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Relationship Between Toxicogenomic and Environment and Lung Cancer

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1. Introduction

Tobacco-smoke is associated with 75-90% of LC cases and ~50% occurs in developing countries (Parkin, et al. 2005; Parkin, et al. 1994). Several studies have demonstrated that organic and inorganic compounds are related with the development of cancer, including LC (Bradley and Golden 2006; De Palma, et al. 2008; Stavrides 2006). The association between cigarette smoking and various types of respiratory cancer has been demonstrated (U.S.EPA 2001) and many carcinogenic compounds present in cigarette smoke, such as polycyclic aromatic hydrocarbons (PAHs), have also been identified in airborne particles in different cities around the world (Adonis, et al. 1997; Gil, et al. 1995; Minoia, et al. 1997; Monarca, et al. 1998).

Cancer rates could further increase by 50% to 15 million new cases in the year 2020, according to the World Cancer Report (World Cancer Report, 2003). The report also reveals that cancer has emerged as a major public health problem in developing countries, matching its effect in industrialized nations. During 2008, more than 1.61 million of new LC cases and 1.31 million of deaths were reported worldwide (Ferlay et al, 2010), accounting for 13% of the total. More than half (55%), of the cases occurred in the developing countries. In South America the estimation for 2008, showed an incidence rate for men of 20.4 /100,000 inhabitant and a mortality rate of 18.8 /100,000 inhabitant (WHO, 2008). For the same period, the women showed a rates/100,000 inhabitant of 8.4 for incidence and 7.4 for mortality.

PAHs are metabolized to reactive DNA binding diols epoxides by phase I enzymes as cytochrome P4501A1 (CYP1A1) and detoxified by phase II enzymes as GSTs, before reaching their target. Adonis et al (2005), have proposed that the contribution of individual variations in metabolic activities of each or both phases, in regulating the clearance of DNA toxic

metabolites, are at least partially related to the individual host susceptibility to PAHs. Several polymorphisms have been described in CYP1A1, however, 3′noncoding region (Msp1, CYP1A1*2A) has been the most studied (Gil et al, 1992; Adonis et al. 1997, Quiñones et al, 2001; Adonis et al, 2005a, 2005b, Ji et al, 2012).

Additionally, the association between inorganic Arsenic (iAs) and skin, bladder, and lung carcinogenesis has been well-established. It is estimated than 220 million in 105 countries are exposed worldwide to iAs (Murcott, 2012). In the Northern Chilean region of Antofagasta, the population (close to 500,000 inhabitants) have been chronically exposed to As concentrations as high as 870 µg l^{-1} from 1958 through 1970, with some towns still registering concentrations of 600 µg l^{-1} by the year 2000 (Smith et al, 1998). This has occurred despite international guidelines placing the maximum tolerable arsenic exposure at 10 µg l^{-1} (IARC, 2004).

As occurs naturally in soil, water and air, the main sources of environmental As exposure in Chile are copper smelters and drinking water (Queirolo et al., 2000; De Gregori et al, 2003). Chile, with a population close to 17.1 million (http://data.worldbank.org/country/chile) and a total life expectancy at birth of 78 years, shows a high rate of lung cancer (LC), especially in the North of Chile. During 2007, Chile showed more than 2,500 LC cases and 1,900 deaths. Between 1990 and 2008 the LC national mortality rate/100.000 inhabitants for both gender increased since 10.8 to 14.6; while for the period 2003–2007, the Antofagasta region (Northern Chile) showed a rate mortality of 30.8/100.000, the second rate mortality higher after skin cancer (Vallebuona, 2011). To stratify by gender, the Antofagasta region for the same period showed for men the highest LC rate mortality/100.000 (53.1), higher than prostate (38.9), and skin (50.4).

The mechanism by which As causes cancer is still no clear and has been speculated that its carcinogenicity potency in humans might be modulate by concurrent exposure to other agents that modify the risk of cancer to As, as environmental pollution containing high levels of carcinogen compounds (Adonis et al, 2005) and or smoking (Thomas et al, 2001), as well as both genetic and epigenetic processes (Salnikow and Zhitkovich, 2008). As is metabolized through a series of reductions of pentavalents to trivalent species, followed by oxidative methylations to yield pentavalent methylated species. It is well recognized that inorganic As is methylated to monomethyl As acid (MMA) and dimethyl As acid (DMA) with a methyl group from S-adenosylmethionine. As reduction of As to the trivalent form is a prerequisite for oxidative methylation, pentavalent arsenicals are reduced by endogenous thiols such as glutathione (GSH) or by As^V reductases (Kala el al., 2000; Radabaugh & Aposhian, 2000, Zakharyan et al., 2001; Radabaugh et al., 2002). Liver is the main site of methylation, but methylation activity is present in all tissues (Vahter, 2002). Arsenic3 methyltransferase (As3MT) is the key enzyme in the biotransformation of iAs. As3MT catalyzes the transfer of a methyl group from S-adenosyl-methionine to trivalent arsenicals resulting in the production of Monometil As(MAs) and dimethylated arsenicals *3 (DMAs). MAs is a susceptibility factor of iAs induced toxicity, before to be excreted in the urine.

As was mentioned before, the high incidence of lung cancer (LC) has been associated with cigarette smoking, however genetic diversity and environmental pollution must also be considered as risk factors, especially in those cities highly exposed to environmental carcinogens. Although, there are many advances in the disease control, very little is known about

changes in detection of pre neoplastic, pre invasive lesions or in the initial steps in LC development. LC has been described as one of the most aggressive diseases with multiples genomic changes or alterations in specific fragments of the DNA, some of them including genes associated with the cell system preservation within normal parameters. Some of the changes might promote a transformation from a normal cell into a tumour cell in a multistage process.

The genomic age, the new images technologies and informatics, probably will demand in a short time from the government, health professionals (clinic and basic), public and private institutions, etc., new actions to improve the public health.

Cancer diagnosis can be done in different stages of the disease. The cancer diagnosis has as main objectives to know the localization (place) and also to identify the stage (stage) and the histological characterization (kind). The knowledge of the place, stage and kind will help to define the treatment and will determine the survival and the clinic success. For LC the current technologies are arriving late, indeed more than 85% of the LC patients died after the diagnosis (McWilliams et al, 2009; Nakamura et al., 2001; Kennedy et al., 2007; Woolner et al., 1984). The main reason of the high mortality is related with the small fraction of the patients detected in early stages. This is mainly related with absence of symptoms in an early stage.

Obviously, the early diagnosis will load in the clinic success in term of treatment and survival. The use of new technologies as complementary tools might chance or improve the early detection and the treatment success. The use of the advanced technologies might reduce the mortality, thus it is possible not only detect early stages but also to have early treatment.

Early detection has a direct relationship with the population screening, in order to identify risk population, healthy population and or asymptomatic population, especially for LC asymptomatic in the early stages. Smoking habit is probably the main risk factor for LC, however additional factors might contribute to the risk of LC. Among the LC risk factors is valid to mention environmental pollution, occupational exposure, life style and genetic factors. That mean, that among the population is possible to find people with a high risk of LC, which would be the main candidates to a screening programme to detect pre neoplastic lesions or early stages.

Within some of the news or advances technologies can be mentioned, Quantitative Automatic Cytology (QAC), Autofluorescence-reflectance bronchoscopy (AFB) and the tumor biomarker DR70, used in a pilot study for early detection of LC, in a high risk population. These technologies have been studied in Chile in a LC risk population, exposed to high levels of air pollutants (Santiago) and on the other hand to high historical exposure to As in drinking water (Antofagasta). Part of these results, were presented in an oral presentation, titled "Pilot Study for Lung Cancer and Pre neoplastic Lesions", in the XXX1 Word Congress of Internal Medicine (WCIM, 2012), where the work got the Award for the First Best Research Work. This is a report of an ongoing prospective bimodality cancer surveillance study for high risk LC volunteers. This study has been done in 364 people, exposed naturally to environmental pollution and where the biomarkers, QAC and DR70 (Onko Sure), were used as tools in the detection of preneoplastic lesions (PNL) and neoplastic lesions (NL). The study has also included Autofluorescent Bronchoscopy (AFB) as an additional technique, to detect pre neoplastic or pre

invasive lesions in the voluntaries with high likehood of malignance for LC, according to AQC and DR70.

2. Materials and methods

The people that were interested and met the criteria for the inclusion factors were enrolled as voluntaries (Figure 1).
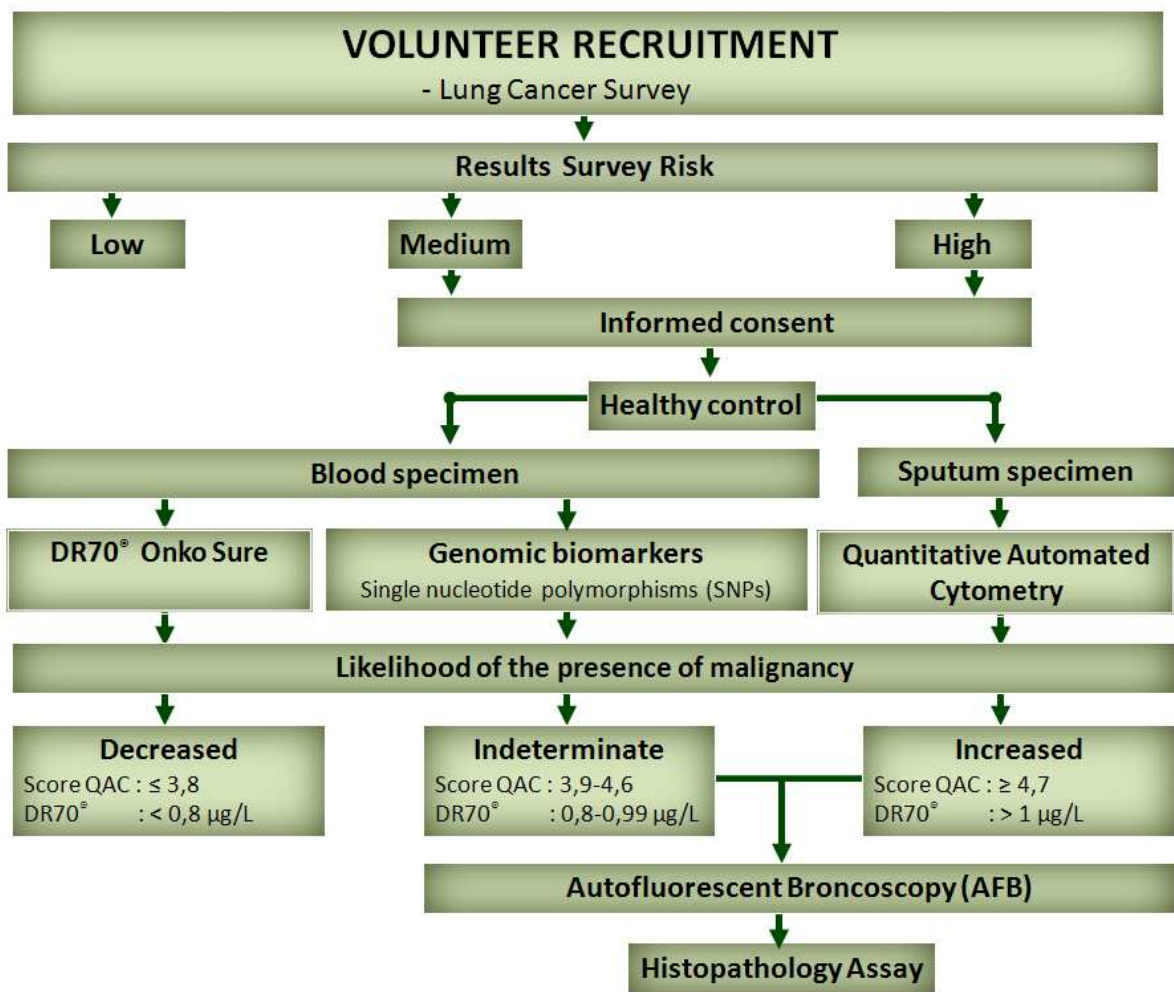

Figure 1. Diagram of the enrolling process for recruiting the voluntaries for LC prevention.

The high risk subjects for LC were recruited via advertisement in newspaper, radio and internet (University of Chile web) and were included in this study from two regions of Chile, Metropolitan (Santiago city) and Antofagasta. Enrolment was based on a LC Risk Survey (Witteman et al., 2011) by Washington University School of Medicine, which was modified by our group. Subjects were eligible for enrolment procedure if they met the following criteria's: male/female aged 40 years or older; family history of LC, non-smokers and ever smokers

exposed naturally to environmental air pollution (Metropolitan region) or to arsenic in drinking water (Antofagasta region) for at least 10 years.

In addition, we enrolled subjects who were suspected of having lung cancer (N=12) based on their clinical symptoms, with the final diagnosis completed in the study. These patients did not have previous tests performed on them such as cytology or CT and did not have any treatment. However, only 12 of them were in agreement to undergo to the AFB, while all of them underwent a CT to rule out or confirm the LC.

The voluntaries were classified according to their risk score, which allowed for classification of the subjects in three risk categories: low, medium and high. Subjects with medium or high risk were invited to participate in the study. The LC risk predictor was applied to 508 volunteer participants, obtaining 364 subjects with medium to high-risk for LC. Of those, 224 were from Santiago and 140 were from the Antofagasta's region.

Sputum generation was induced by inhalation of 3% saline solution, after administration of two puffs of salbutamol. Patients were instructed to cough and expectorate before their Specimens were collected in 50 mL centrifuge tubes, followed by adding enough SedFix solution. Each tube was mixed with DTT solution and was shaken at 1800 rpm overnight. After centrifugation, a cell pellet was obtained; re suspended in an ethanol solution and was placed on slides. The cell DNA was stained with Feulgen thionin and was analysed by an automated cytometry-based scoring system, as described by Kemp et al. (Kemp et al, 2007).

Furthermore, blood samples were taken in serum separator tubes in the morning before ingestion of any meal. The tubes were left at room temperature for 30 minutes. The serum was separated from the cells by centrifugation at 3,500 rpm for 10 min. Each serum specimen was diluted 1:200 with the diluent buffer and was tested along with the calibrators according to AMDL Diagnostics Onko Sure protocol (Radient Pharmaceuticals) as described in Adonis et al. (2005) and Hatton et al. (2006).

Participants with positive DR70 test (threshold>1.0), or an AQC score ≥4.6 were invited to have an AFB, using the Onco-LIFE device (Novadaq Inc., Richmond, Canada), under local anaesthesia. The airways were examined by, White Light Broncoscopy (WLB) and Auto Fluorescence Broncoscopy (AFB) and the visual findings were classified as normal, abnormal or suspicious, as described by Lam et al. (1998). Endobronchial mucosal biopsies were taken from all areas that were suspicious under WLB or AFB. In addition, surveillance biopsies were taken from epithelium with normal appearance in all subjects. An average of 2-3 biopsies was taken from each of the participants.

Categorical variables were analysed by Fisher's exact test. Sensitivity, specificity, likelihood ratio (LR), Predictive Positive Value (PPV) and Predictive Negative Value (PNV) were calculated with 95% CI. Statistical significance was accepted at $p<0.05$. The sensitivity and specificity were assessed for each test and was used in analysis as a single test; two-test parallel combination and two-test series combination (Sullivan and Thomson, 2000, Kruskal, 1998).

3. Results

As show the Figure 2, 27.4% of subjects display an AQC with an increased likelihood of malignancy (score equal or higher than 4.6) and 26.1% showed undetermined likelihood of malignancy (score between 3.9 and < 4.6). Among samples with increased AQC (27.4 %), 15.75% and 11.68% were negative and positive (≥ 1 µg/mL) for DR70, respectively. Samples with undetermined AQC showed 22.01% and 4.07% of negative and positive DR70 values, respectively. Among 46.5% of samples that showed decreased likelihood of malignancy, 42.11% were negative and 4.34% were positive for DR70. In the latter group, additional clinical tests confirmed several cancers including colon, breast and one case of prostate cancer, currently under study.
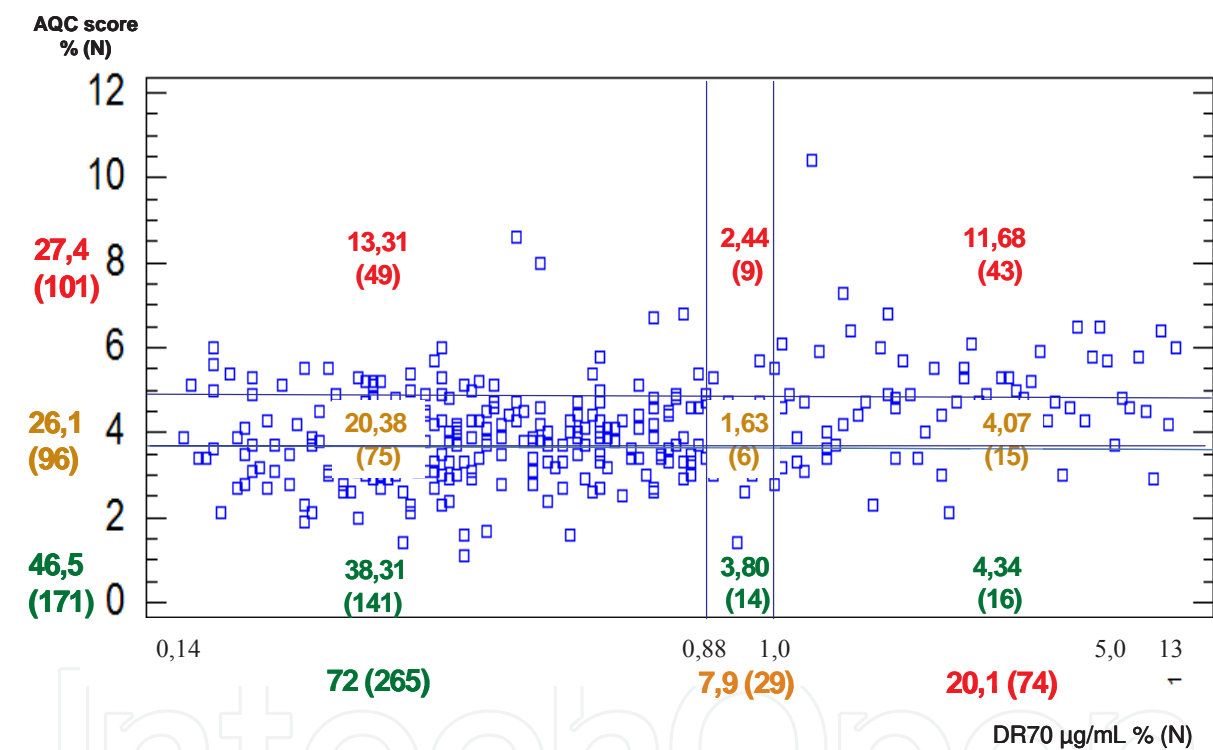

Figure 2. AQC and DR70 correlation and percentage (N) for each LC risk interval. Likehood LC risk: AQC: ≥ 4.6, increased; ≥ 3.9 ≤ 4.6 undetermined; ≤ 3.9 decreased DR70: ≥ 1.0, increased; ≤ 1.0 decreased

The data shows that DR70 itself might contribute to confirm tumour diagnosis and to identify patients with advanced LC, with high sensitivity (Table 3). However, the test would be better identifying negative LC cases with a high specificity and Negative Predictive Value (NPV). AQC for itself resulted with high sensitivity and specificity for LC with higher NPV than Negative Predictive Value (PPV), confirming especially negative tests, with high precision. Related to PNL, both biomarkers might be used as complementary tools to confirm negativity for PNL, showing a higher specificity (98.6%) than both test for itself, keeping a high NPV (97.2%).

On the other hand, AFB identified PNL (metaplasia and dysplasia) better than the White Light Bronchoscopy (WLB) by itself. As show the Figure 3, AFB detected a zone with a score of 0.67, suspicious of malignance and normal according to WLB. The Histopatholo‐ gy assay informed for the biopsy taking in this zone, increasing in the coarse epithelial and desmosomes and cellular flatting and maturation in the upper layer; in comparison to a normal epithelium (Figure 3B), without cytology atypias; classifying the biopsy as a Squamous metaplasia (Figure 3A).
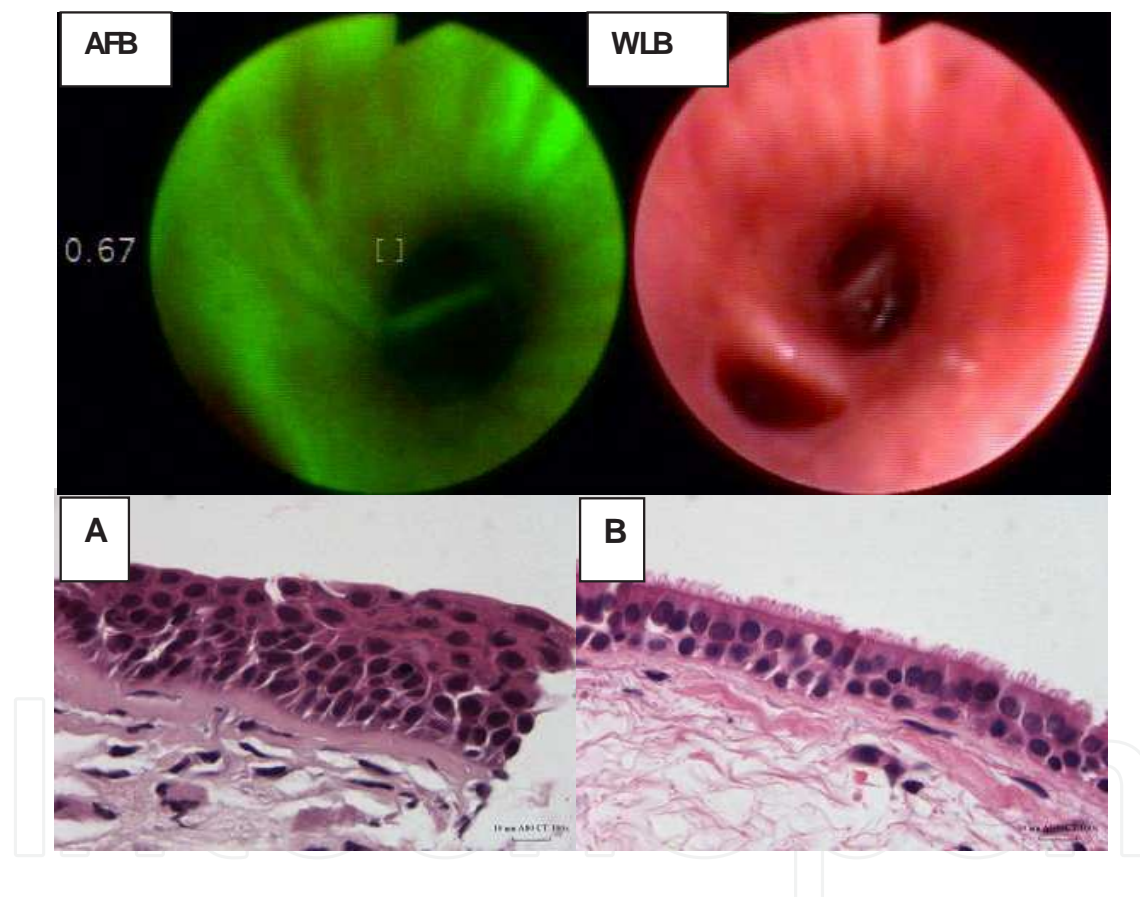

Figure 3. AFB and WLB and Histopathology assay for a Squamous Metaplasia Volunteer A80. Squamous Metaplasia (B) Volunteer A109. Normal Epithelium Stain HE 100x. CeteCáncer. INNOVA CORFO. Thesis Avaria P. MSc, Faculty of Medicine, University of Chile, 2012

The Figure 4 shows an AFB with a score of 0.83 and suspicious of malignance. The Histopa‐ thology assay informed a considerable cellular increasing in the 2/3 lower, pleomorphism, nuclear heterogeneity, chromatin density increased without mitosis, classifying the biopsy as mild dysplasia (Figure 4A).
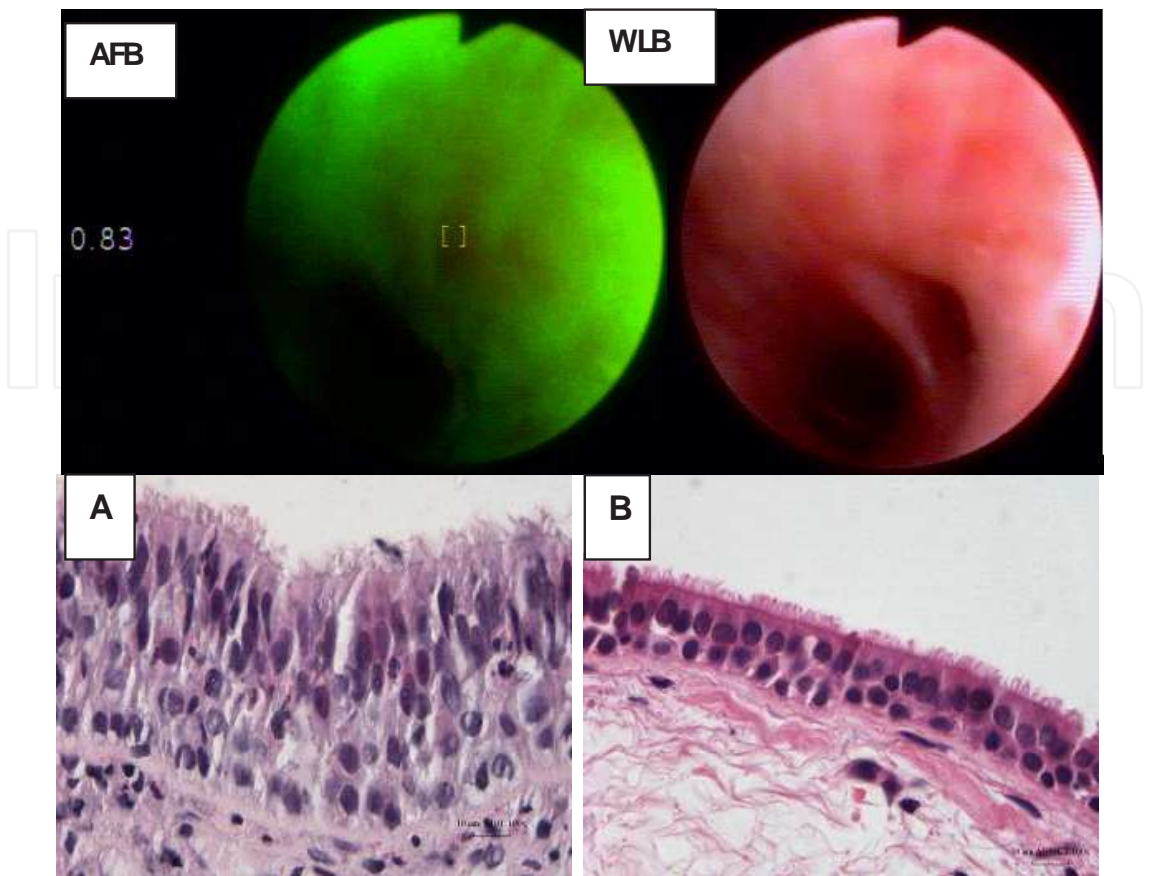
Figure 4. AFB and WLB and Histopathology assay for a Mild Dysplasia Volunteer A101. Mild Dysplasia (B) Volunteer A109. Normal Epithelium Stain HE 100x. CeteCáncer. INNOVA CORFO. Thesis Avaria P. MSc, Faculty of Medicine, University of Chile, 2012

Fifty percent of the samples, classified as suspicious (12%) by AFB, were confirmed as metaplasia (33%) or dysplasia (17%) by histopathology. The rest of the samples classified by AFB as suspicious were classified by the histopathology as inflammation (25%) and hyperplasia (25%). Non one was related with a normal histopathology sample (Figure 5).

Biomarkers	Sensitivity % (IC 95%)		Specificity % (IC95%)	
	LC	PNL	LC	PNL
DR70	95.8	27.3	91.9	91.9
	(784,9- 99,0)	(6.0 -61.0)	(88.1-94.8)	(88.1-94.3)
AQC	64.0	90.9	89.4	89.4
	(42.5- 82.0)	(58.7- 99.8)	(85.2-92.7)	(85.2-92.7)

Table 1. Sensitivity and Specificity for DR70 and AQC, for LC and PNL

The data shows that DR70 itself might contribute to confirm tumour diagnosis and to identify patients with advanced LC, with high sensitivity (95.8%) and specificity (91.9%) (Table 1). AQC

for itself resulted with high sensitivity and specificity for LC, but showed a higher sensitivity and specificity than DR70 for PNL, 90.9% and 89.4%, respectively. Additionally, both biomarkers might be used as complementary tools to confirm negativity for LC and or PNL. In conclusion, as a pre screener for LC, both biomarkers test might be employed with at high specificity/high sensitivity as complementary tools to detect LC. For PNL, both tests would be better confirming negativity than subjecting presence of PNL.
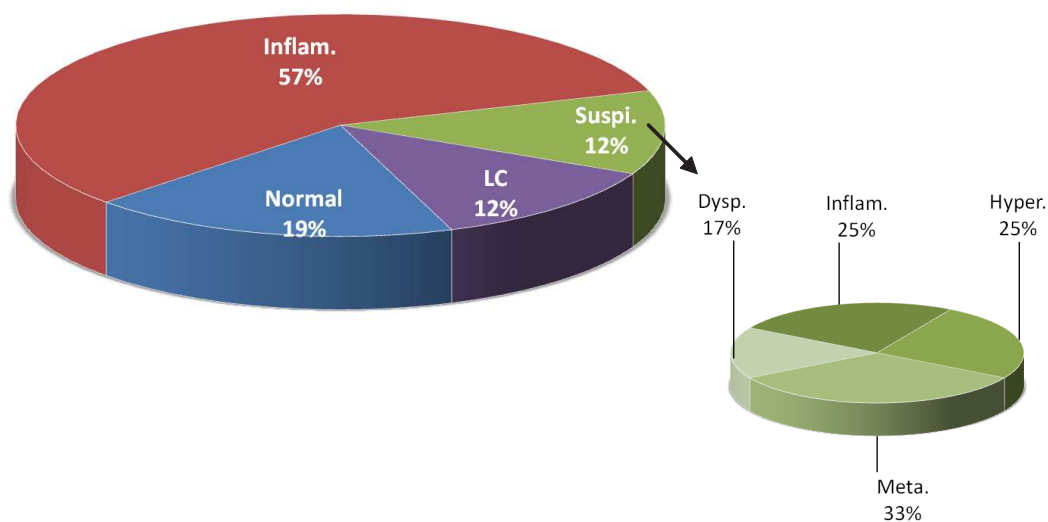

Figure 5. Relationship between Autofluorescence Broncoscopy (AFB) and Histopathology Assay (HA)

4. Discussion

This is the first study in Latin America to complement image techniques with cellular and molecular biomarkers, to detect LC and PNL. These results provide scientific and clinical information for Chilean health authorities to include early detection of LC in the AUGE government programme, that provide additional health services for patients.

Our results presented here clearly demonstrate the reliability of both biomarkers to select good candidates for detect LC or pre neoplasic lesions (PNL). These results suggest that both AQC and DR70 might provide transcendentally information not only to confirm or suggest diagnostic for LC, but also to surveillance screening of LC after treatment. It is as important to

confirm diagnostic as predict a patient's response to certain cancer therapies or determine whether cancer has returned.

These results obtained via biomarkers in blood and sputum were quite promising in elucidating risk to LC, especially when the biomarkers studied are non invasive and inexpensive assays and could be an effective methods also to monitor the biological effects of environmental and occupational carcinogens.

Additionally, we presented evidences that showed that AFB is more sensitive than WLB to detect early lesions, thereby allowing their localisation for biopsy or interventional procedures. Then the association of WLB and AFB would increase the sensitivity to detect PNL.

Is well known that resolution of CT scan and other images technologies improves each year, allowing to detect more nodules, but not necessarily LC in early stages. Additionally, is well known the high frequency of lung nodules with undetermined significance and that LC often causes no symptoms until it is spread outside the lung. From this point of view, non invasive biomarkers and AFB might contribute as a first step in detecting pre- neoplastic a pre- invasive lesions. Screening in people with high LC risk including those with smoking habit and or nodules non necessary cancerous, might means a higher survival and improve the life quality. The screening in people with high risk of LC might be a good preventive way to improve the survival to 5 years, especially when different studies have shown an important association between genetic host characteristics and exposure to environmental carcinogens.

Genetic differences in metabolic activation and detoxification of environmental carcinogen, like PAHs and or As, may partially explain host susceptibility to chemically induced cancers (Daly et al., 1994; London et al, 2000, Kang et al, 2012).

Adonis et al (2005) showed the association of combined genotypes of cytochrome CYP1A1 (Msp1) and glutathione-S-transferase GSTM1 and lung cancer risk, for a population historically exposed to As in the Antofagasta region. This study showed in the healthy group, a CYP1A1 *2A allele frequency for MspI of 0.41, whereas for lung cancer group 0.46. No statically significant difference was observed between the healthy group and lung cancer group ($p = 0.437$, CI $=-0.224$ to 0.124). However, the CYP1A1 *2A genotype was associated with an increased relative lung cancer risk O.R. = 2.08 (95% CI = 1.04–4.03, $p = 0.04$). In addition, 35% of healthy group and 39% of the lung cancer group were homozygote for the null variant allele of GSTM1. For men the CYP1A1 *2A genotype was associated with a highly significant estimated relative lung cancer risk O.R. = 2.60 (95% CI = 1.07–5.94, $p = 0.0334$). The relative lung cancer risk for the total sample with the CYP1A1 *2A/null GSTM1 genotype was 2.51 (O.R. = 2.51, CI = 1.07–5.40, $p = 0.0322$), which one increased when the sample was stratified by smoking habit (O.R. = 2.98, CI = 1.10–7.10, $p = 0.0497$) (Adonis et al, 2005).

These results suggest that patients with previous history of iAs exposure as the Antofagasta population, and with smoking habit might be have an additional factor related to genetic susceptibility to lung cancer. The cancer mortality rate in region II for iAs- associated cancers, as lung cancer, at least might be partly related to differences in As biotransformation. Individuals with the CYP1A1*2A and/or the combined CYP1A1*2A and GSTM1 null genotype might have a greater capacity to metabolically active PAHs and lower capacity to conjugate

with glutathione and clearance of As, which may result in a higher risk of lung cancer or respiratory tract illness.

One of the main conclusion is there is an interaction between CYP1A1 polymorphism, GSTM1 null genotype and LC risk, especially in people exposed to carcinogenic compounds like to As and PAHs. In conclusion, genetic biomarkers such as CYP1A1 and GSTM1 polymorphisms in addition to other genetic biomarkers and other biomarkers like to QAC and DR70 and clinical tools might provide relevant information to identify individuals at high risk to lung cancer as prevention and protection actions of public health.

Acknowledgements

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