Loss of heterozygosity in the short arm of human chromosome 3 in sporadic lung cancer*

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SUMMARY

Introduction: Loss of Heterozygocity (LOH) in the short arm of human chromosome 3 (3p) is a frequent event in different types of sporadic tumors, including lung cancer (LC).

Aim: To determine 3p LOH in LC samples using 17 microsatellite markers.

Methodology: In a pilot study on volunteers, thirteen LC biopsies (tumor tissue) and 4 ml of blood (normal tissue) from the same patient were collected. DNA extraction and Polymerase Chain Reaction (PCR) were performed with 17 microsatellite markers to analyze LOH. Amplified fragments were run on 6% denaturalizing polyacrilamide gels and were visualized by using silver stain. Descriptive analysis was performed for each region on the 3p chromosome.

Results: All tumors were informative for one or more of the analyzed markers. LOH was found in one or more loci in eleven samples (84.6%). The markers with major LOH were UBE1L (23.1%), D3S1317, D3S1300, D3S1284, D3S1274, D3S3049, and D3S1577 (15.4%). Three samples showed microsatellite instability (changes in the length of the microsatellite) in different loci. The percentages of LOH for the regions of 3p were: 17.6 % for 3p24-25, 11.62% for 3p21-22, 20% for 3p13-14, and 18.42% for the 3p12 region.

Conclusions: Chromosomal regions with allelic loss were identified where probably other GSTs involved in the development of the LC are localized. It should increases sample size and marker number in order to narrow a minimal region and to identify a unknown gene involved in LC.

Pérdida de heterocigocidad en el brazo corto del cromosoma 3 humano en cáncer esporádico de pulmón

RESUMEN

*Introducción***:** La pérdida de heterocigocidad (LOH) en el brazo corto del cromosoma 3 (3p) humano es un evento frecuente en diferentes tipos de tumores esporádicos, incluyendo cáncer de pulmón (CP).

Objetivo: Determinar la LOH de 3p en muestras de CP, con 17 marcadores microsatelitales.

Metodología: En un estudio piloto en voluntarios, se recolectaron 13 biopsias de CP (tejido tumoral) y 4 ml de sangre periférica (tejido normal) del mismo paciente, se extrajo el ADN y se realizaron reacciones en cadena de la polimerasa (PCR)

con 17 marcadores microsatelitales para analizar LOH. Los fragmentos amplificados se corrieron en geles de poliacrilamida desnaturalizante al 6% y se visualizaron por medio de la coloración de tinción de plata. El análisis descriptivo se realizó para cada región estudiada en el cromosoma 3p.

Resultados: Todos los tumores fueron informativos para uno o más de los marcadores analizados. Se encontró LOH en uno o más loci en 11 muestras (84.6%). Los marcadores con mayores LOH fueron UBE1L (23.1%), D3S1317, D3S1300, D3S1284, D3S1274, D3S3049 y D3S1577 con 15.4%. Tres muestras presentaron inestabilidad microsatelital (cambios en la longitud del microsatélite) en diferentes loci. Los porcentajes de LOH para las regiones de 3p fueron: 17.6 % para 3p24-25, 11.6% para 3p21-22, 20% para 3p13-14 y 18.4% para la región 3p12.

Conclusiones: Se identificaron regiones cromosómicas con pérdida alélica donde es probable que se localicen otros GST involucrados en el desarrollo de CP, diferentes de los ya identificados como *VHL, RASSF1A, FHIT y DUTTI*, entre otros. Se debe aumentar el número de muestras y de marcadores para delimitar una región mínima e identificar algún gen no descrito implicado en la carcinogénesis de pulmón.

Lung cancer (LC) represents 12.6% (1'448,000) of new cancer cases in the world; it is the most frequent cause of mortality due to cancer in males (848,132 per year) and the second in females (330,786 per year)[1]. In Colombia nearly 3868 cases/year are diagnosed in a proportion of two men for one woman and there are approximately 3895 deaths per year, which places it as the third cause of deaths due to cancer[2]. LC originates in the respiratory epithelium and it is classified as Small-Cell Lung Cancer (SCLC) and Non-Small-Cell Lung Cancer (NSCLC). Cigarette smoking is the main risk factor causing LC and it is involved in up to 71% of deaths due to LC in Western countries[3]. In addition to tobacco, there are other factors involved in the development of LC related to life style and occupational risk factors that imply being in contact with carcinogens[4].

Tumor Suppressor Genes (TSG) are implied in carcinogenesis, like also proto-oncogene and DNA repair genes; the existence of TSG is suspected when observing frequent loss of specific regions in a specific chromosome. If we correlate absence of locus with tumor development, it can be supposed that its presence avoids tumorigenesis. For a cell to acquire tumor phenotype, loss of TSG function is required, which may or may not be accompanied by over expression of proto-oncogene and there is also accumulation of additional mutations in other genes[5,6].

Karyotypes and analysis of Loss of Heterozygosity (LOH) are routine procedures to detect DNA deletion and serve as TSG location indicators. Several authors postulating LOH occurs because there is loss of the whole chromosome due to inappropriate chromosome segregation during mitosis or that it is also produced because genetic alterations change chromosome structures. Other authors suggesting mitotic recombination and gene conversion are additional mechanisms causing LOH[7]. Detecting genetic changes in lung epithelium is important in order to identify patients at high risk of developing LC. Pre-neoplastic and pre-invasive lesions possess changes like LOH in 3p, 9p, 8p, and 17p arms and mutations in some oncogenes and TSG. Recent studies suggest that these changes are also observed in bronchial epithelium of normal appearance in smokers and former smokers and are even detected in sputum samples obtained from these patients, which serve as molecular biomarkers for early detection of LC[8]. The aim of this study is to determine LOH in human chromosome 3p in 13 samples of sporadic LC using 17 microsatellite markers.

METHODOLOGY

This pilot study was carried out with 13 volunteer patients diagnosed with sporadic LC, without chemotherapy and/or radiotherapy treatment from whom we obtained an informed signed consent previously approved by the respective ethics committees. Through a structured interview, we obtained information on their individual life styles, including cigarette and alcohol consumption. 4 ml of peripheral blood was obtained from each patient (normal control), together with a biopsy of the tumor, which was classified according to the American Joint Committee on Cancer (AJCC). 10 mg of tumor tissue were separated and homogenized with Proteinase K (Sigma, USA), and lymphocytes were isolated from peripheral blood through a centrifuge

Table 1
Characteristics of 3p microsatellite markers

Primer name	Forward sequence (5'-3')	Reverse sequence (5'-3')	T (°C)	Size amplified (bp)	Distance in cM*
D3S1304	TTCGCTCTTTGATAGGC	ATTTCATTTGTAATTTACTAGCAG	50	250-270	19.61
D3S1537	CTATAAAATGGCTATACCCAG	CTATTTTTGGACCCAGTAACC	55	212	23.18
D3S1317	TACAAGTTCAGTGGAGAACC	CCTCCAGGCCATACACAGTCA	58	160	27.68
UBE1	GGAGCCAGCTCAGAGCATAG	CCAGTGCAAACAGGAACCAAG	58	108-117	67.94
D3S2407	TGTACCCTATAGAAGTAAGACCAGG	GGAGTTCAAGGTTACAGTCAGC	55	214	67.94
CAP1	TAGATGAGGAAACTGAGGCC	GGCATAACAGCCATTTGGCA	60	170	70.89
D3S1568	CCATGAACAGAACCTCCCTA	CCGCTGTCCTGCTGTAAG	58	280	70.89
D3S1621	CCAAGGAAGGGTTTTACTTA	CCTCACTACTCCTGGAATTG	55	140	70.89
D3S1289	AAAGCAACTTGTAAGAGAGCA	CTCCTAGATATAATCACTGGCA	55	197-215	73.73
D3S2408	ACCTGCTAACACTGACAGTGC	ACACAGATACCAATGGGTGG	55	189	76.58
D3S1300	AGCTCACATTCTAGTCAGCCT	GCCAATTCCCCAGATG	56	217-241	82.20
D3S1284	GCCTTGGGGGTAAATACTCT	GGAATTACAGGCCACTGCTC	58	155	102.10
D3S1274	TTATACATCAGTCTCTGGGAAACAC	TACTGTGCATATAGGTTCCTGTGA	55	130	107.18
D3S3049	AAAGACACAAGGGGTTTTAGG	TTGCACATTCCATGAACATC	52	170	107.18
D3S1577	TCAAAAGTTGCATCGC	TCCATTACAATCCCCTG	50	230	107.43
D3S2494	AGTTCCTAACATGTGTCAGGTAAG	CTGGCAGTCTCCAAACATCAGAAC	56	252-253	124.16
D3S2510	TTGCCACGGATGTAAGGAAGACAATG	TGATTCAGACCCAGTTAACAG	55	500	125.00

*www.ncbi.nlm.nig.gov/entrez/query.fcgi?db=unists   Distances from telomere to centromere

gradient of FICOLL HISTOPAQUE® 1077 (Sigma, USA). Afterwards, the DNA was extracted from the paired samples of tumor and normal tissue with the Wizard® Genomic DNA Purification kit (Promega, USA). The DNA was quantified and diluted to a 50 ng/µl concentration.

LOH in 3p was determined using 17 microsatellite markers synthesized by Integrated DNA Technologies, Inc, USA (Table 1) and optimized in previous studies[9,10]. Standard Polymerase Chain Reaction (PCR) was carried out in 25µl final volume containing 50 ng/µl of genomic DNA; 1X Ammonium buffer; 1.25 mM of $MgCl_2$; 12.5 pmol of each primer pair; 0.04 mM of each deoxynucleotide (dTTP, dGTP, dCTP, and dATP); 0.5 U of *Taq* Recombinant polymerase (Fermentas Life Sciences, USA). Amplification was done on a PT-100 thermocycler (Sensoquest, Germany) with the following thermal profile: initial denaturation at 94°C for five min, followed by 35 cycles consisting of denaturation at 94°C for 30 s, a specific annealing temperature for each set of primer for 30 s, (Table 1), and an extension at 72°C for 1 min; followed by a final extension at 72°C for 10 min.

The amplified products were run on 6% denaturalizing polyacrilamide gels. The gels were preheated at 1700 volts for 30 min. Formamide loading buffer in a 5:1 ratio was added to the PCR products, which were denaturized at 95°C for 5 min and loading into the gel. Electrophoresis was carried out in a vertical chamber (Sequi-Gen, Bio-Rad, USA) during 4-6 hours at constant voltage depending on size of the fragment to accomplish an adequate separation. Then, silver nitrate staining was carried out. Briefly, the gel was washed in 10% acetic acid for 30 min with constant agitation, rinsed in ddH_2O for 2 min; then it was incubated into 1% nitric acid during 15 min and again rinsed with ddH_2O. Thereafter, the gel was submerged in a silver nitrate and formaldehyde solution for 30 min and rinsed with ddH_2O for 2 min. Then, it was introduced onto a pre-cold developing solution (4ºC) of sodium carbonate,
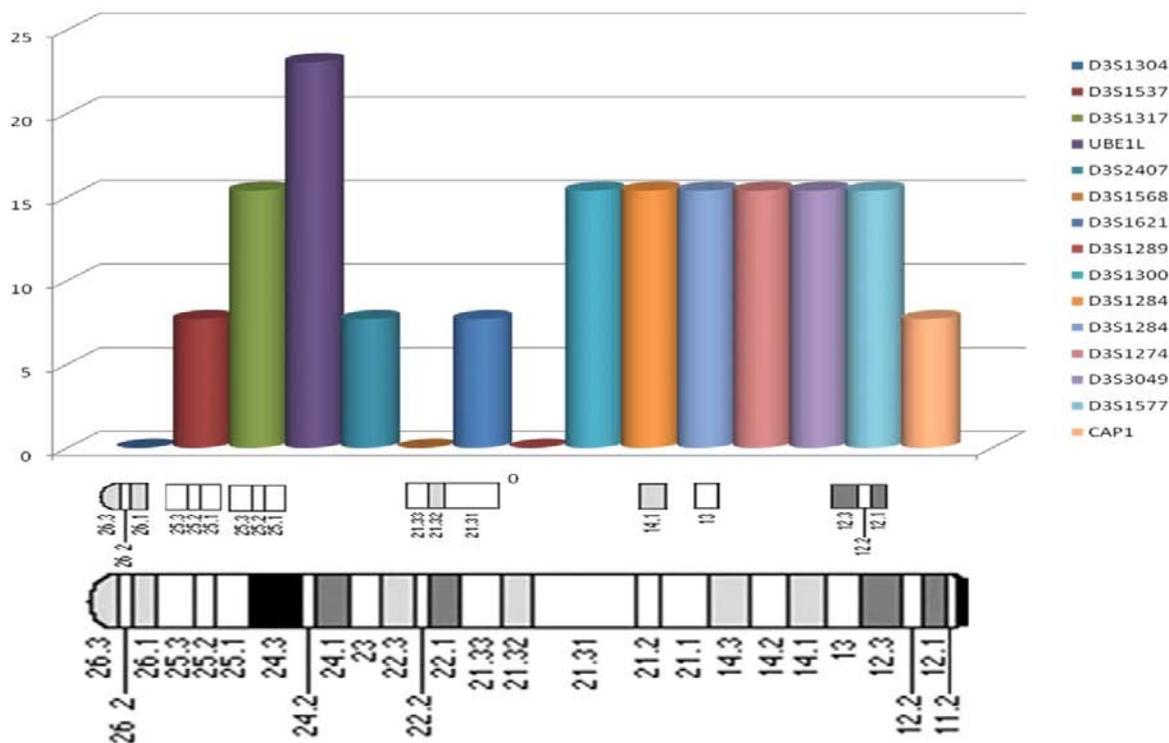
Figure 1. Percentages of LOH for 17 microsatellite markers in 13 samples of human lung cancer

formaldehyde, and sodium thiosulphate until the bands were visualized. Finally, the reaction was stopped with 10% acetic acid and rinsed with running water. The gel was transferred to Whatman 3MM paper and vacuum dried for 90 min at 80°C. LOH was assigned if an allele was absent or exhibited an altered signal in tumor sample with relationship to normal DNA allelic ratio. A descriptive analysis was conducted for each region studied in chromosome 3p.

RESULTS

Patient characteristics are described in Table 2. Patients were in an age range between 49 and 78 years, with a mean of 67 years of age. All were active smokers from an early age, with consumption over 20 cigarettes/day (high consumption). Of these, nine individuals (69.2%) regularly consumed alcoholic beverages (moderate consumption, between 50 and 100 g/day). Two samples (15.4%) were classified as SCLC and ten (77%) were classified as NSCLC. NSCLC samples were classified according to the cellular type involved in malignant transformation; six samples were squamous

cell carcinoma, two bronchogenic squamous cell carcinomas, one adenocarcinoma, and one squamous cell carcinoma. One of the samples presented non-malignant dysplasia; however, molecular study showed important changes which were considered for analyses.

All tumors were informative for one or more of the markers analyzed. In 11 of 13 samples (84.6%) LOH was found in one or more loci. The markers with major LOH were: UBE1L (23.1%), D3S1317, D3S1300, D3S1284, D3S1274, D3S3049, and D3S1577 with 15.4% (Figure 1). LOH percentages for each 3p regions were calculated as: number of LOH in the region/number of informative cases in the same region. The percentages obtained were: 17.6% for 3p24-25, 11.6% for 3p21-22, 20% for 3p13-14, and 18.4% for 3p12 region. In three samples, NT44, NT45, and NT48, microsatellite instability (MSI) and LOH were simultaneously found (Figures 2 and 3). Two squamous cell carcinoma-type NSCLC tumors did not present allelic loss for any loci. One patient, NT55, was found with allelic loss of microsatellite marker D3S1577, flanked by markers CAP1 and D3S3049, both showed allelic retention (Figure 4).

Table 2
Patients and tumor characteristics

Code	Sex	Age	Cigarette consumption	Alcohol consumption	Histopathological diagnosis
NT 43	F	60	High	No	Non-malignant dysplasia
NT 44	M	73	High	Moderate	NSCLC
NT 45	F	49	High	No	SCLC
NT 46	M	73	High	Moderate	NSCLC
NT 47	M	74	High	Moderate	NSCLC
NT 48	M	76	High	Moderate	NSCLC
NT 49	F	61	High	No	NSCLC
NT 50	M	67	High	Moderate	NSCLC
NT 51	M	78	High	No	SCLC
NT 52	M	64	High	Moderate	NSCLC
NT 53	M	70	High	Moderate	NSCLC
NT 54	M	68	High	Moderate	NSCLC
NT 55	M	62	High	Moderate	NSCLC

F: female;   M: Male;   NSCLC: non-small-cell lung cancer;   SCLC: small-cell lung cancer
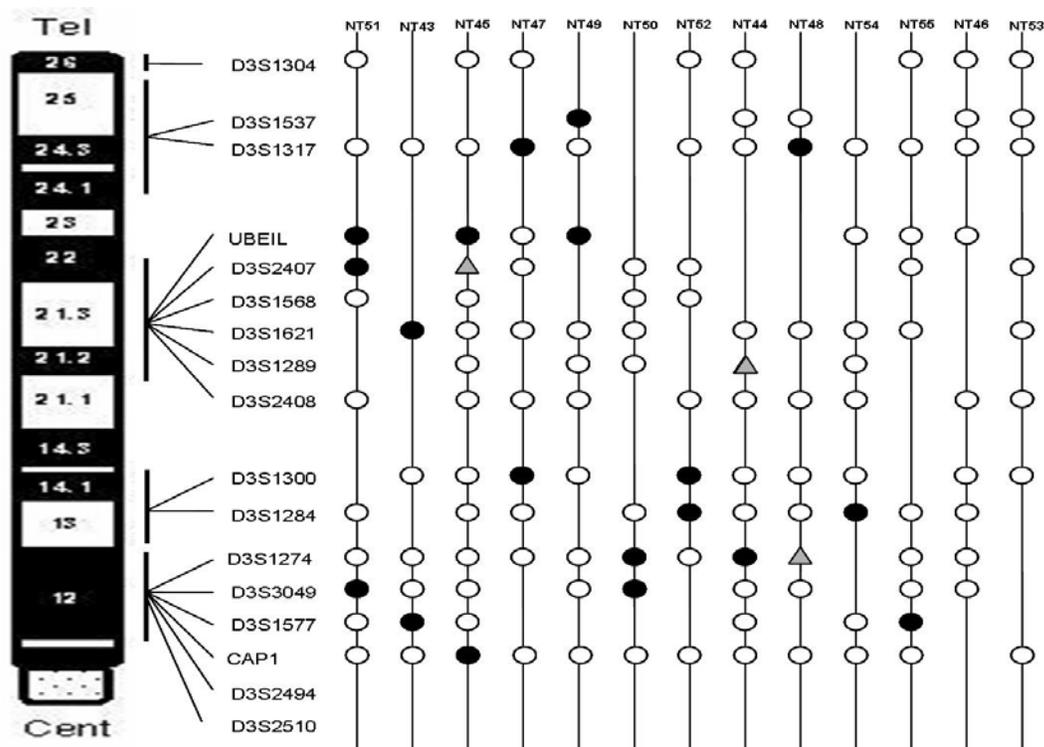

Figure 2. LOH and MSI patterns detected in 13 cases of lung cancer

● Loss of heterozygosity;   ○ Retention of heterozygosity; | Not informative;   ▲ Microsatellite instability
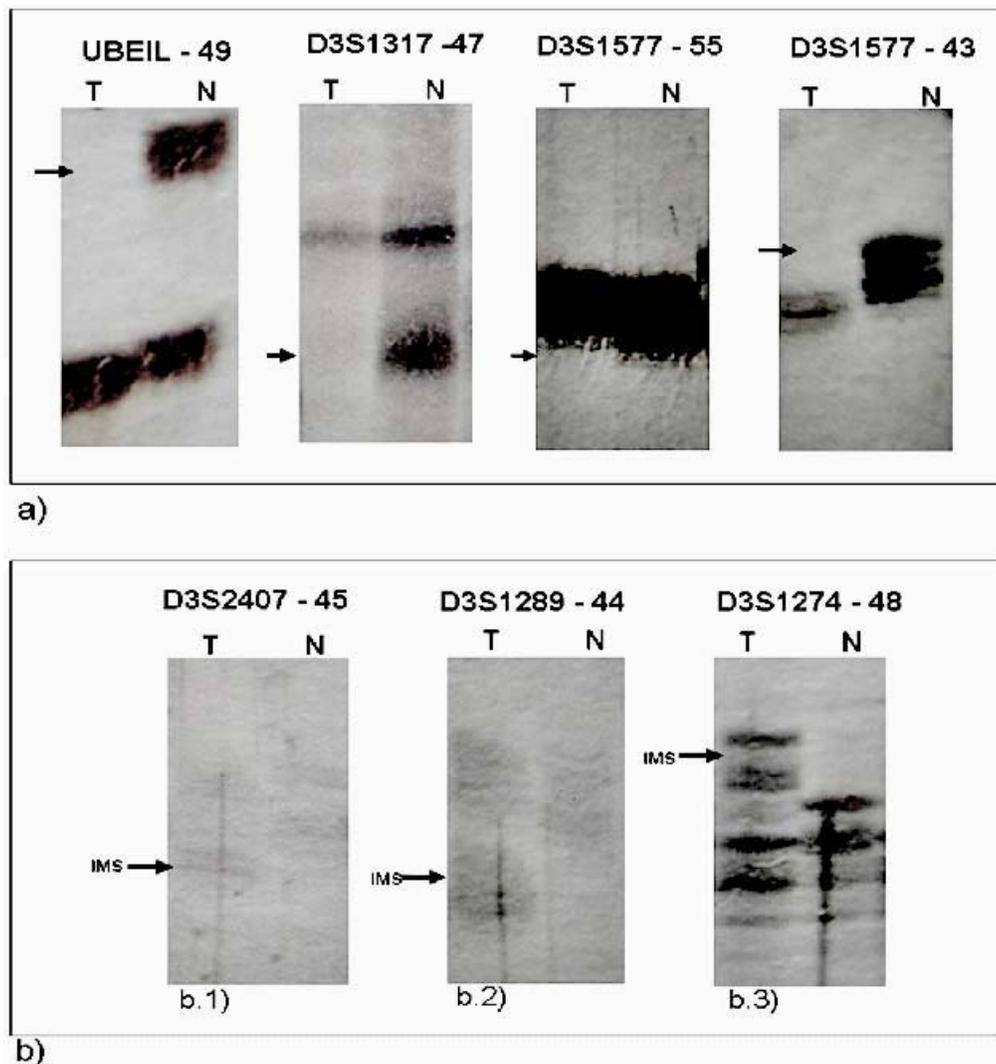
Figure 3. a) Examples of LOH in patients with LC. b) Examples of MSI; b.1) and b.2) located in the 3p21-22 region and b.3) in the 3p12 region. Arrows indicate chromosomal alteration

DISCUSSION

Tobacco smoking is the main risk factor for developing LC and other types of cancer[11,12]. Furthermore, consumption of alcoholic beverages is also an important factor in lung, mouth, larynx, pharynx, stomach, and liver tumorigenesis when consumption overpasses 100 g/day[4]. Patients studied were active smokers for several years (high consumption) and were moderate alcohol drinkers. A meta-analysis performed by Lubin *et al.*[13], calculated that 25-68% of cancers associated to upper aero-digestive tract are related to alcohol consumption and the incidence increases in up to 80% when is combined with cigarette smoking. The same study reports that the combined effect of alcohol and cigarette smoking increases the risk of developing LC up to 80 times, which indicates the multiplicative effect of both. It is suggesting the first carcinogenic event is caused by tobacco and gives the affected cell a selective advantage that together with alcohol consumption favors accumulation of new cell mutations and alterations that in the end would manifest as a tumor phenotype.

In the 3p24-25 region, three LOH (18.8%) were found of the total found in 13 patients. Previous reports
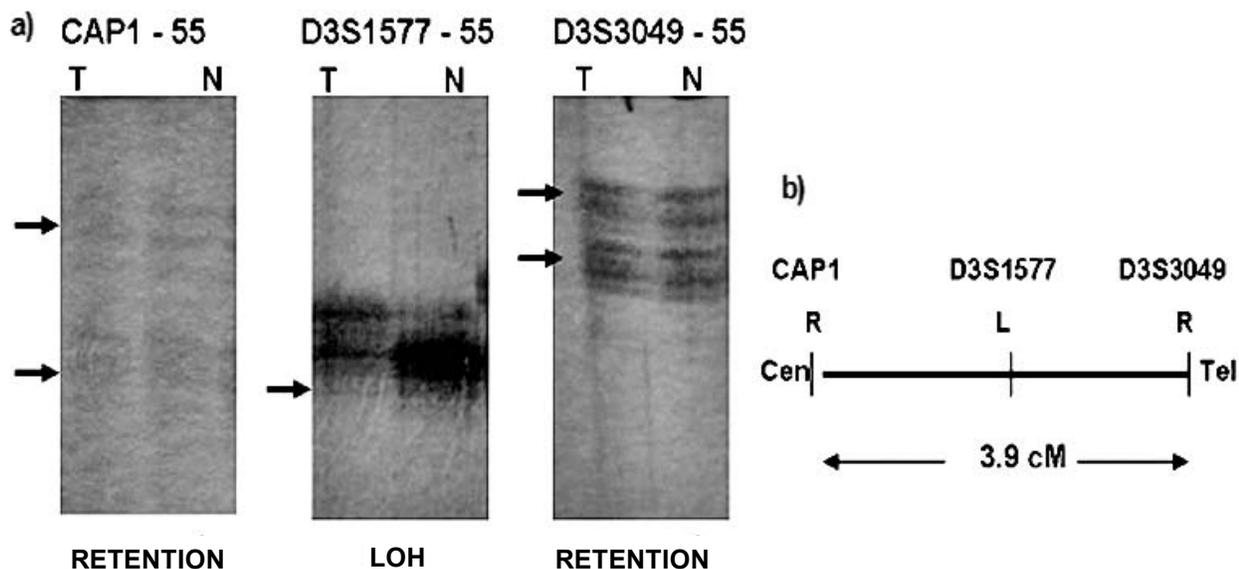
Figure 4. Case NT55. a) The central image shows LOH in the D3S1577 marker, flanked by the microsatellite markers CAP1 and D3S3049, which present retention. b) Schematic representation of the location of the microsatellite markers and the distance in cM

located and identified some genes related to tumor development as *VHL, VHI,* and *RARβ*, which are flanked by microsatellites D3S1537 and D3S1317. Miyakis *et al.*[14]*,* demonstrated that mutations and LOH in *VHL* gene are not common events in NSCLC; nevertheless, they found LOH in regions adjacent to this gene in squamous cell carcinoma and adenocarcinomas at 74.6% and 49.3%, respectively. It is postulated that other still not identified genes located in this region would be genetically altered and involved in tumorigenesis. Our results are agree with the high frequency of LOH in this region; although, it is not comparable with previous study probably due to the low number of microsatellites employed to cover this 4.5 cM region.

For the 3p21-22 region, we used six microsatellite markers covering a distance of 16.58 cM, where five LOH (11.7%) were found. This region contains the Lung Cancer Tumor Suppressor Gene Region 1 (LCTSGR1), where genes as *RASSF1A, HYAL2/LUCA, FUS1/TUSC2, 101F6/CYB561D2,* and *CPR2L/CPRL2/ TUSC4*, are located. Previous studies reporting *RASSF1A* gene presents LOH in up to 75% of NSCLC[15]. Other authors reported that *HYAL2/LUCA, FUS1/ TUSC2, 101F6/CYB561D2,* and *CPR2L/CPRL2/ TUSC4* genes presented genetic changes (LOH, point mutations, deletions, and epigenetic changes in different

types of cancers and tumor-cell lines); however, they did not identify LOH in LC biopsies[5,16]. The results herein reported show a high frequency of LOH; even though, we cannot guarantee that it corresponds to *RASSF1A* gene or to another gene located in this region, which will be the next step to study in our samples.

In 3p13-14 region with a distance of 19.9 cM between markers D3S1300 and D3S1284, four cases of LOH (20%) were identified. The most fragile site of the genome is located in this region and the *FHIT* gene is located here, which is involved in development of different types of cancer. Zabarosky *et al.*[17]*,* refer to some studies demonstrated that up to 50% of LC cases present LOH in *FHIT* gene. They also reported that another gene located in this region, *FOXP1,* presents loss of expression in 51% of cancers analyzed; however, they did not report LOH in LC samples. Our results are similar to those reported for this region. 3p12 region, contains the Lung Cancer Tumor Suppressor Gene Region 2 (LCTSGR2), where the *DUTT1* gene is located; to analyze this region, we used five markers covering a 17.82-cM distance and found the greatest number of LOH, 7 (18.5%) for this study. Dallol *et al.*[18]*,* demonstrated that hypermethylation occurs in *DUTT1* gene in up to 4% of LCs and observed LOH for some markers in 3p12 region. Our results show a smaller

difference than that reported in similar studies[19]. For this region, patients NT43, NT51, and NT55 showed LOH in a microsatellite marker flanked by two markers that showed retention. It suggesting this region is very important in the development of tumorigenesis and there could be a yet unidentified candidate TSG (Figure 2).

There were three cases of microsatellite instability (MSI) in markers D3S2407, D3S1289, and D3S1274 (Figure 2). MSI are changes in the length of the microsatellite marker by insertions or deletions of repetitions in tumor tissue when compared with normal tissue; these alterations may occur due to defects in the DNA repair system (Mismatch Repair - MMR). Nevertheless, others studies identified microsatellite alterations like LOH and MSI in 80.5% of LC samples for chromosomes 3p, 5q, 8p, 9p, 9q, and 17q. Additionally, studies reporting genes involved in DNA repair do not present changes in their expression pattern in SCLC cellular lines and that MSI is a bio-marker for early detection of LC, mainly in NSCLC[20].

In conclusion, chromosomal regions were found with high frequency of LOH where TSG involved in LC development are probably located. This work will permit conducting another study in which the number of samples is increased and comparing to a control group with similar characteristics as the cases, together with increasing the number of informative microsatellite markers for the Colombian population to narrow a minimal region to a few hundred base pairs to permit identifying some yet unknown gene important in lung carcinogenesis, because it was determined that LOH phenomenon is a critical and early genetic event for onset and development of the disease. Detecting LOH in tissues of normal appearance surrounding tumor tissue or in tissues with early stages of the disease will lead to early diagnosis and to efficient preventive and curative treatment. Finally, it must be stressed that this is a pioneering study on LOH conducted in a Colombian population.

Conflict of interest. None of the authors has conflicts of interest related to this study.

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