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High frequency of p16 promoter methylation in non-small cell lung carcinomas from Chile

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ABSTRACT

The inactivation of tumour suppressor genes by aberrant methylation of promoter regions has been described as a frequent event in neoplasia development, including lung cancer. The p16 gene is a tumour suppressor gene involved in the regulation of cell cycle progression that has been reported to be inactivated by promoter methylation in lung carcinomas at variable frequencies around the world in a smoking habit dependent manner. The purpose of this study was to investigate the methylation status of the promoter region of the p16 gene in 74 non-small cell lung carcinomas from Chile. The frequency of p16 gene inactivation by promoter methylation was determined as 79.7% (59/74). When we considered histological type, we observed that p16 promoter methylation was significantly higher in squamous cell carcinomas (30/33, 91%) compared with adenocarcinomas (21/30, 70%) (p=0.029). In addition, no association between p16 promoter methylation and gender, age or smoking habit was found (p=0.202, 0.202 and 0.147 respectively). Our results suggest that p16 promoter hypermethylation is a very frequent event in non-small cell lung carcinomas from Chile and could be smoking habit-independent.

Key terms: lung carcinoma, methylation, promoter region.

INTRODUCTION

Lung cancer (LC) is the most common cause of cancer-related mortality in men and women around the world [Pisani et al., 2002; Greenlee et al., 2001]. In Chile, LC is the second cause of death in men and third in women [Szot, 2003]. LC is classified into 2 major types: small-cell lung carcinomas (SCLCs) and non-small cell lung carcinomas (NSCLCs). In Chile, 75% of LC cases are NSCLCs and squamous cell lung carcinomas (SQCs) range between 25% to 50% of the cases and have been associated to chronic smoking habit [Wistuba et al., 2002; Shields, 2002]. LC is the most deadly and aggressive cancer, and the prognosis is limited by the difficulties of diagnosing early stages. The conventional analysis of sputum, cytological or bronchioalveolar lavage associated to thorax radiological screening has not improved the survival of this disease [Cassidy et al., 2007]. The

rapid advancement in molecular techniques may help to identify novel new diagnostic markers with high sensitivity and prognostic value for early detection of LC in high-risk subjects, such as chronic smokers [Wistuba, 2007].

It has been demonstrated that aberrant CpG island methylation in the promoter region of tumour suppressor genes (TSGs) is an important epigenetic mechanism of gene silencing [Callinan and Feinberg, 2006]. The TSGs inactivation by hypermethylation is a common event described in human cancer, including LC [Esteller, 2003; Herman and Baylin, 2003]. In lung carcinomas, more than 30 silenced genes have been reported to be methylated such as p16^{INK4a} (p16), RAR-β (retinoic-acid receptor β) and E-cadherin [Zochbauer Muller et al., 2001]. The p16 TSG located on 9p21 chromosome encodes a cyclin dependent kinase, a key protein regulator of progression through the G1 phase of the cell cycle [Berger and Bardeesy, 2007]. The p16 protein plays an important role in the binding and inhibition of cyclin D kinase activity and then regulating the phosphorylation of the retinoblastoma protein (p105Rb) [Wikenheiser-Brokamp, 2006].

Recent studies demonstrated that aberrant p16 promoter methylation is an early and critical event in the NSCLCs development [Hilbe et al., 2004], and it has been observed in serum and sputum of chronic smokers without clinical disease [Belinsky, 2007]. It has been reported that aberrant p16 promoter methylation is smoking habit associated, however TSGs methylation in non-smoker subjects has been detected [Liu et al., 2006; Nakata et al., 2006]. In addition, p16 promoter methylation has been shown to correlate with the progression of the malignancy in LC patients [Tanaka et al., 2005]. Taken together, p16 inactivation by promoter hypermethylation may be a useful precocity marker of LC development in high-risk subjects such as heavy smokers. Moreover, methylation is a reversible epigenetic event, thus the use of some drugs such as 5-deoxyazacytidine would be a very important future tool for the treatment of LC patients [Momparler, 2005]. We previously reported a high p16 promoter methylation

prevalence in SQCs from Santiago de Chile [Adonis et al., 2006], and now our goal is to increase the size of analyzed samples including Adenocarcinomas (ACs) and Large cell carcinomas (LCCs) and establish clinical associations with other clinical parameters such as smoking habit.

MATERIALS AND METHODS

Patients and Samples

Primary tumour specimens were obtained from 74 patients undergoing surgical resection or biopsy of SCLC. There were 33 SQCs, 30 ACs, 8 LCCs and 3 specimens without histological information. Twenty-two samples were fresh frozen tissue and 52 specimens corresponded to archival paraffin embedded tissue. The specimens collected prospectively (fresh tissue) were snap-frozen at -80°C after initial histopathological examination and were diagnosed with stage I to III at Instituto Nacional de Enfermedades Respiratorias, Santiago, Chile. The paraffin embedded tissue specimens were diagnosed in the Hospital Salvador, Santiago, Chile during the period between 1998 and 2003.

DNA extraction and Methylation-Specific PCR

Ten μm-thick sections of each formalin-fixed paraffin-embedded sample were used. The samples were treated with 1 mL of xylene, and then with 1 mL of ethanol. After centrifugation, the pellet was resuspended in digestion buffer (50 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0, 0.5% Tween 20) containing 200 μg of Proteinase K (Life Technologies, Inc) and incubated during 24 hours at 56°C. Then the solution was heated to 100ºC for 10 min and centrifuged to 10,000 rpm for 10 min. Phenol-chloroform extraction was made in all the samples and the DNA was precipitated with double volumes of ethanol [Coombs et al., 1999]. Genomic DNA was purified from frozen tumours by digestion with 200 μg/mL Proteinase K (Life Technologies, Inc) for incubation at 50ºC overnight, followed by extraction using the Wizard genomic DNA

purification kit (Promega Corp., Madison, WI), following the instructions of the manufacturer. After the DNA purification, the adequacy of the samples for PCR was determined by amplification of the beta-globin gene. The primer sequences are indicated in Table I. For analysis of methylation patterns within the 5'-CpG island of the promoter region and upstream region of exon 1 of p16 gene, 1μg of purified genomic DNA from each sample was used. The DNA was denatured with sodium hydroxide and modified with sodium bisulfite (Sigma Chemical Co., St Louis, MO), as described previously [Herman et al., 1996]. The modified DNA was purified using the Wizard Cleanup system (Promega Corp., Madison, WI). In order to amplify p16 methylated and non-methylated regions a nested MSP protocol was used as described [Fan X et al., 2002; Herman JG et al., 1996]. Primer sequences for both the methylated and the unmethylated form of the p16 promoter region, annealing temperature, and the expected PCR product sizes are summarized in Table I. In order to amplify p16 promoter fragment in the first PCR (outer primers), 2 μL of modified DNA were used and the reaction mix contained 1X PCR buffer (16.6 mM $(NH_4)_2SO_4$, 67 mM Tris pH: 8.8, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 1.2 mM dNTPs, 1.6 mM each primer, 2.5 U Taq Gold DNA polymerase (Applied Biosystem) and 5.2% DMSO in a final volume of 25 μL. The PCR conditions were initial denaturation at 94ºC for 7 min, 35 amplification cycles of denaturation at 94ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72ºC for 30 s. Using 1 μL of the first PCR product, a second PCR using inner methylated and unmethylated specific primers and 40 cycles of amplification was made. The annealing temparature was adjusted at 55ºC. Genomic DNA from gallbladder treated with SssI methyltransferase (New England Biolabs) served as a positive control. Negative controls without DNA were included. The hypermethylation status of the p16 promoter region was determined by analysis of PCR products in 8% polyacrylamide gels after silver nitrate staining.

Statistical analysis

Statistical analyses were performed using Fisher's exact test and two sided p-values were presented. A *P* value less than 0.05 was considered statistically significant.

RESULTS

In this study we determined the frequency of p16 promoter hypermethylation in NSCLCs from Chile confirmed by histology. We used a MS-PCR method that allows to detect methylated and unmethylated regions in the genome [Adonis et al, 2006]. The primers used in this report are described in Table I. All the used NSCLC specimens for methylation analysis were beta-globin gene positive because of the positive amplification of a 110 bp fragment in the

TABLE I

Primers sequences used in this study

Gene	Primer sequence	Annealing T (ºC)	Size band (pb)
p16[INK4a]-E	AGAAAGAGGAGGGGGTTGGTTGG (forward) ACRCCCRCACCTCCTCTACC (reverse)	56	193
p16[INK4a]- M	TTATTAGAGGGTGGGGCGGATCGC (forward) GACCCCGAACCGCGACCGTAA (reverse)	55	150
p16[INK4a]- UM	TTATTAGAGGGTGGGGTGGATTGT (forward) CAACCCCAAACCACAACCATAA (reverse)	55	151
β-globina	ATAGACCAATAGGCAGAGAGAGTCA (forward) TCAAGGTTACAAGACAGGTTTAAGG (reverse)	50	110

samples. The clinico-pathological features of the analyzed samples and controls are described in Table II. In Figure I the specific amplification of two representative samples and controls are shown. The results of p16 promoter methylation status in all the samples are shown in Table III. Our results indicated that 59/74 (73%) lung carcinomas were p16 promoter methylation positive. In addition, the p16 promoter methylation frequency considering histological type was 91% (30/33) for SQC, 70% (21/30) for AC and 88% (7/8) for LCC. The difference between SQCs and ACs was statistically significant (p=0.029). No association between p16 promoter methylation and age (p=0.202), gender (p=0.202) or smoking habit (p=0.147) was found. The differentiation grade in NSCLC was not related to p16 promoter methylation status (Table 4). However in AC we found that p16 promoter methylation was more frequent in highly differentiated (7/8, 87.5%) compared with moderately (7/12, 58.3%) or poorly (6/9, 66.7%) differentiated carcinomas. These differences were not statistically significant (Table 4, p=0.163).

TABLE II

Clinicopathological features of the patients used in this study

		Number of subjects (%) LC
Total		74 (100)
Gender	Male	49 (66)
	Female	25 (34)
Age (years)		66± 9
Histological type	SQC	33 (45)
	AC 30	(41)
	LCC 8	(11)
	ND 3	(4)
Smoking	Non-smoker	11 (15)
	Smoker	58 (78)
	ND	5 (7)
Differentiation	High	15 (20)
	Moderate	30 (41)
	Poor	25 (34)
	ND	4 (5)

ND: non determined

DISCUSSION

Epigenetic alterations such as CpG island methylation of promoter regions of TSGs have been reported to be a very frequent event in lung cancer development [Esteller M, 2002]. In 1995 the frequent p16 gene inactivation by methylation in diverse cancers was reported [Herman et al., 1995] and this methylation implied loss of p16 transcriptional expression that was reversible after treatment with 5-deoxyazacytidine [Merlo et al, 1995]. After these findings, other reports showed that the p16 promoter region was methylated in lung cancer at frequencies between 20 to 70% [Belinsky et al., 1998; Nuovo et al., 1999]. Previously, we reported that the p16 promoter is methylated in 86% of patients with SQC, a high prevalence compared with other studies around the world [Adonis et al., 2006]. For this reason, we decided to increase the size of our initial study, including other histological types as ACs and LCCs. In this report, a very frequent p16 promoter methylation in NSCLCs from Chile was found (79.7%). In addition, when we stratified by histological type we observed that p16 promoter methylation was higher in SQCs (90.9%) compared with ACs (70%) and this difference was statistically significant (p=0.029). Overall, the p16 promoter methylation has been reported more frequently in SQCs compared with ACs [Belinsky, 2004; Digel et al., 2005] and accordingly with the frequent central location of SQCs compared with the peripheric location of ACs [Travis et al., 2004; Wistuba, 2007]. This higher p16 promoter methylation in SQCs is probably related to smoking habit which is considered a risk factor for SQC development.

Some reports described a significant association between methylation of some genes such as p16 or MGMT and smoking habit [Liu et al., 2006; Nakata et al., 2006; Toyooka et al., 2003]. However in our study we found that p16 promoter methylation was independent of smoking habit. Moreover, p16 promoter methylation was more frequent in non-smokers compared with smokers, although this

difference was not statistically significant (91% vs. 72% respectively, p=0.147). This finding is compatible with another study [Sanchez-Cespedes M et al., 2001] where the authors showed that tumours from non-smokers arise exclusively through inactivation of p16 gene by promoter hypermethylation. However the frequencies of p16 promoter methylation were lower in both smokers and non-smokers compared with our report. In addition, it has been reported that MGMT gene (O6-methylguanine-DNA methyltransferase) methylation was more frequent between non-smokers compared with smokers [Pulling et al., 2003].
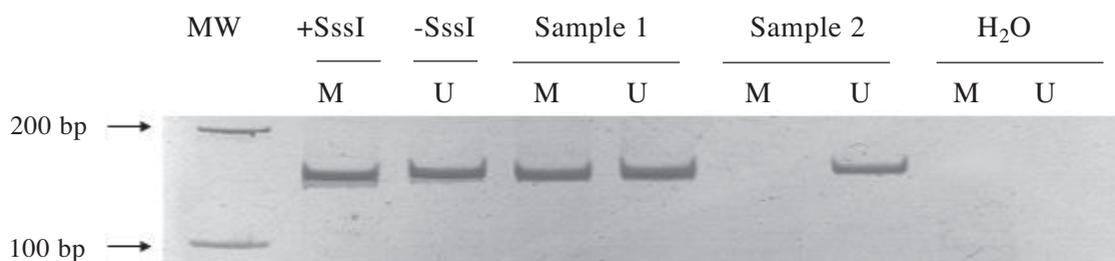

Figure 1: MS-PCR for P16 promoter methylation in NSCLCs specimens. Bisulphite modified DNA was amplified with p16 specific primers to detect methylated (M) and unmethylated (U) alleles. MW: Molecular weight marker; +SssI: Genomic DNA treated with SssI enzyme; -SssI: Genomic DNA untreated with SssI enzyme; Sample 1 and 2: Specimens positive and negative for methylated condition; H_2O: distilled water used as negative control.

TABLE III

P16 promoter methylation status and clinicopathological features in NSCLC patients

Variable		N	Methylated	(%)	P value
Total	(NSCLCs)	74	59	79.7	
Age	≤ 70	49	38	77.6	0.202
	> 70	25	21	84.0	
Gender	Male	49	38	77.6	0.202
	Female	25	21	84.0	
Smoking	Nonsmokers	11	10	90.9	0.147
	Smokers	54	39	72.2	
	≤ 100 pack/year	11	10	90.9	
	> 100 pack/year	43	33	76.7	
	ND	9	7	77.8	
Histology	AC	30	21	70.0	0.029*
	SQC	33	30	90.9	
	LCC	8	7	87.5	
	ND	3	1	33.3	
Differentiation	High	15	13	86.7	0.241
	Moderately	30	23	76.7	
	Poor	22	18	76.0	
	ND	7	4	57.1	

ND: Non determined

TABLE IV

P16 promoter methylation status and differentiation grade in SQCs, ACs and LCCs

		Total	Methylated	(%)	P value
ADC	High	8	7	87.5	0.163
	Moderate	12	7	58.3	
	Poor	9	6	66.7	
SQC	High	7	6	85.7	
	Moderate	17	15	88.2	
	Poor	8	8	100	
LCC	High	0	0	0	
	Moderate	1	1	100	
	Poor	5	4	80	

In 2002, Belinsky et al. reported that exposure to particulate carcinogens such as diesel exhaust, carbon black and beryllium metal is associated with aberrant p16 promoter methylation [Belinsky et al., 2002]. In addition, the carcinogenic role of polycyclic aromatic hydrocarbons (HAP) present in smoke fumes and environmental contamination in lung cancer development has been well established [Gil et al., 2000; Hecht, 1999]. The carcinogenic mechanism implies adduct formation in the genetic material and is highly dependent of host factors [Hecht, 1999]. These observations are relevant to this study because the most of the patients come from Santiago (Salvador Hospital and Thorax Hospital), one of the most polluted cities worldwide [Adonis et al., 2000; Gil et al., 2000]. These compounds include polycyclic aromatic compounds as benzo-α-pyrene which is a potent carcinogen. How the carcinogens present in the air, including cigarette fumes, may be involved in aberrant TSG methylation such as p16 gene, remain to be elucidated. The very high frequency of p16 promoter methylation detected in this study compared with previous reports [Belinsky, 2004] and moreover, the very high p16 promoter methylation prevalence in nonsmokers (91%, Table II) allows us to speculate that other environmental factors acting synergistically or not, are involved in this epigenetic alteration. These other factors may be related to cigarette smoke exposure which is difficult to evaluate in the population within environmental pollution and contamination such as ocurs in Santiago de Chile.

The p16 promoter methylation has been suggested as an early detection marker of lung cancer. In fact, p16 promoter methylation has been detected in early lesions and preneoplastic lesions of the lung. In SQC, p16 promoter methylation has been suggested to occur in squamous dysplasia and metaplasia and then it is possible to detect it in high-risk individuals such as heavy smokers [Wistuba, 2007] when no clinical evidence is observed. It has been reported that p16 methylation may be detected in serum or sputum from heavy smokers three years previous to clinical detection by conventional methods [Belinsky et al., 2006] as radiology or sputum cytology. Future studies should be focused on this area.

In conclusion, our report showed high p16 promoter methylation prevalence in NSCLCs from Chile that was significantly more frequent in SQCs compared with ACs. The p16 promoter methylation was smoking habit-independent.

REFERENCES

ADONIS M, GIL L (2000) Polycyclic aromatic hydrocarbon levels and mutagenicity of inhalable particulate matter in Santiago, Chile. Inhal Toxicol 12: 1173-83

ADONIS W, AGUAYO F, CORDERO E, RODRÍGUEZ L, CASTILLO D and GUZMÁN L (2006). Evaluación de hipermetilación del gen p16INK4 _ en cáncer escamoso de pulmón en pacientes chilenos, Rev Ch Enf Resp, 22: 7-12

BELINSKY SA, NIKULA KJ, PALMISANO WA, MICHELS R, SACCOMANNO G, GABRIELSON E, BAYLIN SB, HERMAN JG (1998) Aberrant methylation of p16 (INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A 95: 11891-11896

BELINSKY SA, SNOW SS, NIKULA KJ, FINCH GL, TELLEZ CS, PALMISANO WA (2002) Aberrant CpG island methylation of the p16(INK4a) and estrogen receptor genes in rat lung tumours induced by particulate carcinogens. Carcinogenesis 23: 335-339

BELINSKY SA (2004) Gene-promoter hypermethylation as a biomarker in lung cancer. Nat Rev Cancer 4: 707-717

BELINSKY SA, LIECHTY KC, GENTRY FD, WOLF HJ, ROGERS J, VU K, HANEY J, KENNEDY TC, HIRSCH FR, MILLER Y, FRANKLIN WA, HERMAN JG, BAYLIN SB, BUNN PA, BYERS T (2006) Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. Cancer Res 66: 3338-3344

BELINSKY SA, GRIMES MJ, CASAS E, STIDLEY CA, FRANKLIN WA, BOCKLAGE TJ, JOHNSON DH, SCHILLER JH (2007) Predicting gene promoter methylation in non-small-cell lung cancer by evaluating sputum and serum. Br J Cancer 96: 1278-1283

CALLINAN PA, FEINBERG AP (2006) The emerging science of epigenomics. Hum Mol Genet 15 Spec No 1: R95-101

CASSIDY A, DUFFY SW, MYLES JP, LILOGLOU T, FIELD JK (2007) Lung cancer risk prediction: a tool for early detection. Int J Cancer 120: 1-6

COOMBS NJ, GOUGH AC, PRIMROSE JN (1999) Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic Acids Res 27: e12

DIGEL W, LUBBERT M (2005) DNA methylation disturbances as novel therapeutic target in lung cancer: preclinical and clinical results. Crit Rev Oncol Hematol 55: 1-11

ESTELLER M (2002) CpG island hypermethylation and tumour suppressor genes: a booming present, a brighter future. Oncogene 21: 5427-5440

ESTELLER M (2003) Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv Exp Med Biol 532: 39-49

FAN X, INDA MM, TUÑON T AND CASTRESANA JS (2002) Improvement of the methylation specific PCR technical conditions for the detection of p16 promoter hypermethylation in small amounts of tumour DNA. Oncol Rep 9: 181-183

GIL L, KING L, ADONIS M (2000) Trends of polycyclic aromatic hydrocarbon levels and mutagenicity in Santiago's inhalable airborne particles in the period 1992-1996.Inhal Toxicol 12: 1185-204

GREENLEE RT, HILL-HARMON MB, MURRAY T AND THUN M (2001) Cancer statistics, 2001. CA Cancer J Clin 51: 15-36. Erratum in: CA Cancer J Clin 2001;51: 144

HECHT SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 91: 1194-1210

HERMAN JG, MERLO A, MAO L, LAPIDUS RG, ISSA JP, DAVIDSON NE, SIDRANSKY D, BAYLIN SB (1995) Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 55: 4525-30

HERMAN JG, GRAFF JR, MYÖHÄNEN S, NELKIN BD AND BAYLIN SB (1996) Methylation –specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA 93: 9821-9826

HERMAN JG, BAYLIN SB (2003) Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349: 2042-2054

HILBE W, DIRNHOFER S, GREIL R, WOLL E (2004) Biomarkers in non-small cell lung cancer prevention. Eur J Cancer Prev 13: 425-436

LIU Y, LAN Q, SIEGFRIED JM, LUKETICH JD, KEOHAVONG P (2006) Aberrant promoter methylation of p16 and MGMT genes in lung tumours from smoking and never-smoking lung cancer patients. Neoplasia 8: 46-51

MERLO A, HERMAN JG, MAO L, LEE DJ, GABRIELSON E, BURGER PC, BAYLIN SB, SIDRANSKY D (1995) 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1: 686-692

MOMPARLER RL (2005) Epigenetic therapy of cancer with 5-aza-2'-deoxycytidine (decitabine). Semin Oncol 32: 443-451

NAKATA S, SUGIO K, URAMOTO H, OYAMA T, HANAGIRI T, MORITA M, YASUMOTO K (2006) The methylation status and protein expression of CDH1, p16(INK4A), and fragile histidine triad in nonsmall cell lung carcinoma: epigenetic silencing, clinical features, and prognostic significance. Cancer 106: 2190-2199

NUOVO GJ, PLAIA TW, BELINSKY SA, BAYLIN SB, HERMAN JG (1999) In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. Proc Natl Acad Sci USA 96: 12754-12759

PISANI P, BRAY F, PARKIN DM (2002) Estimates of the world-wide prevalence of cancer for 25 sites in the adult population. Int J Cancer 97: 72-81

PULLING LC, DIVINE KK, KLINGE DM, GILLILAND FD, KANG T, SCHWARTZ AG, BOCKLAGE TJ, BELINSKY SA (2003) Promoter hypermethylation of the O6-methylguanine-DNA methyltransferase gene: more common in lung adenocarcinomas from never-smokers than smokers and associated with tumour progression. Cancer Res 63: 4842-4848

SANCHEZ-CESPEDES M, DECKER PA, DOFFEK KM, ESTELLER M, WESTRA WH, ALAWI EA, HERMAN JG, DEMEURE MJ, SIDRANSKY D, AHRENDT SA (2001) Increased loss of chromosome 9p21 but not p16

inactivation in primary non-small cell lung cancer from smokers. Cancer Res 61: 2092-2096

SHIELDS PG (2002) Molecular epidemiology of smoking and lung cancer. Oncogene 21: 6870-6876

SZOT J (2003) Epidemiological analysis of mortality caused by solid tumours in the Metropolitan Region, Chile, 1999. Rev Med Chil 131: 641-9

TANAKA R, WANG D, MORISHITA Y, INADOME Y, MINAMI Y, IIJIMA T, FUKAI S, GOYA T, NOGUCHI M (2005) Loss of function of p16 gene and prognosis of pulmonary adenocarcinoma. Cancer 103: 608-615

TOYOOKA S, TSUDA T, GAZDAR AF (2003). The TP53 gene, tobacco exposure, and lung cancer. Hum Mutat 2: 229-239

TRAVIS WD, BRAMBILLA E, MULLER-HERMELINK HK AND HARRIS CC (2004) Pathology and Genetics: Tumours of the Lung, Pleura, Thymus and Heart: World Health Organization. Classification of Tumours. Pathology & Genetics. Travis WD, Brambilla E, Muller-Hermelink Hk and Harris Cc (eds). International Agency for Research on Cancer (IARC): Lyon, pp. 9-124

WIKENHEISER-BROKAMP KA (2006) Retinoblastoma regulatory pathway in lung cancer. Curr Mol Med 6: 783-793

WISTUBA II, MAO L AND GAZDAR AF (2002) Smoking molecular damage in bronchial epithelium. Oncogene 21: 7298-7306

WISTUBA II (2007) Genetics of preneoplasia: lessons from lung cancer. Curr Mol Med 7: 3-14

ZOCHBAUER-MULLER S, FONG KM, VIRMANI AK, GERADTS J, GAZDAR AF, MINNA J (2001) Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 61: 249-255