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ALLELIC IMBALANCE ON CHROMOSOMES 1 AND 5 IN COLORECTAL CARCINOMA

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ABSTRACT. Colorectal cancer is one of the most frequent causes of cancer deaths in the world. The characteristic keys of the colorectal cancer are AI/LOH and MSI.
The purpose of this study was to investigate the relevance of LOH and MSI at the APC and PLA2G2A genes in blood and tumors from patients with colorectal cancer.
APC is the first tumoral suppressor gene in colorectal tumorigenesis and its germline mutations is responsible for FAP. The somatic APC mutations are found in the majority of sporadic colorectal tumors and was suggested that loss of APC function play a rate-limiting role in tumor initiation. An alteration in the APC gene is one of the earlier events in carcinogenesis of some adenocarcinomas. $sPLA_2$ is a member of the phospholipase A_2 family, a group of enzymes that catalyze the hydrolysis of membrane glycerophospholipids to generate free fatty acids. It was also suggested that *PLA2G2A* gene is intact, but an AI, or an allelic loss, was found at one of allele and a LOH was identified on *PLA2G2A* regions.
We evaluated the AI/LOH on chromosome 1p and 5q with the ABI PRISM 310 Genetic Analyzer by using a panel of 8 microsatellites loci from human chromosomes 1p and 5q.
We found LOH in 6 samples and MSI in 13 samples.

KEY WORDS. colorectal cancer, PLA_2, APC, LOH, MSI

INTRODUCTION

Colorectal cancer is one of the three leading causes of cancer mortality worldwide. The progression of the cancer is due to an accumulation of mutations,

either inherited (germline) or acquired (somatic), in critical proto-oncogenes and tumor suppressor genes. Each mutation may provide an additional growth advantage to the transformed cells as they dominate their normal counterparts [Sidransky D., 1992; Fearon E.R., 1990].

APC is a large protein of 312 kDa, with a cytoplasmatic and nuclear expression which is an extremely multifunctional protein. The APC gene spans approx. 150 kb of genomic DNA and is fragmented into 15 exons which are spliced into an mRNA transcript of about 8 kb. The exon structure of APC is remarkable by the first 14 exons are relatively small while exon 15 codes for an open reading frame of about 6.5 kb.

APC is the first tumoral suppressor gene in colorectal tumorigenesis and its germline mutations is responsible for Familial Adenomatous Polyposis (FAP) [Groden J., 1991]. The somatic APC mutations are found in the majority of sporadic colorectal tumors regardless of histological stage [Miyoshi Y., 1992]. This suggests that loss of APC function is likely to play a rate-limiting role in tumor initiation in the colorectum [Hardy R.G., 2000; Fearon E.R., 1990].

$sPLA_2$ type IIA is a member of the phospholipase A_2 family, a group of enzymes that catalyze the hydrolysis of membrane glycerophospholipids to generate free fatty acids. Some of the PLA_2 proteins are able to generate arachidonic acid (AA), the substrate used by COX-2 to synthesize PGs. It seem that the group IIA $sPLA_2$ function in a pathway widely considered to be important to tumorigenesis, and is a good candidate gene that modifies the *Apc* gene in the Min (**m**ultiple **i**ntestinal **n**eoplasia) mice. On one hand, a mutation resulting in splice variants of the *Pla2g2a* gene and in different truncated forms of its protein has been suggested to account for the increased number of polyps in mice carrying the Min mutation. On the other hand, mice expressing an active *Pla2g2a* gene are resistant and developed only few polyps. Many studies suggested that *Pla2g2a* is a candidate gene for *Mom-1* [MacPhee M., 1995].The analysis of a mouse/human hybrid panel showed that *PLA2G2A* gene, localized on human chromosome 1, is a candidate gene for the *MOM-1* locus. It was also observed that *PLA2G2A* gene is intact, but an allelic imbalance (AI), or an allelic loss, was found at one of allele and a loss of heterozygosity (LOH) was identified on *PLA2G2A* regions [Cormier R.T., 1997].

The loss of one allele in the clinical sample results from chromosomal deletion or mitotic combination and is commonly thought to represent the second genetic inactivation step in the complete loss of a tumor suppressor gene locus [Knudson Jr.A., 1985].

The presence of a novel allele in the tumour sample was interpreted as microsatellite instability (MSI). Widespread microsatellite instability, manifested as expansion or deletion of many repeat elements in tumor DNA, is particularly common in colorectal tumors. In patients with hereditary nonpolyposis colorectal carcinoma (HNPCC), it is caused by mutations of DNA mismatch repair genes [Kinzlerand K.W., 1996].

The detection of one of these genetic changes (LOH or MSI, or both) in a clinical sample demonstrates the presence of a tumoral cell clone population that share altered genetic information.

The purpose of this study was to investigate the relevance of loss of heterozygosity (LOH) and microsatellite instability (MSI) at the APC and PLA2G2A genes in blood and tumors from patients with colorectal cancer. The study loci were: D1S2644, D1S199, D1S2843 and D1S234 for human chromosome 1p, D5S421, D5S489, D5S656 and D5S82 for human chromosome 5q.

MATERIAL AND METHODS

All patients have been provided from Clinical Hospital "Sfantul Ioan" Bucharest. These had a median age of 60 years (range 55-74) and the most frequent symptoms were intestinal occlusion, bleeding, anaemia.

Biological material. Samples were obtain with the consent of 5 patients (19 samples), consisting of histopatologically confirmed colorectal adenomas. Tumors were removed by endoscopies and stored at –70 ^{0}C. Immediately after sectioning DNA analysis studies have been performed to indicate whether these tumors have alterations at different loci on the chromosomes 1p and 5q. Some of the tumors were transversal divided in three regions (top, middle, bottom), to see more precisely where the abnormalities can take place.

DNA analyses. After surgery, fresh tumors were cutted in approximately 2mm^3 from top, centre and bottom of resection piece and frozen immediately at -80^{0}C. DNA was extracted from blood and tissue samples using Wizard® Genomic DNA Purification kit (Promega).

We evaluated the AI/LOH on chromosomes 1p and 5q with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) by using a panel of eight microsatellite markers, which surround the *PLA2G2A* locus (Tab.1), on chromosome 1p and *APC* locus (Tab.2), on chromosome 5q. The samples from blood and frozen tissues were amplified by PCR with the fluorescent-labelled forward primer, using a Taq platinum polymerase (Invitrogen) for eight microsatellite markers. The PCR consisted of an initial activation step of Taq platinum polymerase for 10 minutes at 95^{0}C, followed by 30 cycles of 30 seconds at 95^{0}C, 30 seconds at variable temperatures, and 1 minutes at 72^{0}C. A final extinction step was at 72^{0}C for 1 hour and than 4^{0}C hold. The variable temperatures have been depended of the amplified microsatellites loci. It was performed a PCR multiplex for D1S2644, D1S2843, D1S199 microsatellites loci and another for D5S489, D5S656, D5S82, D5S421 where the temperature was 60^{0}C and 55^{0}C, respectively. One last microsatellite loci, D1S234 was amplified at 57^{0}C.

Products analysis Aliquots of 0.5 to 1µl of the microsatellites PCR product were mixed with 0.5µl ROX 350-labeled sizing standards (Applied Biosystems, USA) in 12µl deionised formamide and denatured at 95°C for three minutes than removed on ice for other three minutes. PCR amplicons from tumor and normal samples were separated individually on the ABI PRISM™ 310 Genetic Analyzer

(Applied Biosystems, USA). Resulting electrophoregrams were analyzed with GeneMapper ID v3.1 software for molecular size and peak heights.

Allelic imbalance was determined using the following ratio: $(T_1:T_2)/(N_1:N_2)$, where 1 and 2 are the first and the second peaks of alleles identified in the tumor/blood DNA samples from patients with colorectal cancer [Canzian F., 1996; Ramburan A., 2005]. A ratio less than 0.6 or higher than 1.5 was regarded as allelic imbalance with loss of one of alleles. The presence of a novel allele in the tumor sample was interpreted as microsatellite instability [Ramburan A., 2005; Cawkwell L., 1995].

RESULTS AND DISCUSSION

The biologic material was represented by blood and tumor of each patient which was first exanimate at Clinical Hospital "Sfantul Ioan" Bucharest. In our previous studies we observed that the immunoreactivity of $sPLA_2$ type IIA was modified either by losing the fluorescent signal in SM cells and presence of signal in the epithelial border of the crypts, or by losing the signal on the whole section. A close association of losing $sPLA_2$ type IIA from SM cells and losing APC signal from epithelial cells was observed in some of the samples [Mihalcea A., 2004; Ionica E., 2003].

By DNA examination of 19 normal and tumoral samples from 5 patients we analyse the alterations of eight loci, four on the short arm of chromosome 1 and other four on the long arm of chromosome 5. We found loss of heterozigosity (LOH) in 6 samples and MSI in 13 samples (Tab.3). All the samples showed mutation at one of two alleles from different loci and apparition of new alleles on whole 1p or 5q length.

On patient 1 (74 years old) we observed that all the microsatellites loci that were analysed on chromosome 1 are altered. On chromosome 5 only one microsatellite, which belong to *APC* gene was modified. With D1S2843 exception, the typical alteration was MSI (Fig.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 hereditary predisposition [Loukola A., 1999].

On patient 5 (23 years old) which was diagnosed by surgery as having multiple (>100) adenomatous polyps, our analyses at D5S421 loci indicate that allelic alteration appear on the blood and tumor samples of the patient (Fig.2). This loci are long enough to cover the entire *APC* gene. Our finding could indicate that *APC* gene have an inherited mutation that could lead to the multiple adenomas like in FAP patients. FAP is an autosomal dominant disorder which carry mutation that inactived APC protein. The result is a truncated protein incapable of binding the proteins involved in Wnt signalling pathway.

CONCLUSION

In conclusion, our study has shown that LOH/MSI of the PLA2G2A and APC locus is a more frequent aberration then the once observed at the other microsatelites

loci. We can suppose that could be a significant correlation between these two genes that could be useful in diagnosis of colorectal cancer.

By our study we conclude that the markers that we used for analysing abnormalities on chromosomes 1 and 5 in colorectal cancer can be useful for diagnosed the stage of the tumor. We supposed that PLA2G2A could be a tumor suppressor gene and seems to be involved in the first steps of colorectal tumorigenesis.

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REFERENCE

LOUKOLA ANU, REIJO SALOVAARA, PAULA KRISTO, ANU-LIISA MOISIO, HELENA KÄÄRIÄINEN, HEIKKI AHTOLA, MATTI ESKELINEN, NIILO HÄRKÖNEN, RISTO JULKUNEN, EERO KANGAS, SEPPO OJALA, JUKKA TULIKOURA, ERKKI VALKAMO, HEIKKI JÄRVINEN, JUKKA-PEKKA MECKLIN, ALBERT DE LA CHAPELLE, and LAURI A. AALTONEN. 1999. Microsatellite Instability in Adenomas as a Marker for Hereditary Nonpolyposis Colorectal Cancer, *Am.J Pathol.*, **155**, 6, 1849 -1853

MIHALCEA, AURA V. MURESAN, CAMELIA-DOINA VRABIE, C. COPAESCU, ANCA DINISCHIOTU, MARIETA COSTACHE, C. DRAGOMIRESCU, ELENA IONICA, Phospholipase A_2 expression in colorectal cancer, *in press*

CANZIAN, F., SALOVAARA, R., HEMMINKI, A. 1996. Semiautomated assessment of loss of heterozygosity and replication error in tumor, *Cancer Res.*, **56**, 3331-7

CAWKWELL, L., LI, D., LEWIS, F., 1995. Microsatellite instability in colorectal cancer: improved assessment using fluorescent polymerase chain reaction, *Gastroenterology*, **109**, 465-71.

CORNIER, R.T., HONG, K.H., HALBERG, R.B., HAWKINS, T.L., RICHARDSON, P., MULHERKAR, R., DOVE, W.F., LANDER, E.S., 1997. Secretory phospholipase *Pla2g2a* confers resistance to intestinal tumorigenesis, *Natur.Genet.,* **17**, 88-91

DIETRICH, W.F. *et al.* 1993. Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse, *Cell* **75**, 631–639

IONICA, E., C. VRABIE, E. CONDAC, C. COPAESCU, A. DINISCHIOTU, M. COSTACHE. 2002. The modification of APC expression in colorectal cancer, *Bul. Anal Soc Nat Biol Cel* , **VII** (**1**), 49-56.

FEARON, E.R., VOGELSTEIN, B. 1990. A genetic model for colorectal tumorigenesis, *Cell* **6**1, 759-767

GRODEN, J., et al. 1991. Identification and caracterization of the FAP gene, *Cell* **66**, 589-600.

HARDY, R.G., MELTZER, S.J., JANKOWSKI, J.A. 2000. ABC of colorectal cancer: Molecular basis for risk factors. *BMJ*, **321**, 886-889

KATAOKA, M., OKABAYASHI, T., JOHIRA, H., NAKATANI S,, NAKASHIMA A,, TAKEDA, A., NISHIZAKI, M., ORITA, K., TANAKA, N. 2000. Aberration of p53 and DCC in gastric and colorectal cancer, *Oncol Rep*. **7**, 99-103

KINZLERAND, K. W., VOGELSTEIN B. 1996. *Cell* **87**, 159

KNUDSON, JR, A., 1971. G. Mutation and cancer: statistical study of retinoblastoma, *Proc. Natl. Acad. Sci. U.S.A.* **68,** 820-823

KNUDSON, JR, A., 1985. G *Cancer Res.* **45**, 1437

LENGAUER, C, KINZLER, KW, VOGELSTEIN, B. 1998. Genetic instabilities in human cancers, *Nature* **39**6, 643-649

MACPHEE, M., CHEPENIK, K.P., LIDDELL, R.A., NELSON, K.K., SIRACUSSA, L.D., BURCHBERG, A.M. 1995.. The secretory phospholipase A_2 gene is a candidate for the *Mom 1* locus, a major modifier of the APC^{Min} induced intestinal neoplasia, *Cell,* **81**, 957-966

MARRAAND, G., BOLAND, C.R. 1996. *Gastroenterol. Clin. North Am.* **25,** 755

MYOSCHI, Y., et al. 1992. Somatic mutation of the APC gene in the colorectal tumors: mutation cluster regeon in the APC gene , *Hum. Mol. Genet,* **1**, 229-233

RAMBURAN, A., OLADIRAN, F., SMITH, C., HADLEY, G.P., GOVENDER, D. 2005. 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

SHOEMAKER, A.R. 1998. et al., A resistant genetic background leading to incomplete penetrance of intestinal neoplasia and reduced loss of heterozygosity in ApcMin/mice. *Proc. Natl. Acad. Sci. U. S. A.* 95, 10826–10831

SIDRANSKY, D., MIKKELSAN, T., CAVENEE, W. 1992. Vogelstein, *Nature* **355**, 841

Table 1. Microsatellites loci on chromosome 1p

Marker	Primer sequence	Chromosome location	Product size (bp)
D1S2644	(F) TGCAACCCACCTGAATGA	1p36.13	215 - 231
	(R) ACGTGAAGTGCCAGCACA		
D1S199	(F) GTGACAGAGTGAGACCCTG	1p36.21-p36.11	94 - 116
	(R) CAAAGACCATGTGCTCCGTA		
D1S2843	(F) GGGCTGGGCATTACACAAC	1p36.13-p35.3	178 - 187
	(R) ATCAAATTGGCTTCTCACCG		
D1S234	(F) TGAGCCCAGGAGGTTGAGG	1p35.3-p36.2	226 - 238
	(R) AAGGCAGGCTTGAATTACAG		

F, forward; R, reverse

Table 2. Microsatellites loci on chromosome 5q

Marker	Primer sequence	Chromosome location	Product size (bp)
D5S421	(F) TGGAAATAGAATCCAGGCTT	5q22-q23	152 - 170
	(R) TCTATCGTTAACTTTATTGATTCAG		
D5S489	(F) TTCACTTGTTGATGGGCT	5q15-q21	184 - 192
	(R) ACCAGACTTGTATATGTGTGTGT		
D5S656	(F) GCTAAGAAAATACGACAACTAAATG	5q21.3	185 - 203
	(R) CATAATAAACTGATGTTGACACAC		
D5S82	(F) ATCAGAGTATCAGAATTTCT	5q14-q21	169 - 179
	(R) CCCAATTGTATAGATTTAGAAGTC		

F, forward; R, reverse

Table 3. Loss of heterozygosity/microsatellite instability based on electropphoregrams

Patient	Sections		D1S2644	D1S199	D1S2843	D1S234 PLA2	D5S421 APC	D5S489	D5S656	D5S82
1	N1				+				+	+
	T1	top			+				+	+
		middle					nd		nd	+
		bottom					nd		nd	nd
2	N2		nd	+	+	+		+		+
	T2	top	+	+	+	+		+		+
		middle	+	+	+	+	nd	+		
		bottom	+	+	+	+	nd	+		
3	N3		nd	+		nd			+	+
	T3	top	+			+				nd
		middle	+	+			nd		+	+
		bottom	+	+		+	nd		+	+
4	N4		nd	+	+	nd		+	nd	
	T4	top	nd	nd	nd	+		+	nd	
		middle	+	+	+	+		+	nd	
		bottom	nd	+	+	+		+	nd	
5	N5			+	+	+		+	+	+
	T5	under polip		+	+	+		+	+	+
		polip		+	+	+		+	+	+

homozigot MSI LOH (+) heterozigot (nd) nondetected

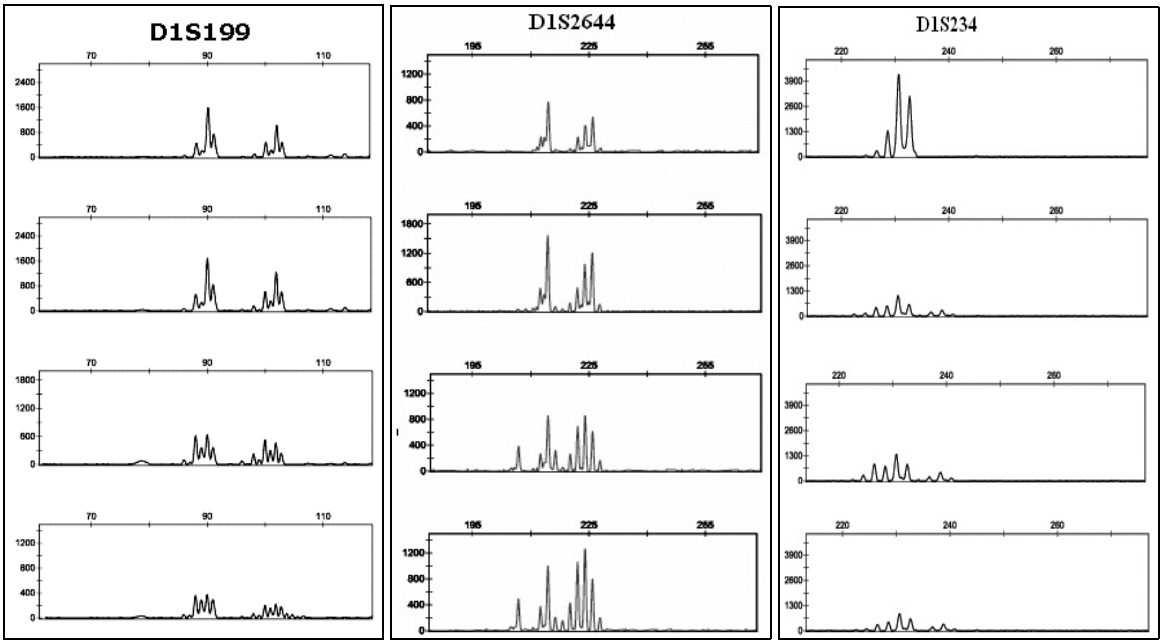

Figure 1. General profile for D1S199, D1S2644 and D1S234 loci at chromosome 1p at the patient 1.

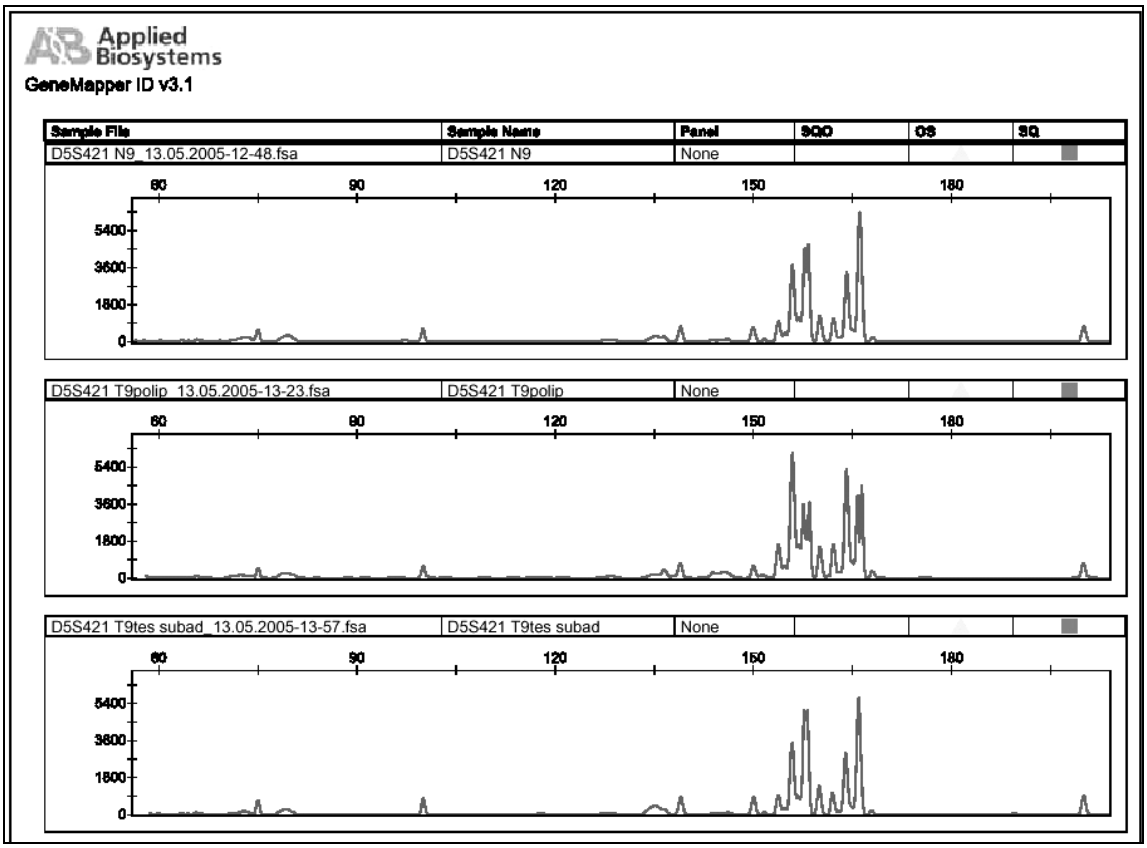

Figure 2. General profile for D5S421 loci at chromosome 5q at the patient 5.