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Loss of heterozygosity at the *BRCA1* locus in Romanian cancer patients

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Abstract

BRCA1 is a tumor suppressor gene and any alteration of its functions can contribute to cancer development and progression. The loss of heterozigosity (LOH), meaning the loss of the normal functional allele in a heterozygous locus of a tumor suppressor such as BRCA1 gene, leads to wild-type gene inactivation and can be correlated with cancer. The role of BRCA1 LOH has already been demonstrated in familial and sporadic breast cancer. Little is known about BRCA1 LOH correlated with other types of familial cancers such as prostate cancer, esophageal cancer or gastric cancer and even less about BRCA1 LOH correlations with the types of sporadic cancers. The aim of this study was to investigate the correlation between distinct BRCA1 alterations and tumor site or cancer progression in Romanian patients with prostate, esophageal and gastric cancer. For this purpose polymerase chain reaction (PCR)-based microsatellite analysis was used to detect LOH occurring in regions mapping for BRCA1 gene. From all the five analyzed microsatellites, three (D17S855, D17S856 and D17S1322) presented loss of heterozygosity in the DNA extracted from the tumor tissue of different patients. 28% of all analyzed patients, presented loss of heterozygosity of at least one microsatellite marker corresponding to BRCA1 gene locus.

Key words. *BRCA1* gene, loss of heterozygosity**,** prostate cancer, esophageal cancer, gastric cancer

Introduction

BRCA1 (BReast CAncer type 1 gene) is a tumor suppressor gene situated on chromosome 17q21 having a length of 100kb and encoding an 1836 amino acids protein [1,2]. It has been demonstrated that BRCA1 protein plays an important role in cell cycle regulation acting as a tumor suppressor [3]. *BRCA1* gene is involved in apoptosis, cell cycle control and genome stability acting upon DNA repair and damage control [4-9]. The role of *BRCA1* in DNA repair explains in fact its main function as a tumor suppressor gene. Any alteration of *BRCA1* functions can contribute to cancer development and progression. However, the loss of these important and fundamental functions of *BRCA1* cannot explain the tissue or gender specificity of cancer risks [10]. Also, sporadic cancer is rarely associated with somatic mutations of BRCA1 which are supposed to be involved in genome instability [11].

BRCA1 was initially correlated with cancer susceptibility by MC King in a study on hereditary breast cancer families [2]. It is shown that mutations in *BRCA1* gene are correlated with 50% of the breast cancers and 80-90% of the breast-ovarian cancers [2, 12, 13].

The development and progression of cancer is associated with some events that include alteration of some genes which regulate cell growth and differentiation: activation of oncogenes and inactivation of tumor suppressor genes. This is the reason why the loss of

heterozigosity (LOH), meaning the loss of the normal functional allele in a heterozygous locus of a tumor suppressor such as *BRCA1* gene, leads to wild-type gene inactivation and can be correlated with cancer [14].

The role of *BRCA1* LOH has already been demonstrated in familial and sporadic breast cancer. In this case the genotype and phenotype features shared by these two groups of breast cancer are similar [15]. Little is known about *BRCA1* LOH correlated with other types of familial cancers such as prostate cancer, esophageal cancer or gastric cancer and even less about *BRCA1* LOH correlations with the types of sporadic cancers.

Prostate cancer is the most frequent cancer in males and the second cause of cancer death [16]. Tumor heterogeneity makes difficult the understanding of the complex molecular genetic mechanisms that lead to the progression of prostate cancer [17]. A small number of studies have revealed the specific allelic imbalances, especially the loss of heterozygosity in the tumor associated DNA of prostate cancer patients [18-23]. LOH discovery in the 17q21 chromosome region corresponding to *BRCA1* gene is associated with a three fold increased risk of mortality caused by prostate cancer [24]. In the same study, *BRCA1* LOH was found in 44% of prostate tumors. Haplosufficiency of *BRCA1* gene is correlated with the presence of circulating tumor cells in the peripheral blood of prostate cancer patients, so it might indicate the metastatic stage of the disease [25].

Esophageal cancer stands among the ten most common cancers in the world and is a fatal disease. The genetic modifications that lead to cancer development and progression are not well understood [26]. LOH studies on esophageal cancer patients revealed a few tumor suppressor genes, including *BRCA1* that are often lost in this type of cancer [27-31]. Also, the LOH detected at 17q21 were significantly more frequent in the tumors of female than male patients [27]. Previous studies also detected a very high incidence of LOH in Japanese and Chinese patients [32].

Gastric cancer has been ranked as the second most common cancer in the 1990s [33]. Even if this cancer incidence is declining, its aggressiveness still leads to just 20% overall survival rate after 5 years from the diagnosis [34]. The locus corresponding to *BRCA1* gene stands among the most frequent five chromosomal loci where LOH is usually found in gastric cancers. The previous LOH studies on young gastric cancer patients stated that *BRCA1* gene could be responsible of an early onset of this disease [35]. Also, Semba *et al.* (1998), found LOH in the region corresponding to *BRCA1* gene in 40% of the gastric cancer analyzed patients [36].

The aim of this study was to investigate the correlation between distinct *BRCA1* alterations and tumor site or cancer progression in Romanian patients with prostate, esophageal and gastric cancer. For this purpose polymerase chain reaction (PCR)-based microsatellite analysis was used to detect LOH occurring in regions mapping for *BRCA*1 gene. The results were statistically correlated with the clinical data for all the investigated patients.

Materials and methods

Patients

Pairs of tumor tissue and blood samples were obtained from patients who underwent surgery at the "Theodor Burghele" Clinical Hospital, Fundeni Clinical Institute, Emergency Clinical Hospital and Emergency Universitary Hospital, all from Bucharest. Patients gave informed consent according to institutional guidelines prior to surgery. All esophageal and gastric tissue samples were diagnosed and staged according to TNM system classification and prostate tissue samples were classified on Gleason and Mostofi system by the pathologists in

the Pathological Department of Hospitals involved in the study. For each patient, data on age at diagnosis, clinical stage, histological tumor type, stage of tumor were recorded. The number of the patients involved in this study was 25, one female, 24 males, with a mean age of 60 years (range: 40-77 years). Ten patients were diagnosed with prostate cancer, ten with esophageal cancer, and five with gastric cancer.

DNA extraction

DNA from tumoral tissue samples was isolated with Wizard Genomic DNA Purification Kit (Promega) and the DNA from peripheral blood separated from whole blood samples was extracted with QIAmp®DNA Blood Minikit (Qiagen). The concentration and quality of DNA was determined spectrophotometrically.

Multiplex PCR

The multiplex PCR was performed using GoTaq® Flexi DNA Polymerase Promega kit on a Corbett Research Termocycler, for the amplification of five highly polymorphic markers belonging to the region of chromosome 17q corresponding to the locus of *BRCA*1 gene (D17S250 with a length of 151bp, D17S800 which has 242bp, D17S855 which has 143bp, D17S856 with a length of 228bp, D17S1322 which has 130bp). Two types of multiplex PCR were performed: a duplex PCR using two primer pairs corresponding to D17S250 and D17S1322 markers and a multiplex using three primer pairs for D17S800, D17S855, and D17S856. For the PCR amplification, all the forward primers were marked with TET (green), 6-FAM (blue), or HEX (black) dye-labelled markers. Briefly 2 µl (approx. 20ng) genomic DNA from peripheral blood and tumor tissue in each case was mixed with 5x Buffer, 1.5mM $MgCl_2$, 0.4 mM dNTP mix, Taq DNA polymerase (1unit), 5pM primers and water up to a final volume of 25 µl. The duplex PCR conditions were as follows: after an initial 3 min denaturation step at 95°C, 30 amplification cycles were performed, each consisting of a 30 s step at 95°C, a 30 s step at 55°C, and a 1 min elongation step at 72°C. Amplification was completed with a final incubation step at 72°C for 20 min. For the multiplex PCR using three different primer pairs the conditions were the same but using a melting temperature of 60°C instead of 55°C.

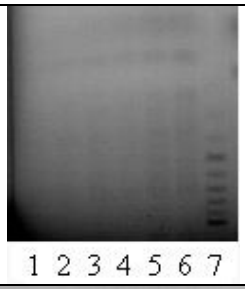
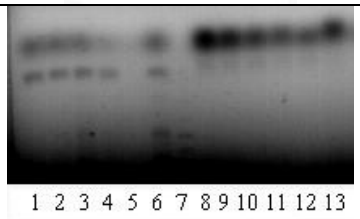
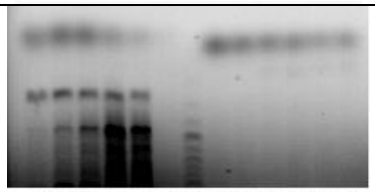
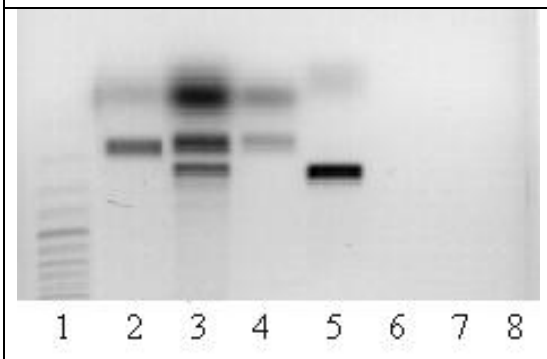
Detection of LOH

PCR products were prepared for capillary electrophoresis with GeneScan-500 ROX TM, used as a size standard marker, and analyzed by capillary gel electrophoresis with fluorescent detection technique (ABIPrism 310 Genetic Analyser). Briefly, 12.5 µl deionized formamide were combined with 0.6 µl GeneScan-500 ROX TM and 1 µl PCR product in a Genetic Analyzer sample tube. Before loading on the Genetic Analyzer, the samples were denaturated for 3 minutes at 95°C in a heat block. The results were visualized and analyzed with GeneMapper 3 software.

Results and Discussions

The optimization of PCR conditions for each target DNA was accomplished using five specific primer pairs marked with three dye-labelled markers (TET, 6-FAM, HEX), for the five microsatellites of interest: D17S250, D17S800, D17S855, D17S856, D17S1322. The amplification of the target DNA fragments was verified through agarose gel electrophoresis (2%), and the optimal annealing temperature specific to each primer pair was established (Fig. 1-3).

Two types of multiplex PCR were used in order to visualize simultaneously the product of several regions, one duplex PCR for D17S1322 and D17S250, and one multiplex PCR for D17S800, D17S855, D17S856. Figure 4 reveals the electrophoretic profile of the amplified DNA fragments corresponding to the two types of multiplex PCR used in our study.

	**Figure 1.** Electrophoretic profile of gradient temperature PCR for intragenic BRCA1 microsatellite D17S1322. Lines1-6 – gradient amplified DNA fragments (60-55°C); line 7- 100bp molecular mass marker
	**Figure 2.** Electrophoretic profile of gradient temperature PCR for intragenic BRCA1 microsatellites D17S800 and D17S855. Lines1-6 – gradient amplified DNA fragments of D17S800 (60-55°C); Lines8-13 – gradient amplified DNA fragments of D17S855 (60-55°C); line 7- 100bp molecular mass marker.
	**Figure 3.** Electrophoretic profile of gradient temperature PCR for intragenic BRCA1 microsatellites D17S856 and D17S250. Lines1-6 – gradient amplified DNA fragments of D17S856 (60-55°C); Lines8-13 – gradient amplified DNA fragments of D17S250(60-55°C); line 7- 100bp molecular mass marker.
	**Figure 4.** The electrophoretic profile of the amplified microsatellites D17S250, D17S800, D17S855, D17S856, D17S1322 through multiplex PCR. Line 1 - 100bp molecular mass marker; line 2 – D17S1322 (130bp) and D17S250 (151bp) DNA fragments amplified through duplex PCR; line 3 - DNA fragments corresponding to D17S855 (143bp), D17S856 (228bp), D17S800 (242bp) microsatellites amplified through multiplex PCR; line 4 - DNA fragment of D17S855 microsatellite (143bp); line 5 - DNA fragment of D17S856 (228bp) and D17S800 (242bp) microsatellites amplified through duplex PCR.

The amplified DNA fragments of interest were analyzed on ABIPrism 310 Genetic Analyser. Loss of heterozygosity was detected based on the analysis made with GeneMapper v3.1 software and the loss of alleles could be determined precisely by calculating the ratio of the peak heights of normal and tumor alleles according to a previously described formula [37]. LOH was correlated with the loss of a wild type allele in tumor DNA compared with the allelic profile observed in peripheral blood DNA set as normal type.

$$LOH = \frac{(\text{peak height of tumor allele 1}) / (\text{peak height of tumor allele 2})}{(\text{peak height of normal allele 1}) / (\text{peak height of normal allele 2})}$$

The ratio of the peak heights is calculated only for the heterozygous loci, where the two alleles corresponding to the locus of interest are different in size, measured in base pairs (bp). In case of homozygosity the two alleles have the same size, thus the corresponding peaks have the same height and are overlapped (Fig. 5).

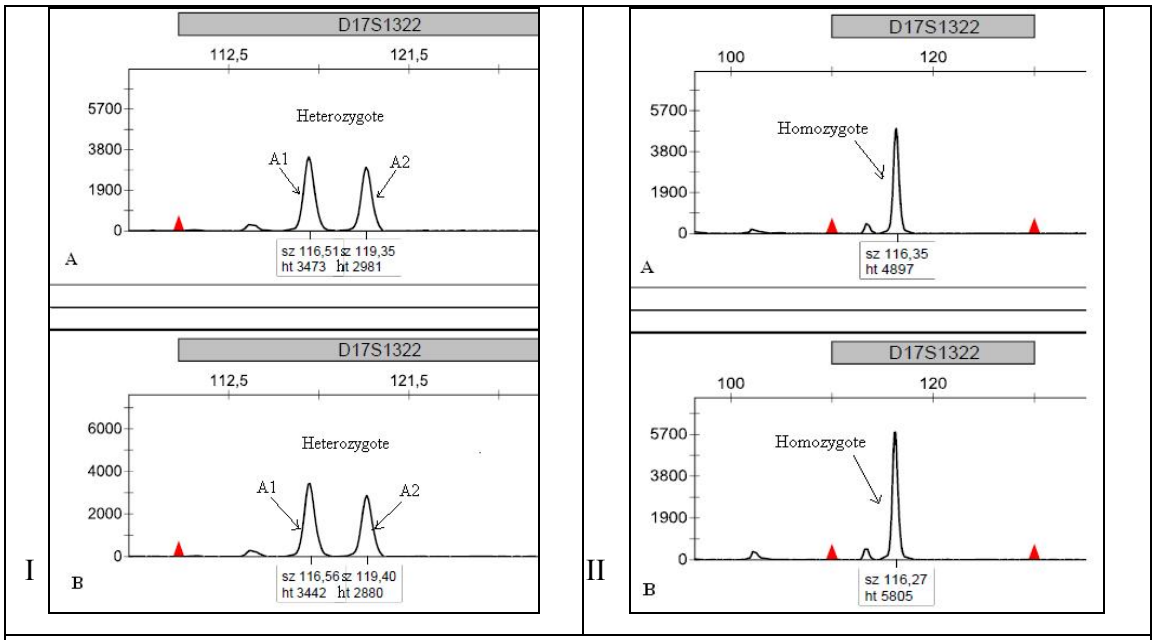


Figure 5. The histogram showing the heterozygous (I) and homozygous allele profile (II) specific to D17S1322 microsatellite locus. A – peripheral blood; B – tumor tissue. Ratio of the peak heights of normal and tumor alleles in case of heterozygous profile = 1.02

From all the five analyzed microsatellites, three (D17S855, D17S856 and D17S1322) presented loss of heterozygosity in the DNA extracted from the tumor tissue of different patients. Seven patients (28%) out of 25 analyzed patients, presented loss of heterozygosity of at least one microsatellite marker corresponding to *BRCA1* gene locus. Also, seven patients were heterozygous for all the analyzed loci, without presenting any LOH (Table 1). Considering all the studied microsatellites, the number of determined heterozygous loci for all patients was 99 (79.2%). LOH corresponding to BRCA1 region was found in 9 out of 99 heterozygous loci (9.09%). There were 26 (20.8%) homozygous loci, all set as non-informative for the present study.

Table 1. Loss of heterozygosity corresponding to the investigated microsatellites in the region of *BRCA1* gene.

Patient no.	Tumor type	Sex	Age (years)	Tumor stage	D17 S250	D17 S800	D17 S855	D17 S856	D17 S1322
1	esophageal tumors	M	54	IV	+	+	+	+	LOH
2		M	56	IIB	++	+	++	++	+
3		M	54	IV	++	++	++	++	++
4		M	50	IIB	++	++	LOH	+	LOH
5		M	59	IB	++	+	++	++	++
6		F	49	I	++	+	+	++	++
7		M	62	IV	++	++	++	+	+
8		M	55	IIA	++	++	++	++	++
9		M	56	IV	++	++	++	+	++
10		M	40	I	++	++	LOH	++	+

11	prostate tumors	M	58	II-III	++	++	++	++	++
12		M	68	II	++	++	++	+	++
13		M	63	III	++	++	++	+	++
14		M	69	III-IV	++	+	+	LOH	++
15		M	59	III	++	++	++	+	+
16		M	75	III	++	++	++	+	++
17		M	66	III	++	++	++	+	++
18		M	62	III	++	+	++	+	+
19		M	58	II	++	++	LOH	+	++
20		M	58	II-III	++	++	++	++	++
21	gastric tumors	M	61	IV	++	++	LOH	++	++
22		M	60	IV	++	++	LOH	LOH	++
23		M	64	IIIA	++	++	++	++	++
24		M	70	IV	++	++	++	++	++
25		M	77	III	++	++	++	++	++

M – male; F – female; LOH – loss of heterozygosity; **(++)** – heterozygote; **(+)** – homozygote

The most affected microsatellite locus was D17S855 (Fig. 6), five patients presenting LOH corresponding to this locus in their tumor tissue. Loss of heterozygosity corresponding to this microsatellite locus was correlated with all types of investigated tumors. Two patients were found with LOH for D17S856 (Fig. 7) and other two for D17S1322 (Fig. 8) in the tumor tissue. Loss of heterozygosity corresponding to D17S1322 could be correlated only with tumors of the esophagus.

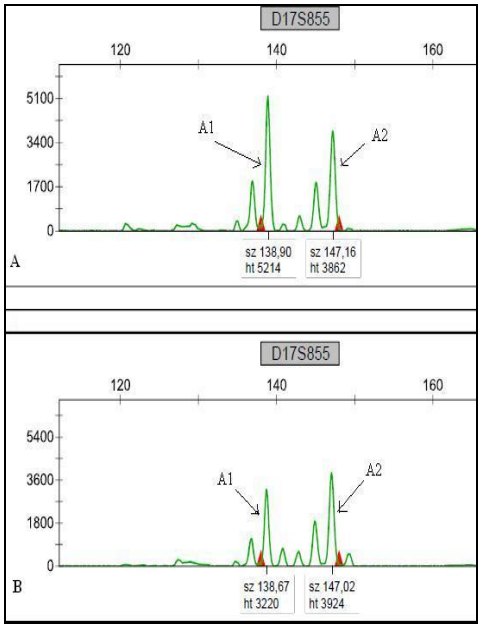


Figure 6. The histogram representing LOH corresponding to the microsatellite locus D17S855. A – peripheral blood; B – tumor tissue; ratio of the peak heights of normal and tumor alleles =0.6

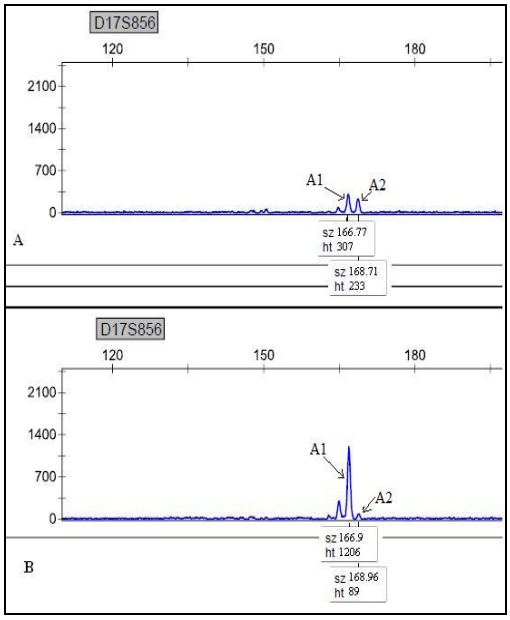


Figure 7. The histogram representing LOH corresponding to the microsatellite locus D17S856. A – peripheral blood; B – tumor tissue; ratio of the peak heights of normal and tumor alleles =10.28

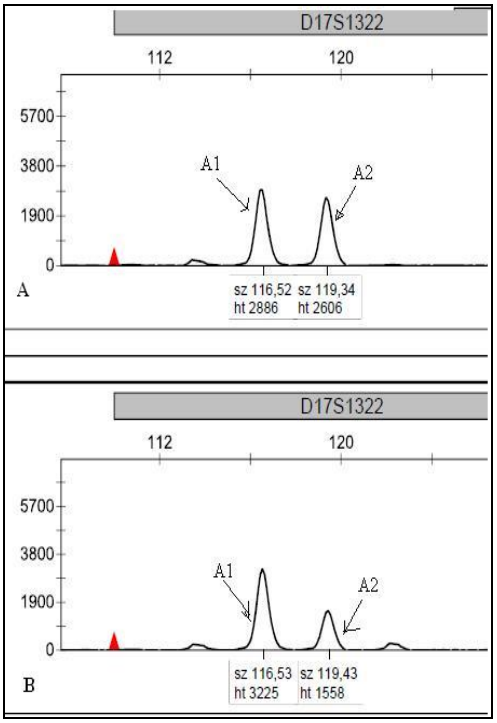


Figure 8. The histogram representing LOH corresponding to the microsatellite locus D17S1322. A – peripheral blood; B – tumor tissue; ratio of the peak heights of normal and tumor alleles =1.87

Considering the three types of tumors, the highest number of LOH was found in the group of gastric cancer patients (3 patients with LOH out of 5 patients, representing 60%), followed by the esophageal cancer group with a frequency of 4 patients with LOH out of 10 (40%) and the prostate cancer group where LOH was observed in 2 out of 10 patients (20%), (Table 2).

LOH found in *BRCA1* gene region could be correlated with an advanced tumor stage only in patients with gastric cancer, where LOH was detected in patients with stage IV. Our results correlate with those of Chen *et al* (2006), who revealed the association of higher frequency of LOH in D17S855 locus with tumor stage III-IV for gastric cancer Chinese patients [38]. Also, the patients with LOH had the lowest age considering the group of gastric cancer patients. There are few studies on LOH corresponding to *BRCA1* region, the most investigated microsatellite being D17S855. Semba *et al.* (1998), found that 40% of all the investigated gastric cancer tumors had LOH in D17S855 locus [36]. Our study confirms their results, we found that 40% of the studied patients have LOH in this locus, respectively.

Table 2. Correlation between the types of investigated cancers and loss of heterozygosity

Type of cancer	Number of patients	Age (years)	TNM Stage	Differentiation degree (Mostofi classification)	Number of LOH
esophageal cancer	10	40-62	I-IV	-	4
prostate cancer	10	58-75	-	II-IV	2
gastric cancer	5	60-77	III-IV	-	3

Considering the group of patients with esophageal cancer, no correlation could be done between LOH in *BRCA1* gene region and the stage of the tumors. The lack of any correlation between LOH on *BRCA1* locus and lymph node metastasis or depth of tumor

invasion was noticed also by Mori *et al*. in 1994. They also found LOH even in tumors at an early stage [27]. However, the detection of LOH slightly relates to the youngest studied patients (40, 50 and 54 years old).

According to our study, prostate cancer is the least correlated with the existence of LOH corresponding to *BRCA1* locus. LOH in this region of chromosome 17 is not a very informative marker for the prediction of this type of cancer. From all 10 studied patients, only 2 had LOH, one in D17S855 locus and one in D17S856 locus. Some previous studies showed the same low proportion of LOH in *BRCA1* region [13]. Other studies report a higher percent of LOH in D17S855 and D17S856 microsatellite loci, and even in D17S250 microsatellite locus [24].

Conclusions

From all the patients analyzed in our study, seven patients (28%) presented loss of heterozygosity of at least one microsatellite marker corresponding to *BRCA1* gene locus.

LOH corresponding to *BRCA1* region was found in 9 out of 99 heterozygous loci (9.09%).

The most affected microsatellite locus was D17S855, five patients presenting LOH corresponding to this locus in their tumor tissue. Loss of heterozygosity corresponding to this microsatellite locus was correlated with all types of investigated tumors.

The highest number of LOH was found in the group of gastric cancer patients (3 patients with LOH out of 5 patients, representing 60%).

The results of our study are preliminary, the three groups of cancer patients that were investigated is small and this is why some precise comparisons between the three types of cancer related to LOH frequency could not be made. These initial results will guide our further extended studies.

Acknowledgments

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