markers, a PCR reaction was carried out for 10 ng DNA from normal and tumor tissue. The marker-specific fluorescent amplification PCR products were separated on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Fragment analysis was performed using GeneMapper ID v3.1.

Multiplex Ligation-dependent Probe Amplification (MLPA)

The whole APC gene was also investigated for large genomic rearrangements by MLPA using the same samples and also the 3 samples of genomic DNA isolated from blood belonging to a healthy patient (as a control for this case). The MLPA reaction was performed according to the instructions of the manufacturer (Salsa P043 kit; commercially available at MRC-Holland, http://www.mrc-holland.com, Amsterdam, NL). The Salsa P043 kit contains probes for each coding exon of *APC*, three probes for the *APC* promoter region and 11 probes for other human genes located on different chromosomes. Briefly, 100 ng genomic DNA was used as starting material. After hybridization, ligation and amplification, the FAM-labelled PCR products of MLPA reactions were separated on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Fragment analysis was performed using GeneMapper ID v3.1. Data analyses of sample DNA were done by exporting size and peak areas to an Excel file. By using Coffalyzer programme for normalization, relative probe signals were calculated by dividing each measured peak area by the sum of all peak areas of the sample. The ratio of

each individual relative probe area was then normalized to the mean obtained with three control samples.

Results and discussion

During tumorigenesis, the loss of wild-type alleles (inherited from the non-mutation-carrying parents) is frequently observed. Loss of heterozygosity (LOH) on tumor suppressor genes played a key role in colorectal cancer transformation, and LOH analysis of sporadic colorectal cancers could help discover unknown tumor suppressor genes or other microsatellite markers associated with tumor suppressor genes [6, 10]. In this study, LOH scanning was analyzed by GeneMapper ID v3.1 software in 26 sporadic colorectal cancer samples and one with FAP with four polymorphic markers. The ratio of the fluorescence intensity of alleles was studied to identify additional loci involved in colorectal tumorigenesis.

Alleles were defined as the two highest peaks within the expected size range. A ratio of T1:T2/ N1:N2 less than 0.67 or greater than 1.50 was scored as a loss of heterozygosity, indicating that one allele had decreased with 40% or more (Figure 2). Most amplifications of normal DNA producing the two PCR products indicated preserve heterozygosity. A single fragment amplified from normal DNA (homozygote) and fragments not clearly amplified from PCR reactions were scored as not informative. The LOH frequency of a locus was equal to the ratio of the number between allelic loss and informative cases [19 - 21, 28].

At the microsatellite loci designated on chromosome 5 we found LOH/ MSI in 12 of the 27 cases (44.44%) and 6 of these 12 cases have AI on D5S421 loci (Table 1). These results emphasize the significance of these markers, especially of D5S421 involved in the neoplastic process of colorectal cancer. Thus, we hypothesized that the higher LOH frequency of D5S421 might be the reason why the LOH frequency appears on the coding region of the *APC* gene. Also, our previous data from immunohistochemical studies (data not show), are correlated with microsatellite analysis. Also, in one case (patient no. 6, D5S421 locus) we cannot make an assessment referring to MSI, because we have not the sample control for this patient (Table 1).

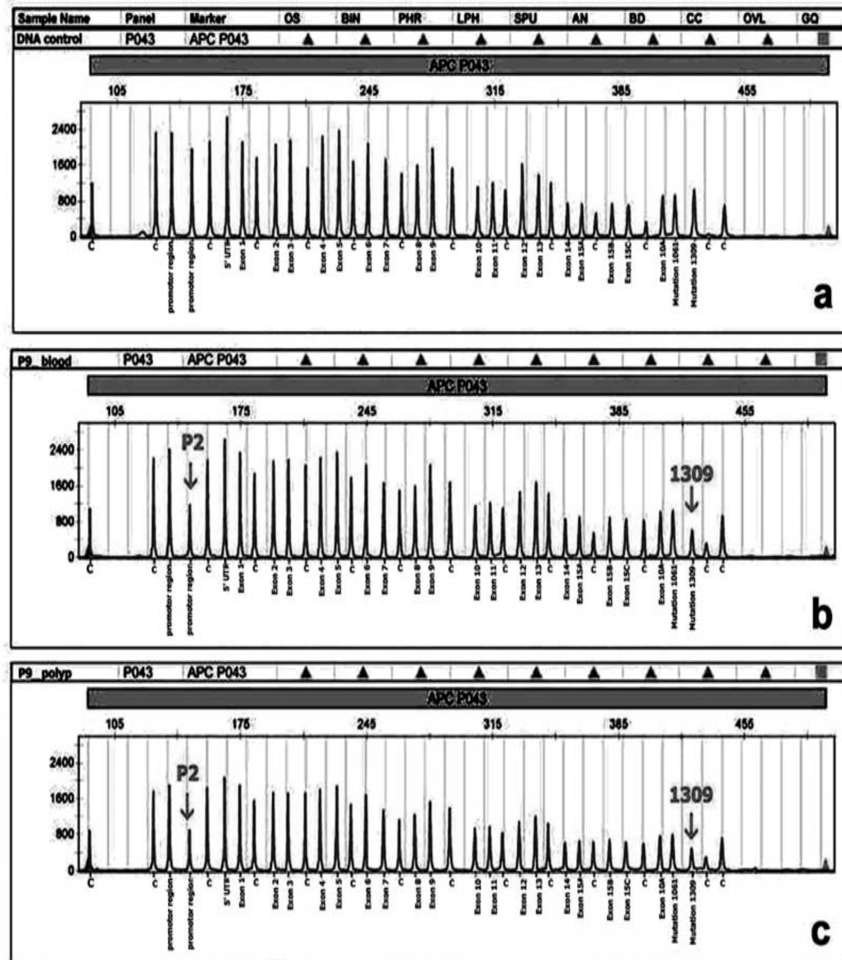
MSI in adenomas appears to be a relatively specific pointer for HNPCC. As MSI is very rare in sporadic adenomas, screening such lesions routinely for MSI may not be a high priority. However, MSI analysis in adenomas is likely to be useful in the cases where clinical features or family history suggest a hereditary predisposition [7, 14, 17].

All 27 patients included in the first round and the subsequent 3 controls were then submitted to MLPA analysis. Deletion was suspected when the peak area was reduced with more than 40% to 50% compared with normal controls [15].

The samples of all unrelated patients were directly analyzed by MLPA and no positive cases were found in the blood DNA samples, with the exception of one patient diagnosed with FAP. This patient showed two deletions, in blood and in the tumor, in the promoter 2 region and mutation at codon 1309, although the individual did not show microsatellite loci alteration (Figure 1). These results are in accordance with those obtained by Lamlum [11], according to which, occasionally, in certain tumors in patients with *germline* mutations at the level of codon 1309, either the MCR locus or the 3' and 5' region of APC gene, do not associate with the allelic loss at the level of adenomas. This same fact is observed in the case of patient 19 (P19) whose deletion, detected through MLPA at the E12 - E15 level, a region also including the MCR situ, is not supported by an allelic loss in any of the other microsatellite markers assayed. Although in this case no *germline* mutations are identified, we could extrapolate the same reasons as Lamlum [11], starting from the premise that APC is often cited as the first tumor suppressor gene affected both by familial and sporadic tumours [18].

Table 1 Comparative result of microsatellites and gene analysis in APC region
(**LOH** – loss of heterozygosity; **MSI** – microsatellite instability; **(++)** – heterozygote; **(+)** – homozygote; nd-non-detected).

Patient code	Subsection code	Detection of mutations on chromosome 5q in *APC* region				
		Microsatellites analysis				Gene analysis
		D5S421 *APC*	D5S82	D5S489	D5S656	MLPA
1	N1	++	++	+	++	unmodified
	T1	MSI	++	+	++	
2	N2	+	++	++	+	unmodified
	T2	+	++/ LOH	++	+	
3	N3	++	++	+	++	unmodified
	T3	MSI	++	+	++	
4	control for MLPA reaction					
5	control for MLPA reaction					
6	T6	++/ MSI ?	++	+	++	unmodified
7	T7	++	++	+	++	unmodified
8	N8	++	+	++	++	unmodified
	T8	++	+	++	++	
9	N9	++	++	++	++	Partial deletion on Prom2 and mut 1309
	T9	++	++	++	++	
10	N10	++	++	++	++	unmodified
	T10	++	++	++	++	
11	N11	++	++	++	+	unmodified
	T11	++/ LOH	++	++	+	
12	N12	+	++	++	++	unmodified
	T12	+	++	++	++	
13	T13	+	++	+	+	unmodified
14	N14	++	++	+	+	unmodified
	T14	++	++	+	+	
15	N15	++	++	+	++	unmodified
	T15	++/ LOH	++	+	++/ LOH	unmodified
16	N16	++	++	+	++	unmodified
	T16	++	++	+	++	
17	N17	+	++	+	+	unmodified
	T17	+	++	+	+	
18	N18	++	++	++	++	unmodified
	T18	++	++	++/ LOH	++	Partial deletion E10 – E15
19	T19	++	++	++	+	Partial deletion E10, E11, E14 - E15, + mut: 1061 and 1309
20	N20	+	++	++	+	unmodified
	T20	+	++/ MSI	++	+	unmodified
21	N21	+	++	++	++	unmodified
	T21	+	++	++	nd	unmodified
22	control for MLPA reaction					
23	N23	++	+	+	++	unmodified
	T23	++/ LOH	MSI	+	++/ LOH	Partial deletion E12 – E15 + mut: 1061 and 1309
24	N24	++	++	++	+	unmodified
	T24	++	nd	++	+	unmodified
25	N25	+	++	++	+	unmodified
	T25	+	++/ MSI	++/ LOH	+	
26	N26	++	++	++	+	unmodified
	T26	++	++	++	+	
27	control for MLPA reaction					
28	N28	++	++	+	+	unmodified
	T28	++	++	+	+	unmodified
29	N29	++	++	++	++	unmodified
	T29	++	++	++/ LOH	++	Partial deletion E12 – E15 + mut: 1061 and 1309
1 m	N1 m	++	++	+	++	unmodified
	T1 m	++/ LOH	++/ LOH	+	++/ LOH	Partial deletion E12 – E15 + mut: 1061 and 1309
2 m	N2 m	++	+	++	+	unmodified
	T2 m	++	+	++	+	



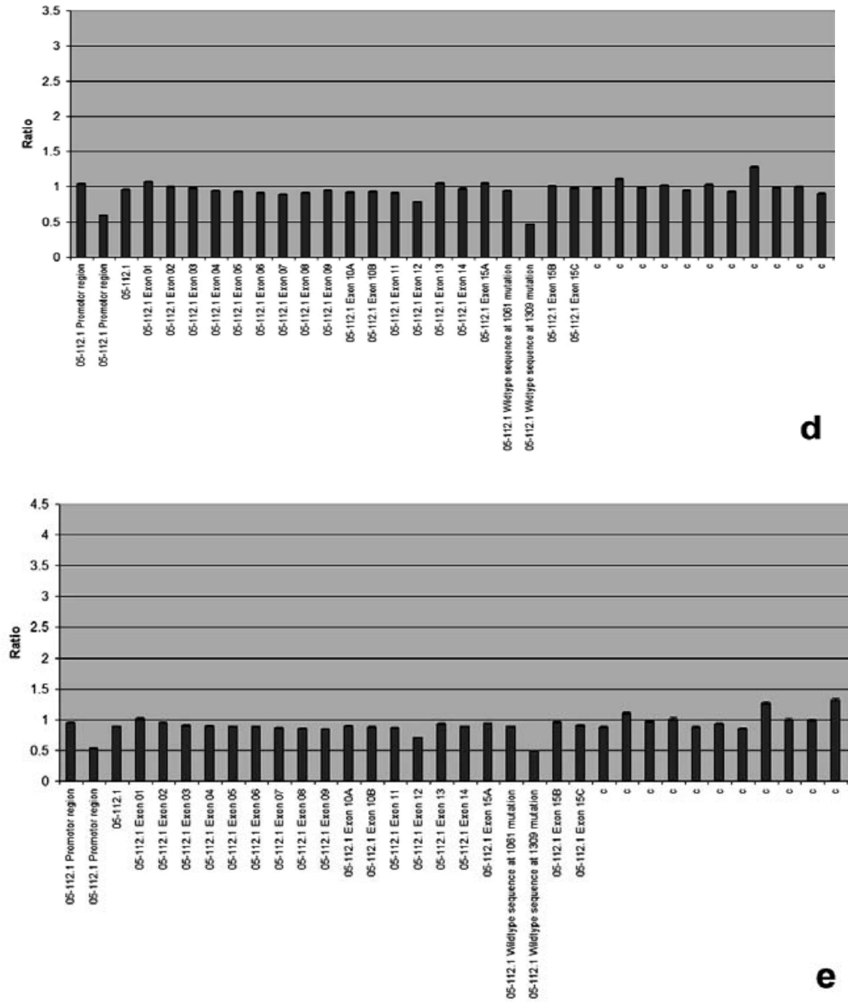


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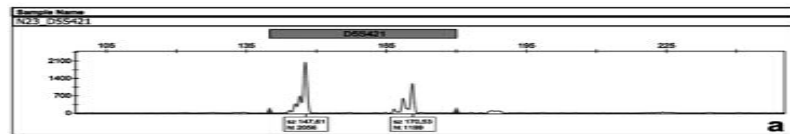
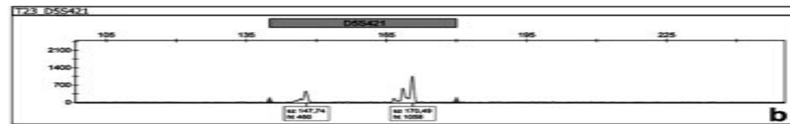
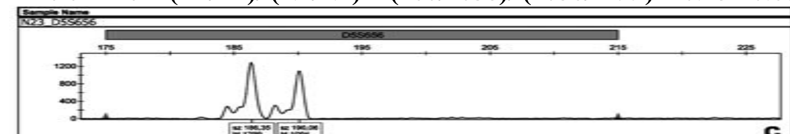
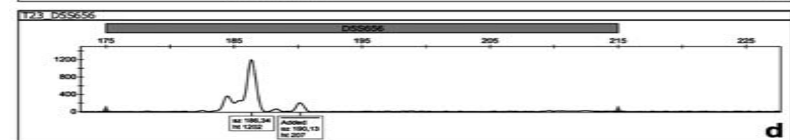
Figure 1. The genetic profile of patient 9 by MLPA method. a) Chromatogram profile of normal DNA control; **b)** Chromatogram profile of blood DNA sample; **c)** Chromatogram profile of DNA tissue sample (polyp); **d)** Normalization for blood sample by Coffalyzer programme; **e)** Normalization for tissue sample by Coffalyzer programme.



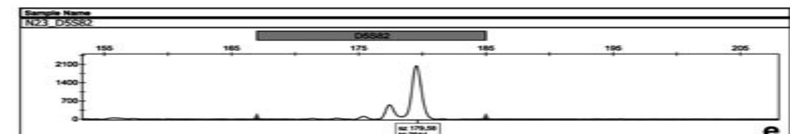


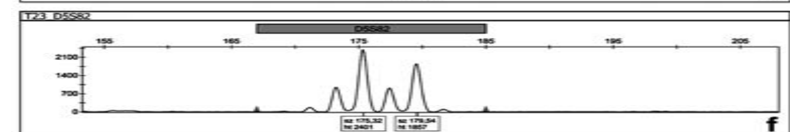
Typical peak of LOH for P23 on D5S421:
Allele ratio = (T1/T2)/ (N1/N2) = (460/1058)/ (2056/1199) = 0.25< 0.67





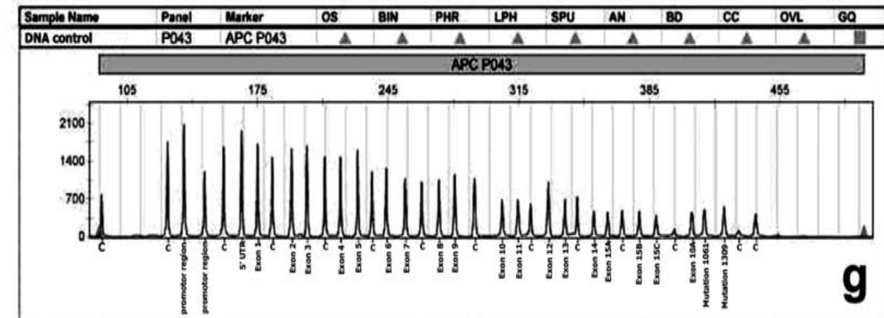
Typical peak of LOH for P23 on D5S656:
Allele ratio = (T1/T2)/ (N1/N2) = (1202/207)/ (1299/1094) = 5> 1.5





Typical peak of MSI for P23 on D5S82:
Allele ratio = (T1/T2)/ (N1/N2) = (1202/207)/ (1299/1094) = 5> 1.5

Applied Biosystems
GeneMapper ID v3.1



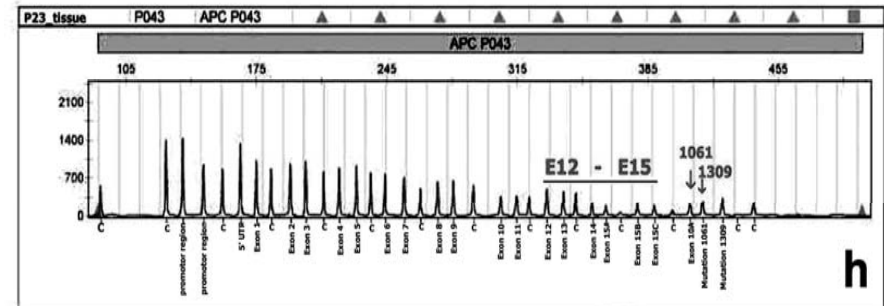


Figure 2. Comparison between microsatellites alteration and the MLPA method for the *APC* gene on patient 23
a-b microsatellites alteration on D5S421 locus – **(a)** from blood sample and **(b)** from tissue sample (middle region of tumor); **c-d** microsatellites alteration on D5S656 locus – **(c)** from blood sample and **(d)** from tissue sample (middle region of tumor); **e-f** microsatellites alteration on D5S82 locus – **(e)** from blood sample and **(f)** from tissue sample (middle region of tumor); **g-h** Chromatogram profile of normal DNA control – **(g)** compare with DNA from tumor sample **(h)**

In the case of patient 18 we encountered the loss of heterozygosity in the D5S489 locus as well as a partial deletion of the region between E12 – E15, detected through MLPA. In the cases of patients P23, and P 1m, a similar mutational profile presents itself as a result of both analysis techniques, as opposed to patient 29 in whose case the deletions detected through the MLPA technique are identical with those of the other patients, but the loss of heterozygosity (LOH) is present only for the D5S489 microsatellite marker. These results are also conformant with those present in the literature according to which no less than one mutation at the level of the MCR situ, also associated with allelic loss, may be found in some of the sporadic adenomas as well as in some of the familial ones [11, 22].

Overall, while fully supported by the data extant in the literature in the field, sustaining the involvement of *APC* in the DNA repair process or in maintaining the integrity of the genome, these results do not explain the low rate of allelic loss in the adenomas with *germline* mutations beyond codon 1300, regarded as a "second target" in the case of most Mendelian segregation tumors. On the other hand, there is no knowledge referring to the homology or interaction of any protein involved in the repair of the DNA or in maintaining genomic integrity with the *APC* gene region, associated with allelic loss; however it is widely deemed that this gene region is important from a structural viewpoint in the degrading and stabilization of β-catenine [2, 12].

Other studies have shown that allelic loss is the most widely known „second target" in patients with mutations at the level of codon 1309, which shows that spontaneous mutations lead to allelic loss during deletions, chromosome non-disjunctions and mitotic recombinations [11].

Various mechanisms have been proposed for explaining the origin of most genomic alterations including unequal *crossing-over* through mediated recombinations of homologous retrotransposons and random replications [4]. Thus, modifications at the level of the Alu sequence lead to recombinations that seem to be the most frequent cause of large deletions at the level of the *APC* gene as well as in other tumor suppressor genes [25]. Complex alterations, of the deletion type, at the level of exons 12 – 14 also constitute a result of inadequate splicing, as described by Su [24], and they are also supposed to be mediated by the Alu sequence. However, the complexity of chromosome rearrangements is difficult to explain in terms that would emphasize the mutational mechanisms since, to a great extent, they are only enunciated in a hypothetical manner [4].

Our results show the sporadic character of colorectal cancer in Romania and the clinical data also sustain that diet is one of the major causes of colorectal cancer.

Taking into account as a whole, all these results show that only 44.44% of patients have AI/ LOH at microsatellite loci and, for approximately half of them (18.5% from total no of patients), we observed an association with MLPA. The difference of percentage, confirming the mutational mechanism of the *APC* gene, is still difficult to explain as a consequence of chromosomal rearrangements complexity. These results underline the sporadic character of colorectal cancer in Romania and the clinical data also sustain them.

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References

1. BAKER SJ, FEARON ER, NIGRO JM, HAMILTON SR, PREISINGER AC, JESSUP JM, VANTUINEN P, LEDBETTER DH, BARKER DF, NAKAMURA Y. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*; 244: 217-221. (1989)
2. BERX G, NOLLET F, VAN ROY F. Dysregulation of the E-cadherin/catenin complex by irreversible mutations in human carcinomas. *Cell Adhes. Commun,* 6: 171–184. (1998)
3. CASPARI R, FRIEDL W, MANDL M, MOSLEIN G, KADMON M, KNAPP M, JACOBASCH KH, ECKER KW, KREISSER-HAAG D, TIMMERMANNS G, PROPPING P. Familial adenomatous polyposis: mutation at codon 1309 and early onset of colon cancer. *Lancet* 343:629-632. (1994)
4. CHEN J.M, CHUZHANOVA N, STENSON P.D, FEREC C, COOPER D.N. Metaanalysis of gross insertion causing human genetic disease: novel mutational mechanisms and the role of replication slippage, *Hum Mutat*, 25: 207-221, (2005).
5. CHONG-ZHI ZHOU, GUO-QIANG QIU, FANG ZHANG, LIN HE, ZHI-HAI PENG. Loss of heterozygosity on hromosome 1 in sporadic colorectal Carcinoma, *World J Gastroenterol,* 10(10):1431-1435. (2004)
6. GRODEN J, THLIVERIS A, SAMOWITZ W, CARLSON M, GELBERT L, ALBERTSEN H, JOSLY G, STEVENS J, SPIRIO L, ROBERTSON M. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell;* 66:589-600. (1991)
7. Gylling A, Abdel-Rahman WM, Juhola M, Nuorva K, Hautala E, Ja¨rvinen H J, Mecklin J-P, Aarnio M, Peltoma¨ki P. Is gastric cancer part of the tumour spectrum of hereditary non-polyposis colorectal cancer? A molecular genetic study. *Gut* ;56:926–933. (2007)
8. HADJISAVVAS A, PAPASAVVA T, LOIZIDOU M, MALAS S, POTAMITIS G, CHRISTODOULOU C, PAVLIDES G, PAPAMICHAEL D, KLONIS C, NASIOULAS G, ANASTASIADOU V, KYRIACOU K. Novel germline mutations in the APC gene of Cypriot patients with familial and sporadic adenomatous polyposis. *Clin Genet* 69:404-409. (2006)
9. KATAOKA M, OKABAYASHI T, JOHIRA H, NAKATANI S, NAKASHIMA A, TAKEDA A, NISHIZAKI M, ORITA K, TANAKA N. Aberration of p53 and DCC in gastric and colorectal cancer. *Oncol Rep*; 7: 99-103. (2000)
10. KINZLER KW, NILBERT MC, VOGELSTEIN B, BRYAN TM, LEVY DB, SMITH KJ, PREISINGER AC, HAMILTON SR, HEDGE P, MARKHAM A. Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science*; 251: 1366-1370. (1991)
11. LAMLUM H, ILYAS M, ROWAN A, CLARK S, JOHNSON V, BELL J, FRAYLING I, EFSTATHIOU J, PACK K, PAYNE S, ROYLANCE R, GORMAN P, SHEER D, NEALE K, PHILLIPS R, TALBOT I, BODMER W, TOMLINSON I. The type of somatic mutation at APC in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson's 'two-hit' hypothesis, *Nat Med*, 5: 1071-5, (1999).
12. LATCHFORD A, VOLIKOS E, JOHNSON V, ROGERS P, SURAWEERA N, TOMLINSON I, PHILIPS R, SILVER A. APC mutations in FAP–associated desmoid tumours are non-random but „just right", *Hum. Molec. Genetics*, v16, 1: 78-82, (2007).
13. LENGAUER C, KINZLER KW, VOGELSTEIN B. Genetic instabilities in human cancers. *Nature*; 396: 643-649. (1998)
14. LOUKOLA A, SALOVAARA R, KRISTO P, MOISIO A L, KÄÄRIÄINEN H, AHTOLA H, ESKELINEN M, HÄRKÖNEN N, JULKUNEN R, KANGAS E, OJALA S, TULIKOURA J, VALKAMO E, JÄRVINEN H, MECKLIN J P, DE LA CHAPELLE A, AND AALTONEN L A. Microsatellite Instability in Adenomas as a Marker for Hereditary Nonpolyposis Colorectal Cancer, *Am.J Pathol.*, 155, 6, 1849 -1853. (1999)
15. MICHILS G, TEJPAR S, THOELEN R, VAN CUTSEM E, VERMEESCH JR, FRYNS JP, LEGIUS E, MATHIJS G. Large deletion of the APC gene in 15% of mutation – negative patients with classical polyposis (FAP): a Belgian study. *Hum Mutat* 25:125-134. (2005)
16. MIHALATOS M, APESSOS A, DAUWERSE H, VELISSARIOU V, PSYCHIAS A, KOLIOPANOS A, PETROPOULOS K, TRIANTAFILLIDIS J K, DANIELIDIS I, FOUNTZILAS G, AGNANTIS N J, NASIOULAS G. Rare mutations predisposing to familial adenomatous polyposis in Greek FAP patients. *BMC Cancer*; 5:40. (2005)
17. MIHALCEA A, TICA V, GEORGESCU S.E., TESIO C., DINISCHIOTU A, CONDAC E., COSTACHE M, AND IONICA E. Allelic imbalance on chromosomes 1 and 5 in colorectal carcinoma. *Plovdiv University Press;* P.568-575. (2005)
18. MIYAKI M, TANAKA K, KIKUCHI-YANOSHITA R, MURAOKA M. & KONISHI M. Familial polyposis: recent advances, *Crit. Rev. Oncol. Hematol*., 19: 1–31, (1995).

19. PENG Z, LING Y, BAI S. Loss of heterozygosity on chromosome 3 in sporadic colorectal carcinoma. *Zhonghua Yixue Zazhi*; 81: 336-339. (2001)
20. PENG Z, ZHANG F, ZHOU C, QIU G, BAI S, LIU W, HE L. Loss of heterozygosity analysis to define putative region involved in tumor differentiation and metastases in sporadic colorectal cancer patients. *Zhonghua Waike Zazhi*; 40: 776-779. (2002)
21. RAMBURAN A., OLADIRAN F, SMITH C, HADLEY G.P, GOVENDER D, Microsatellite analysis of the adenomatous polyposis coli (APC) gene and immunoexpression of ß catenin in nephroblastoma: a study including 83 cases treated with preoperative chemotherapy. *J Clin Pathol* 58, 44-50. (2005)
22. STEKROVA J, SULOVA M, KEBRDLOVA V, ZIDKOVA K, KOTLAS J, ILENCIKOVA D, VESELA K, KOHOUTOVA M. Novel *APC* mutations in Czech and Slovak FAP families: clinical and genetic aspects, *BMC Medical Genetics*, 8: 16, (2007).
23. SU LK, STEINBACH G, SAWYER JC, HINDI M, WARD PA, LYNCH, PM, Genomic rearrangements of the APC tumor-suppressor gene in familial adenomatous polyposis, *Hum Genet*, 106: 101-107, (2000)
24. THIRLWELL C, HOWARTH K.M, SEGDITSAS S, GUERRA G, THOMAS H J W, PHILLIPS R K S, TALBOT I C, GORMAN M, NOVELLI M R, SIEBER O M AND TOMLINSON I P M. Investigation of pathogenic mechanisms in multiple colorectal adenoma patients without *germline* APC or MYH/MUTYH mutations, *British Journal of Cancer*, 96, 1729–1734. (2007)
25. WANG Y, FRIEDL W, LAMBERTI C, JUNGCK M, MATHIAK M, PAGENSTECHER C, PROPING P, MANGOLD E. Hereditary nonpoliposis colorectal cancer: frequent occurrence of large genomic deletion in MSH2 and MLH1 genes, *Int J Cancer*, 103: 636-641, (2003).
26. XU SF, PENG ZH, LI DP, QIU GQ, ZHANG F. Refinement of heterozygosity loss on chromosome 5p15 in sporadic colorectal cancer. *World J Gastroenterol*, 9: 1713-1718. (2003)
27. YOSHIHIKO M, SHINYA O*,* KEIZO S, The instability within: problems in current analyses of microsatellite instability. *Mutation Research*, 461 249–263. (2001)
28. ZHANG F, ZHOU C, LING Y, BAI S, LIU W, QIU G, HE L, PENG Z. High frequency of LOH on chromosome 18 in Chinese sporadic colorectal cancer patients. *Zhonghua Shiyan Waike Zazhi*; 19: 320-321. (2002)