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# ENVIRONMENTAL FACTORS AND *p53* MUTATION SPECTRUM IN LUNG CANCER

Anna Bessö


Karolinska Institutet

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# ABSTRACT

The use of molecular biomarkers, such as mutations in the *p53* gene, has raised expectations for improving the resolution power in epidemiological studies. In this thesis we assessed the influence of smoking, environmental tobacco smoke (ETS), residential radon and arsenic on the *p53* mutation prevalence and spectrum in lung tumors. Furthermore, we investigated the lung cancer risk among 316 cases and 727 controls in the area surrounding Rönnskärsverken, a non-ferrous metal smelter, and cases from this study were also included in the analyses of *p53* mutations.

Tumor samples were collected from pathology departments and exons 5-8 of the *p53* gene were analyzed using SSCP or DGGE screening in combination with DNA sequencing or using direct DNA sequencing for a total of 479 lung cancer cases, including 196 cases among never-smokers. Information on smoking, occupational and residential histories was collected through questionnaires and/or interviews of study subjects or next-of-kin. Exposure to residential radon was estimated based on measurements for a 32-year retrospective period in the dwellings of the study subjects.

An increased risk of lung cancer was indicated among men who had lived close to the non-ferrous smelter, primarily among those exposed during the early years of operations, when emissions were high, and for less than 20 years, odds ratio (OR) 2.5, 95% confidence interval (CI) 0.9-7.1, compared to unexposed. In total, we detected 103 mutations in 99 lung tumors (mutation prevalence 21%). Tobacco smoking was associated with an increased *p53* mutation prevalence (OR 2.4, 95% CI 1.1-5.1) and a higher proportion of G to T transversions whereas G to A transitions at CpG sites were more common among never-smokers. A higher *p53* mutation prevalence was suggested also for exposure to residential radon, OR 2.8 (95% CI 0.8-9.3) for cases exposed to a time-weighted average level of more than 400 $Bq/m^3$ compared to those exposed to less than 50 $Bq/m^3$. Cases with exposure to both residential radon (>50 $Bq/m^3$) and a long duration of ETS exposure (≥30 years) showed a clear increase in prevalence of *p53* mutations compared to unexposed (OR 4.9, 95% CI 1.2-21.1). For exposure to arsenic, a possible negative interaction with smoking was suggested (OR 0.5, 95% CI 0.2-1.2). Tumors from smokers without arsenic exposure had a greater variety of base-changes than tumors from smokers with arsenic exposure.

We could not detect any clear exposure specific spectra of p53 mutations in lung tumors associated with exposure to ETS, arsenic or residential radon. Weak associations may have been missed, however. Although mutations in the *p53* gene did not seem to be a useful marker in our studies, our results provide a substantial addition to the available *p53* data on never-smoking lung cancer cases and give new evidence on possible mechanistic pathways in environmentally induced lung cancer.

# LIST OF PUBLICATIONS

This thesis is based on the following original articles and manuscripts, which will be referred to in the text by their Roman numerals.

I. Anna Yngveson, Cecilia Williams, Anders Hjerpe, Joakim Lundeberg, Peter Söderkvist and Göran Pershagen. *p53* mutations in lung cancer associated with residential radon exposure. *Cancer Epidemiology, Biomarkers and Prevention*, 1999, 8, 433-438.

II. Anna Bessö, Fredrik Nyberg and Göran Pershagen. Air pollution and lung cancer mortality in the vicinity of a nonferrous metal smelter in Sweden. *International Journal of Cancer*, 2003, 107, 448-452.

III. Anna Bessö, Susanne Ahlberg, Ulrik Carling, Anders Hjerpe, Magnus Ingelman-Sundberg, Fredrik Nyberg and Göran Pershagen. *p53* mutations in lung cancer cases exposed to arsenic at a non-ferrous metal smelter. *Submitted*.

IV. Anna Bessö, Anders Hjerpe, Kirsti Husgafvel-Pursiainen, Göran Pershagen, Peter Söderkvist and Fredrik Nyberg. *TP53* gene mutations in lung cancer cases exposed to ETS and residential radon. *Submitted*.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AC | Adenocarcinoma |
| DGGE | Denaturing gradient gel electrophoresis |
| ETS | Environmental tobacco smoke |
| IHC | Immunohistochemistry |
| LCC | Large cell carcinoma |
| NNK | 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone |
| NNN | 4´-nitrosonornicotine |
| NSCLC | Non small cell lung cancer |
| *p53*- tumor | A tumor without a detected *p53* mutation |
| *p53*+ tumor | A tumor with a detected *p53* mutation |
| PAH | Polycyclic aromatic hydrocarbons |
| SCC | Squamous cell carcinoma |
| SCLC | Small cell lung cancer |
| SSCP | Single strand confirmation polymorphism |

# INTRODUCTION

Smoking is the major cause of lung cancer but other factors also play a role in the etiology, such as residential radon, environmental tobacco smoke (ETS) and arsenic, which may occur both in the occupational and general environment. These factors are less important than smoking and it can be difficult to assess their etiological role in epidemiological studies. Such investigations may be facilitated by using biomarkers of exposure and subclassification of disease based on etiology. This necessitates access to biological materials from groups of patients with detailed exposure assessment, which makes obtaining adequate sample sizes a challenge. The use of molecular biomarkers, such as mutations in the *p53* tumor suppressor gene, has raised expectations for improving the resolution in epidemiologic studies and facilitating quantitative risk estimation. In Papers I, III and IV included in this thesis the *p53* mutation prevalence and spectrum was evaluated in lung tumors associated with smoking, ETS, residential radon and arsenic exposure. Paper II investigated the lung cancer risk for people living close to an arsenic emitting smelter, who constitute the study base from which cases were selected into Paper III.

# BACKGROUND

## Lung cancer occurrence and pathology

Lung cancer is the most common form of cancer in the world, both with regard to incidence and mortality.[1] In 2002 more than 1.3 million new cases occurred worldwide.[1] In Sweden, almost 3200 new cases of lung cancer were registered in 2004 and 44% of these were women.[2] The current Swedish incidence rates (per 100 000 in 2004) are 28.6 among women and 42.5 among men (age standardized to the Swedish population 2000) (http://www.socialstyrelsen.se/Statistik/statistikdatabas/) and the average rate of increase in incidence has been 2.8% per year among women during the latest 20-year period (1985-2004), while a small decrease during the same period occurred among men.[2] Lung cancer still has quite a dismal prognosis and the overall 5-year survival is only about 10%.[3]

Lung tumors derive from pluripotential cells, i.e. cells that have the ability to mature or differentiate into any of the cells in the lung, which line the tracheobronchial tree or alveoli. Lung cancer is often subdivided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which includes squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC). Squamous cell carcinoma was previously the most common histological type of lung cancer but the proportion of AC has increased. This change in pattern may be due to changes in the composition of the cigarettes and deeper inhalation of filter cigarettes as well as improved histological classification and diagnostic tools.[4] Adenocarcinoma is today the most common histological type in Sweden and accounted for 35% of the new lung cancer cases in 2004 (http://www.socialstyrelsen.se/Statistik/statistikdatabas/). It is also the most common histological type nowadays in the US.[5] Adenocarcinoma often originates peripherally in the lung.[6] Squamous cell carcinoma, the other type of NSCLC, accounted for 21% of new lung cancer cases in Sweden in 2004 (http://www.socialstyrelsen.se/Statistik/statistikdatabas/) and usually originate within a central bronchus, but the incidence of peripheral SCC is increasing.[6] Small cell lung cancer accounted for 14% of the lung cancer cases in Sweden in 2004.

## Risk factors for lung cancer

### Smoking

Smoking is the main risk factor for lung cancer and accounts for almost 90% of all cases, by itself or in combination with other risk factors.[4] The lung cancer risk is related to both duration and intensity as well as age at taking up smoking.[4] The average cumulative probability of death from lung cancer in male and female smokers has been estimated to 24% and 11% respectively, compared to 1.6% and 1.1% in male and female never-smokers when excluding competing causes of death.[4] A rough estimate is that the risk of contracting lung cancer increases 15-fold

from smoking one package of cigarettes a day during at least 10 years, compared to the risk of a non-smoker.[3] Tobacco smoking increases the risk of all histological types of lung cancer. The association between cigarette consumption and AC was weak in earlier studies but has become stronger over time.[4] The carcinogenic effect of cigarette smoking seems to be similar in both women and men, although men have smoked more and male smokers therefore show a higher cumulative probability of death in lung cancer than female smokers.[4] Cigarette smoke condensate has been shown to both initiate and promote carcinogenesis.[4]

Tobacco smoke consists of thousands of compounds, of which 69 had been identified as carcinogens in 2000.[4] Examples of carcinogens are polycyclic aromatic hydrocarbons (PAHs) and tobacco-specific nitrosamines such as 4´-nitrosonornicotine (NNN) and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK).[4] Components in the tobacco smoke have been shown to induce a variety of genetic and epigenetic changes involved in transforming a normal cell into a tumor cell, such as inducing sister chromatid exchanges, DNA strand breaks, oxidative damage as well as mutations in tumor suppressor genes and oncogenes.[4]

## Environmental tobacco smoke (ETS)

ETS is formed when the sidestream smoke from the cigarette, or other tobacco products, mixes with the mainstream smoke and the ambient air.[4] Exposure to ETS is also referred to as exposure to secondhand tobacco smoke, involuntary smoking or passive smoking. ETS contains essentially the same substances as the tobacco smoke inhaled by active smokers but the concentrations and relative proportions of the substances differ.[4] Animal data suggest a carcinogenic effect of ETS[4] and passive smoking is classified as a human carcinogen by IARC.[7] There have been many studies on ETS and the risk of lung cancer, and meta-analyses of epidemiological data have shown an excess risk for lung cancer of approximately 20% for female non-smokers and 30% for male non-smokers ever having lived with a smoking spouse.[8, 9] The excess risk increases with increasing exposure. Exposure to ETS at the workplace is similarly associated with a 12-19% higher risk for lung cancer among never-smokers. Approximately one-fourth of the adult population in Sweden has been exposed to secondhand tobacco smoke.[10] Exposure to tobacco smoke carcinogens seems to affect normal cellular growth and differentiation.[11]

## Radon

Radon (Radon-222) is a radioactive noble gas and a decay product of uranium-238.[12] Radon decays into radioactive metal ions which emit high energy alpha particles that irradiate the bronchial epithelium after inhalation.[12] Uranium is present in the crust of the earth and radon may emanate from the ground, building materials and ground water. Radon in residences is the dominating source of exposure to ionising radiation in most countries, including Sweden. The amount of indoor radon originating from the ground is mainly determined by the radon concentration in the soil air, the permeability of the ground beneath the building, the type of

house foundation and differences in air pressure between the air in soil and indoors.[12] In Sweden the building material is also important for the radon concentration indoors since uranium rich alum shale concrete was commonly used as building material from 1950 to 1975, and is now present in every tenth building. Based on national measurement programs the average radon concentration in dwellings in Sweden is estimated to about 100 Bq/m$^3$. The distribution is approximately log-normal, and differs by a factor of 1000 between houses with the lowest and highest concentrations. The current standard for residential radon in Sweden is 200 Bq/m$^3$.

Underground miners exposed to radon have shown an increased risk of lung cancer, with a linear relationship between estimated cumulative exposure to radon and lung cancer risk.[13] Two recently performed meta-analyses of epidemiological studies have shown an association between exposure to residential radon and lung cancer risk with increased risks of 11% and 16%, respectively, per 100 Bq/m$^3$.[14, 15] A multiplicative interactive effect with smoking was indicated in the meta-analyses as well as in the Swedish nationwide radon study, which was part of one of the meta-analyses.[16] In a Swedish study on residential radon among never smokers a more harmful effect of radon was indicated among those also exposed to ETS.[17]

The mechanisms underlying radiation induced carcinogenesis are not known in detail. The radiation may interact either directly with DNA or indirectly through the action of free radicals.[18] Alpha radiation has been shown to induce chromosomal damage, gene mutations, micronuclei as well as sister chromatid exchange.[18] It has also been suggested that not only directly irradiated cells but also cells close to the irradiated cells are damaged.[18] Mutations induced by alpha radiation are predominantly of deletion and translocation types.[19]

## Arsenic

Arsenic is a metalloid present in the environment in both inorganic and organic forms.[11] It is the inorganic form that is most toxic. Arsenic-contaminated water is a big problem in parts of the world causing skin lesions, cancer and peripheral vascular damage. Exposure to arsenic may also come from arsenic-containing pesticides and fertilizers, as well as from production of copper and lead from sulphite ores where inorganic arsenite trioxide is a by-product. In northern Sweden there is a large smelting plant, Rönnskärsverken, where the emissions of sulphur dioxide and various metals, including arsenic, have been substantial. The production at the smelter started in 1930 and during the early period of operations the emissions of inorganic arsenic are estimated to have been several hundred tons yearly.[20] The emissions have then progressively decreased and were less than a ton in 2004. Similarly, the emissions of sulfur dioxide have decreased from nearly 200 000 tons annually to about 3500 tons in 2004. Emissions of lead, copper and zinc have also decreased substantially. Epidemiological studies have shown that smelter workers exposed to inorganic arsenic have an increased risk of contracting lung cancer.[21-25] Living close to a smelter has also been associated with an excess lung cancer risk.[26-29] Several studies indicate an

interaction between smoking and arsenic exposure exceeding an additive effect,[30, 31] which is also supported by experimental studies.[32, 33]

The carcinogenic action of arsenic remains poorly understood. Different postulated mechanisms involve oxidative stress, genetic changes and signal transduction.[34] Carcinogenicity studies of arsenic are inconclusive due to the low doses used, short duration and few number of animals but in vitro studies have shown that arsenic can induce chromosomal aberrations, affect methylation and DNA repair, induce cell proliferation, transform cells and promote tumors.[11]

## *Lung carcinogenesis*

The formation of a lung tumor is thought to be a multistep process and several pathways need to be disrupted for a normal cell to become malignant. Self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, sustained angiogenesis and unlimited potential to replicate are steps of importance for a neoplasm to form.[4] The carcinogenic process starts with the tumor initiation (Figure 1). The initiated cells may be less sensitive to signals regulating growth and maturation than other cells, giving them a growth advantage. Alterations in oncogenes and tumor suppressor genes, caused both by endogenous processes (such as replication errors or generation of free radicals) and exogenous exposures, increase the probability of clonal expansion and the formation of a tumor.[35] Environmental and occupational exposures to carcinogens can affect any of the steps in the carcinogenic process. According to our current knowledge, the most commonly mutated gene, in lung cancer is the *p53* tumor suppressor gene. Although this thesis focuses on *p53* mutations it is important to remember that the *p53* gene is only one part in a very complex network.

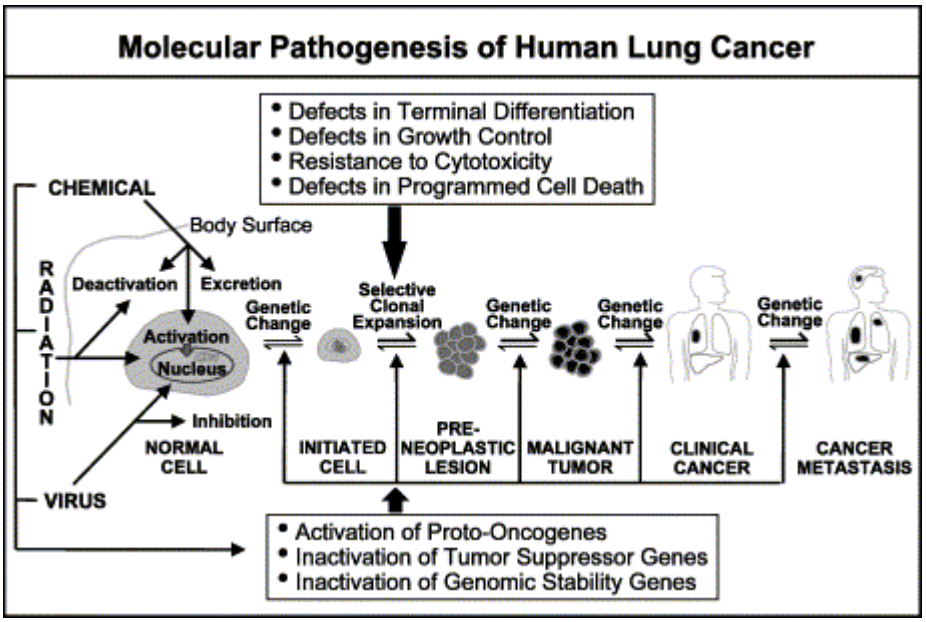


**Figure 1**. A description of the multistep process in cancer development. Reprinted from Hussain et al. 2001 with permission from Elsevier.[36]

## *The p53 tumor suppressor gene*

The *p53* gene, or *TP53* gene, was first discovered in 1979 and was then thought to be an oncogene.[37] Approximately 10 years later it was discovered that the *p53* gene may function as a tumor suppressor gene and it was then named "The guardian of the genome"[38] and became "The molecule of the year" in Science 1993.[39] The human *p53* gene is located on chromosome 17p, has 11 exons and encodes a 53kD protein consisting of 393 amino acids which is located in the nucleus of cells throughout the body.[35] The *p53* gene has several functions in the cell involving gene transcription, DNA synthesis and repair, cell cycle arrest and apoptosis.[35] Germline mutations in the *p53* gene have been identified in patients with Li-Fraumeni syndrome, which is a rare inherited disorder that increases the risk of developing several types of cancer.[35]

The p53 protein is normally expressed at very low levels. When the cells are exposed to stress, different pathways lead to stabilization and accumulation of the p53 protein. The accumulation activates transcription of many genes leading to induction of cell cycle arrest, apoptosis, enhanced DNA-repair and/or inhibited angiogenesis (Figure 2).[35]

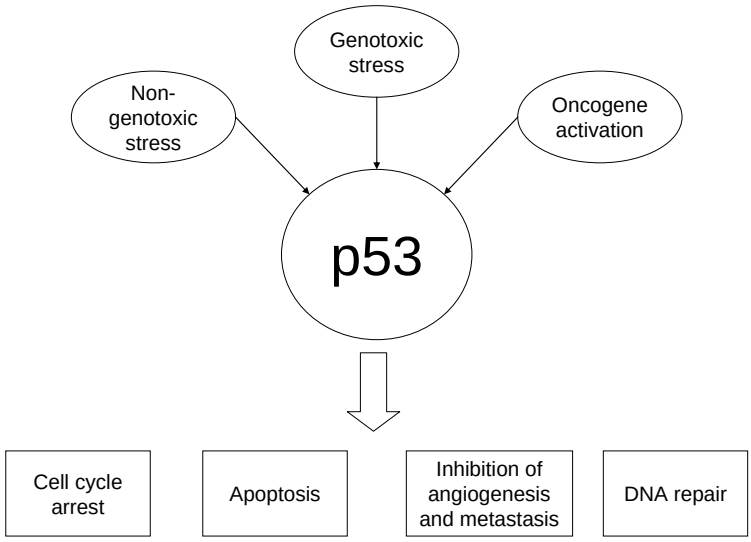


**Figure 2.** Activation of the *p53* gene by stress signals and known response of the activated p53 protein.

Mutations in the *p53* gene occur in all coding exons but mostly in exons 4 to 9 which are highly conserved through the evolutionary history and code for the DNA binding domain.[40] Different mutation spectra in the *p53* gene have been linked to various exposures as summarized below.[41]

**Table 1.** Examples of *p53* specific mutations in various types of cancer associated with different exposures.

| Exposure | *p53* mutation | Codons | Type of cancer |
|---|---|---|---|
| Tobacco smoking (benzo(a)pyrene) | G:C to T:A transversions | 157, 158, 245, 248 and 273 | Lung cancer |
| Aflatoxin $\beta_1$ | AGG→AGT transversions | 249 | Hepatocellular carcinoma |
| Sunlight | CC→TT double base substitutions | | Skin cancer |
| Vinyl chloride | A:T to T:A transversions | | Hepatic angiosarcoma |

Analyses of the IARC *p53* database (http://www-p53.iarc.fr) reveal that G to T transversions are more common in lung tumors from smokers than from never-smokers (30% and 12% respectively)[42] which is supported by a recent study including detailed smoking data.[43] G to T transversions have been shown to be induced by PAHs, which is a group of potent carcinogens present in cigarette smoke. One of the best studied PAHs is benzo(a)pyrene, which is activated to a diol epoxide and induce mutations preferentially on guanine positions at codons 157, 248 and 273.[44] Other hot-spots in lung cancer are codons 158 and 245, which are also binding-sites for PAHs, and codon 249.[45]

The effect of ETS on *p53* mutations is less clear. A previous study suggested an increased mutational prevalence in the *p53* gene and the predominant mutation type was G to A transitions, with 2 of the 4 detected G to A transitions occurring at CpG sites.[46] However, only 9 mutations were detected in never smoking cases making conclusions uncertain. In CpG sites a cytosine nucleotide occurs next to a guanine nucleotide in the sequence of bases in DNA, separated by a phosphate which links the two nucleotides together. Mutations at CpG sites have been suggested to occur as a result of spontaneous deamination of methylated cytosine into thymine.[47] On the other hand, some studies show that methylated CpG dinucleotide sites may also be targets for exogenous chemical carcinogens.[48]

Regarding *p53* mutations and radon exposure, several studies have been performed with varying results.[49-54] However, many of the earlier studies aimed to confirm an initial report[55] suggesting a mutational hot-spot in tumors of miners exposed to radon and are therefore limited to codon 249 and include mostly smokers. The total number of known never-smokers included in these studies was 10.

There is no previous study on *p53* mutation prevalence and spectrum in lung cancer cases exposed to airborne arsenic. There are, however, a number of studies with contradicting results, most of them including only few subjects, concerning *p53* mutations in skin and bladder tumors related to arsenic exposure from medical use or contaminated water.[56-61] Two of these are large epidemiological investigations, one in which the exposure to arsenic was assessed using toenail measurements[57] and the other assessing arsenic exposure based on the concentration of arsenic in drinking water.[56] The former study suggested a relative absence of *p53* mutations with arsenic exposure while in the latter study an increased *p53* mutation prevalence was indicated, although the effect was smaller for cases with the highest exposure than for cases with moderate exposure.

## *Why study the p53 gene in epidemiologic investigations?*

Papers I, III and IV in this thesis were performed to assess the ability to use the spectrum and prevalence of *p53* mutations as a marker of exposure or a subclassification of lung tumors based on etiology in epidemiological studies, as well as to increase the knowledge about lung cancer pathology. The background was a suggested radon-associated *p53* mutational hotspot detected in uranium miners[55] but also other specific *p53* mutations that had been associated with different exposures, as described in Table 1. The hope was that biomarkers, such as mutations in the *p53* gene, could be used to improve the resolution power in epidemiologic studies and facilitate quantitative risk estimation. The prevalence and spectrum of the *p53* gene is interesting to investigate in this respect since the *p53* gene is of great importance in cell regulation, apoptosis and DNA repair. Further, the *p53* gene is the most frequently mutated gene in lung cancer, more than 75% of the mutations are missense mutations and the diversity of point mutations indicate that the *p53* gene could be informative to analyze with respect to etiology- specific mutations.

# AIMS OF THE THESIS

The main purpose of this thesis was to investigate the association between different environmental exposures and the *p53* mutation prevalence and spectrum in lung cancer tumors as well as to evaluate if studies of the *p53* gene in epidemiological investigations can contribute to our knowledge about the carcinogenic mechanisms of environmentally induced lung cancer.

The specific aims were:

- ✓ to investigate how the *p53* mutation prevalence and spectrum differ between smokers and never-smokers (Papers I, III and IV).

- ✓ to evaluate if the prevalence and spectrum of *p53* mutations differ among lung cancer cases with and without exposure to residential radon (Papers I and IV), occupational or environmental arsenic exposure (Paper III) and ETS (Paper IV).

- ✓ to assess the combined effects of tobacco smoking and exposure to residential radon or arsenic, respectively, on the *p53* mutation prevalence (Papers I and III).

- ✓ to investigate the combined effect of ETS and residential radon with regard to *p53* mutations in never-smokers (Paper IV).

- ✓ to investigate if the previously observed increased risk of lung cancer among men in the area surrounding Rönnskärsverken has decreased following reduced emissions and to study the effect of environmental arsenic exposure among women in the area (Paper II).

# MATERIAL AND METHODS

## *Study subjects*

**Paper I** was based on subjects from the nationwide case-control study on residential radon and lung cancer in Sweden.[16] The study included all women and a random sample of men aged 35-74 years with primary cancer of the bronchus or lung diagnosed 1980-1984, who had lived in one of 109 municipalities in Sweden at some time from January 1, 1980, through December 31, 1984 and who had been living in Sweden on January 1, 1947. In total, 1360 subjects were identified from the Swedish Cancer Registry and 84% of the cases had a histopathological classification based on biopsy or autopsy. From this group, all non-smoking lung cancer cases with a time-weighted average radon exposure exceeding 140 Bq/m$^3$ (n=34) or up to 50 Bq/m$^3$ (n=49) were selected. Among smokers, the intention was to randomly select 50 cases from the two exposure categories for each of the major histological types (SCC, SCLC and AC). However, among cases with radon exposure over 140 Bq/m$^3$, each of the three histological groups contained less than 50 smoking cases and consequently all subjects in these groups were included (n=111). Among smoking lung cancer cases with radon exposure up to 50 Bq/m$^3$, 50 SCC cases (58% of the cases of this histological type) and 50 SCLC cases (76%) were randomly selected as well as all available cases with AC (n=39).

**Paper II** used a case-control methodology similar to that used by Pershagen (1985) and was designed as a follow-up of that study, extending the recruitment period for men and adding women. The cases were men and women who had died in Skellefteå Municipality in 1961-1990 and who had received a diagnosis of cancer of the bronchus or lung at any time. Cases were identified through the Regional Cancer Registry at the Oncological Center in Umeå and the National Cause-of-Death Registry at Statistics Sweden. A total of 369 men and 116 women with lung cancer were identified, including 221 male cases from the previous study. Controls were selected from the National Cause-of-Death Registry among people deceased in Skellefteå Municipality during the same time period [1961-1979 (men), 1980-1990 (men) or 1961-1990 (women), respectively] as the cases, but without a diagnosis of cancer of the bronchus or lung. Two controls were selected to each case after matching for sex and birth year. Subjects who had worked at the Rönnskärsverken smelter, according to information from the company or in questionnaire answers, were excluded.

**Paper III** comprised lung cancer cases from the study in Paper II. All never-smoking male lung cancer cases were included (n=11). Among smokers, all male cases who had been resident in either of the two parishes closest to the smelter (the defined arsenic exposed area) for any period from 1930 to 1990 were selected. Further, a random sample of 59 smoking male lung cancer cases was selected among those who had not lived close to the smelter (to obtain 70 unexposed

men including the 11 selected never-smokers). In addition, men occupationally exposed at the Rönnskärsverken smelter for at least five years to an estimated exposure level of arsenic of at least 0.05 mg/m$^3$, were selected from previous studies of lung cancer among workers at the smelter (n=68).[30]

**Paper IV** included never-smokers with a histologically confirmed diagnosis originating from 5 previously performed studies as follow: ***i)*** A study of the relation between ETS exposure and lung cancer among never-smokers in Stockholm County aged 30 years or more. Never-smoking lung cancer cases (ICD-9, code 162) were recruited between October 1, 1989, and September 30, 1995 from the three main hospitals in the area responsible for diagnosis and treatment of lung cancer.[62] ***ii)*** An investigation of the relation between environmental factors and lung cancer among subjects below 75 years of age living in one of 26 municipalities in Gothenburg and Bohus county and Älvsborg county in southwest Sweden. Cases (ICD-7 code 162.1) were recruited between January 1989 and June 1994 among lung cancer cases referred to any of the three main regional hospitals.[63] ***iii)*** Paper II from which all never-smoking female cases were selected.[26] ***iv)*** A case-control study investigating the association between occupational exposure and lung cancer conducted in a Swedish county, Västernorrland, with a large number of paper and pulp mills. Cases were identified using the regional cancer registry in Umeå (ICD-7, code 162.1) from 1978 to 1991 among those deceased before September 1, 1992.[64] ***v)*** Paper I from which all never-smoking cases were selected.[65]

## *Exposure assessment*

Detailed information on smoking, occupational and residential histories was collected through questionnaires and/or interviews with study subjects or next-of-kin. Data from parish registries were used to review and complete the residential histories. Exposure to ETS was assessed in the questionnaires/interviews by asking if the person had lived with a smoking spouse or cohabitant, the amount smoked by the cohabitant, type of tobacco and during what period of time.

In Paper I, radon measurements were performed in all available dwellings in which the study subject had lived during a period of at least 2 years since 1947 to three years before the end of follow-up in 1980-1984. Measurements were performed over a period of three months during the heating season using solid-state alpha track detectors. Time-weighted mean radon concentrations were calculated by dividing the cumulative radon exposure by the total residential time in dwellings for which radon measurements were available. The measurements are described in more detail in Pershagen et al. 1994.[16]

For study subjects included in Paper IV but not originating from Paper I, radon measurements were preformed similarly as in Paper I and included all available dwellings where the study

subjects had lived for at least 2 years during a retrospective period of 32 years ending 3 years before diagnosis. A more detailed description is given in Lagarde et al. 2001.[17]

## *p53 mutation analyses*

Microscopy slides and tumor sample blocks were collected from the pathology departments where the cases were diagnosed. Paraffin-embedded blocks were sectioned and one section was stained with hematoxylin-eosin. The slides were reviewed by a pathologist for assessment of presence of tumor cells and in Papers I and III also for histological classification. Samples included in Paper III and parts *ii*-*iv* of Paper IV were micro-dissected by hand in order to obtain a high proportion of tumor cells.

### SSCP analysis and DNA sequencing

For cases in Paper I and parts *ii*-*v* of Paper IV as well as for cases in part *i* of Paper IV diagnosed before 1992, deparaffinised tumor tissues were digested with proteinase K and genomic DNA was purified by phenol/chloroform extraction. Intronic primers for exons 5-8 of the *p53* gene were used to amplify genomic tumor DNA. For SSCP-analysis,[66] PCR products were labelled by including $^{32}$P-dATP in a secondary PCR-amplification. Radiolabelled PCR products were diluted, denatured and loaded on polyacrylamide and MDE gels (Mutation Detection Enhancement, FMC Bio-Products, Rockland, ME). DNA strands were separated and autoradiographed. Mutations were detected as shifts in the mobility of the bands of separated single strands in the autoradiogram. PCR products showing altered mobility were eluted from the gels and reamplified for sequence determination. Sequencing was performed using Thermo Sequenase with $^{32}$P-radiolabelled dideoxynucleotides from Amersham Life Sciences.

### Direct DNA sequencing

In Paper III micro-dissected paraffin-embedded formalin-fixed tumor tissue samples were deparaffinised using xylene-ethanol and then treated with proteinase K.[67, 68] Exons 5-8 of the *p53* gene were first amplified in a multiplex-PCR reaction and thereafter in a second exon-specific PCR. The PCR products were purified using QIAquick PCR purification Kit, (QIAGEN Gmbh, Germany) and sequenced on a ABI 377 using BigDye Terminator v1,1 Cycle Sequencing Kit (Applied Biosystems, USA). The DNA was sequenced both in the forward and reverse direction. In some samples PCR amplification of exon 5 was difficult and a method employing amplification of smaller overlapping fragments of exon 5 was used. All sequences were checked for *p53* mutations both visually and by using PolyPhred software.[69] All exons 5-8 were successfully sequenced for 69 samples (58%), 3 exons for 26 samples (22%), 2 exons for 11 samples (9%) and 1 exon for 9 samples (8%), in total 115 samples with sequence data out of the 120 samples available for DNA analysis.

## DGGE analysis and DNA sequencing

Cases diagnosed 1992-1995 in part *i* of Paper IV originate from a study where *p53* analyses had already been performed.[46] Briefly, tumor blocks containing at least 50% tumor tissue were selected and DNA was obtained by phenol-chloroform extraction.[70] Exons 4-9 and 11 were screened for *p53* mutations using DGGE-analysis (more thoroughly described in Kannio et al.[70] and Husgafvel-Pursiainen et al.[46]). *p53* mutations were identified using Sequenase Version 2.0 (United States Biochemical) and Thermo Sequenase radiolabeled terminator cycle sequencing (Amersham Life Science, Inc,) with primers described in Kannio et al.[70] and Ridanpää et al.[71]

In all studies, the detected mutations were confirmed in a second analysis using a new independent PCR product to exclude possible artefacts.

## Confirmatory direct solid phase DNA sequencing

In Paper I confirmative, direct solid-phase DNA sequencing[72] was performed of separately generated PCR products as a sensitivity test of the SSCP screening. Primers used for DNA amplification were situated in intronic sequences covering exons 5-8 of the *p53* gene and labelled with biotin to facilitate direct solid-phase sequencing of PCR products by use of paramagnetic beads. The biotinylated amplified fragments were immobilised onto streptavidin-coated solid support and strand specific alkali elution produced a clean template for sequencing. Solid-phase sequencing was performed by a robot with fluorescence-labelled primers and an automated laser fluorescence instrument was used for sequence analysis. Parallel analyses were performed using SSCP analysis combined with traditional DNA sequencing and direct solid phase sequence analysis of the first 49 samples for quality assessment. For the rest of the tumors only those samples showing confirmed altered mobility in polyacrylamide and/or MDE gels in the SSCP analysis were further analysed by the solid phase methodology.

## Immunohistochemistry

As a comparative analysis of the *p53* gene, immunohistochemistry staining was performed for samples included in Paper I. The tumors were mounted on glass slides, deparaffinised, placed in citrate buffer and treated with microwaves for antigen retrieval. The immunohistochemistry staining was performed using a Tech Mate 500 immunostainer and the outcome was then classified as ”reactive” or ”non-reactive” regarding binding of antibodies to the cell nuclei. No antibody binding (representing degree 0) and poor antibody binding (representing degree 1) in combination with focal distribution was considered as non-reactive.

## Sample exclusion

Patients treated with chemotherapy or radiation before tissue collection according to medical records (Papers I, III and part *v* of Paper IV) or reports from the pathology departments (parts *i-iv* of Paper IV) were excluded. For some cases there was no tumor sample available or the DNA

was insufficient or too contaminated with normal cells. In Paper III, one pathology department declined to send samples (n=12) and for 5 samples the direct sequencing did not succeed for any of the exons. The number of cases excluded in the different studies is described in Figure 3.

## Histological classification

In Papers I and III, one haematoxylin-eosin stained slide from each case with an available tumor sample was reviewed by a Swedish pathologist. This slide was taken from the same tumor block and at the same occasion as the sections used for DNA sequencing. In paper IV the histological classification was based on pathology records, information in the Swedish Cancer Registry or an assessment based on both pathology and medical records.

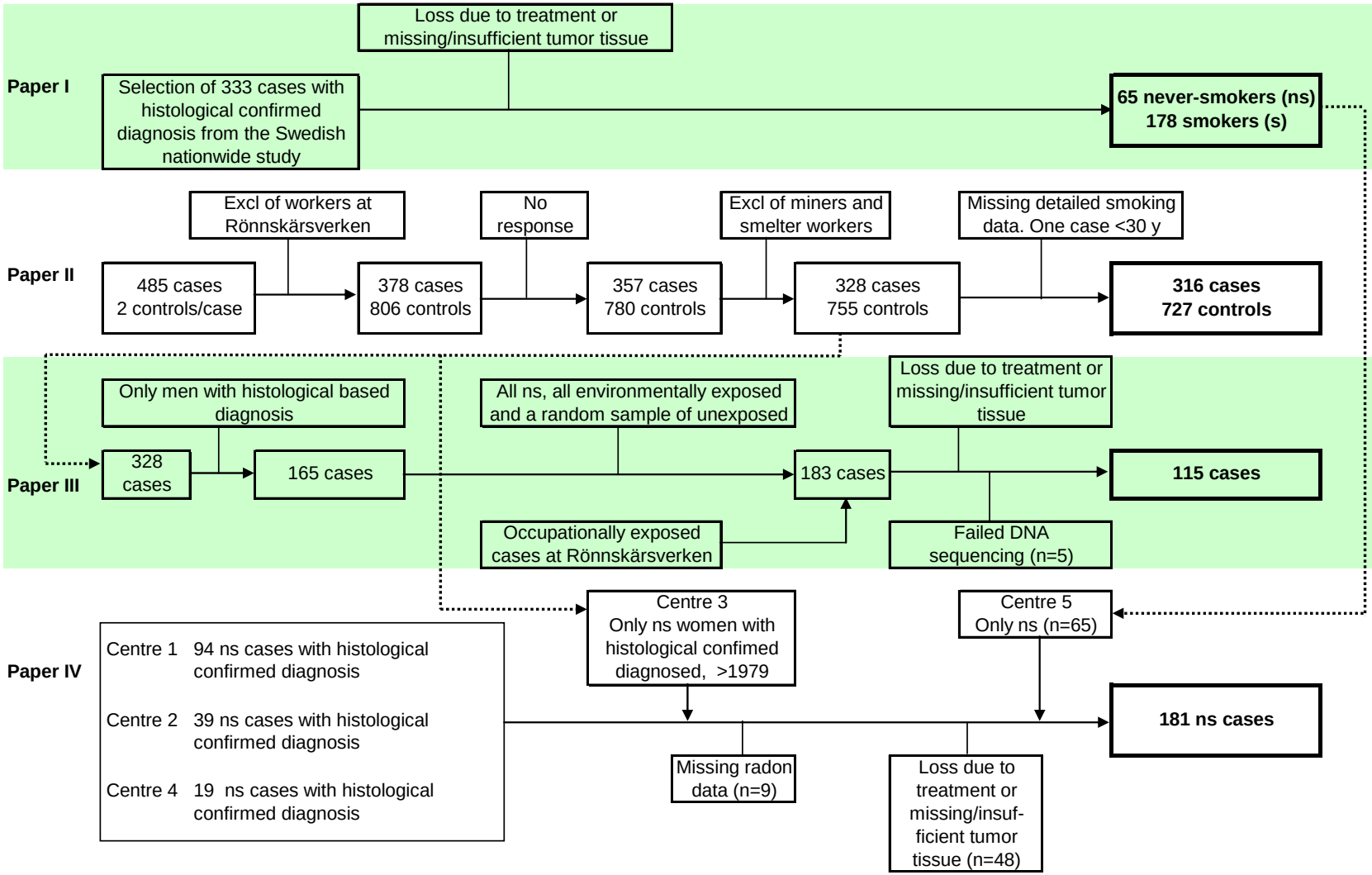


**Figure 3.** A description of the selection and drop-out of cases in Paper I-IV

## Statistical analysis

In Papers I, III and IV the association between exposure to residential radon (Papers I and IV), arsenic (Paper III) and ETS (Paper IV) and the presence of *p53* mutation was evaluated using maximum likelihood estimation of the prevalence ORs with 95% CI, based on unconditional logistic regression analysis. Differences in type of mutations between groups were tested using Fisher's exact test.

In Paper II the relative risk (RR) of lung cancer associated with environmental exposure to air pollution from the smelter was assessed using maximum likelihood estimation of the odds ratio (OR) and 95% confidence interval (CI), based on conditional logistic regression analysis with strata defined by the matching factors birth year, gender and (for men) period of recruitment.

Adjustment for relevant covariates was done by including indicator variables that represented categories of exposure. In Paper II adjustment was also performed for the difference in age between the cases and the controls within a stratum using a continuous variable representing the difference between the age at death for a subject in a matching stratum and the mean age at death for all subjects within that stratum.

# RESULTS

## *Lung cancer risk in the area around Rönnskärsverken*

Living in any of the two parishes closest to the smelter was tended to be associated with an increased risk of lung cancer among men (Table 2). No clear difference in risk could be detected among men deceased during the first (1961-1979) and the second (1980-1990) time periods, RR 1.4 (95% CI 0.8-2.6) and RR 1.3 (95% CI 0.7-2.4) respectively. The increase in risk seemed to primarily concern men exposed in the beginning of operations (before 1940) and particularly among those exposed for less than 20 years. No difference in effect was detected between men exposed before or after 1950.

**Table 2.** Estimated relative risk for lung cancer associated with exposure duration and time period of residence in the Rönnskärsverken area among men deceased in Skellefteå Municipality 1961-1990.[a]

| Men resident in the Rönnskärsverken area | cases/controls | RR/CI[b] |
|---|---|---|
| Never | 153/413 | 1 |
| Ever | 56/89 | 1.4 (0.9-2.1) |
| <20 years | 19/27 | 1.7 (0.8-3.4) |
| ≥20 years | 37/62 | 1.3 (0.8-2.1) |
| First time after 1939 | 16/31 | 1.2 (0.6-2.4) |
| First time before 1940 | 40/58 | 1.5 (0.9-2.5) |
| First time after 1939, <20 years | 8/18 | 1.1 (0.4-3.1) |
| First time after 1939, ≥20 years | 8/13 | 1.2 (0.4-3.2) |
| First time before 1940, <20 years | 11/9 | 2.5 (0.9-7.1) |
| First time before 1940, ≥20 years | 29/49 | 1.3 (0.7-2.3) |

[a] Smelter workers and miners excluded, as well as persons without detailed smoking information.
[b] Estimated relative risk and 95% confidence interval adjusted for smoking habits, occupation and age difference between subjects within each stratum, in a conditional logistic regression analysis stratified by birth year and period of recruitment.

Estimates for the increased lung cancer risk from smoking among men were RR 4.3 (95% CI 2.4-7.7) for former smokers, RR 7.8 (95% CI 4.5-13.6) for current smokers of 1-15 cig/day and RR 22.5 (95% CI 11.4-44.5) for current smokers of more than 15 cig/day compared to never-smokers. The corresponding estimates for women were 3.8 (95% CI 1.5-9.7), 4.1 (95% CI 2.0-8.4) and 9.8 (95% CI 3.2-29.8), respectively. Among men the results were in agreement with an additive effect between smoking and residence in the exposed area, but data were comparatively sparse and the estimates were compatible also with other patterns of interaction. For women, no

overall increase in risk was indicated with residence in the area closest to the smelter. However, the data were limited and precluded more detailed analyses.

## *p53 mutations in lung cancer*

In total, across all studies, we succeeded to analyze 474 lung tumors and detected 103 *p53* mutations in 99 lung cancer cases (21%). All detected *p53* mutations are listed in Appendix. The number of *p53* mutations in relation to some characteristics of the lung cancer cases is given in Table 3. This should be seen more as a description of the different studies than as a comparison, since no adjustment is made for the exposures under study and there is some overlap between Papers I and IV (65 cases).

**Table 3.** *p53* mutations in relation to some characteristics in the different studies.

| | Paper I | Paper III | Paper IV |
|---|---|---|---|
| Smoking status | | | |
| Never smokers | 9/65 (14) | 3/14 (21) | 24/181 (13) |
| Smokers | 49/178 (28) | 23/101 (23) | 0/0 |
| Histology[a] | | | |
| SCLC | 24/73 (33) | 10/33 (30) | 2/12 (17) |
| SCC | 19/59 (32) | 7/46 (15) | 4/26 (15) |
| AC | 12/86 (14) | 6/24 (25) | 15/111 (14) |
| Other | 3/25 (12) | 3/12 (25) | 3/32 (9) |
| Age | | | |
| <60 years | 8/53 (15) | 3/20 (15) | 4/46 (9) |
| 60-69 years | 39/127 (31) | 9/46 (20) | 9/62 (15) |
| >69 years | 11/63 (18) | 14/49 (29) | 11/73 (15) |
| Sex | | | |
| Women | 20/104 (19) | 0/0 | 17/127 (13) |
| Men | 38/139 (27) | 24/115 (21) | 7/54 (13) |

[a] In Papers I and III the histological classification was based on a review of tumor slides from the same tumor samples that were analyzed for *p53* mutations. In paper IV the classification was based on pathology records, information in the Swedish Cancer Registry or an assessment based on both pathology and medical records.

## *p53* and tobacco smoking

This thesis includes 218 smoking and 189 never-smoking lung cancer patients, excluding those with arsenic exposure. Smokers had a higher *p53* mutation prevalence than never-smokers (OR 2.4, 95% CI 1.1-5.1). The *p53* mutation prevalence was 13% (25/189) among never-smokers and 28% (61/218) among smokers. The difference in mutational spectrum is shown if Figure 4.

Smokers had a higher proportion of G:C to T:A transversions, while G:C to A:T transitions at CpG sites were more common among never-smokers.

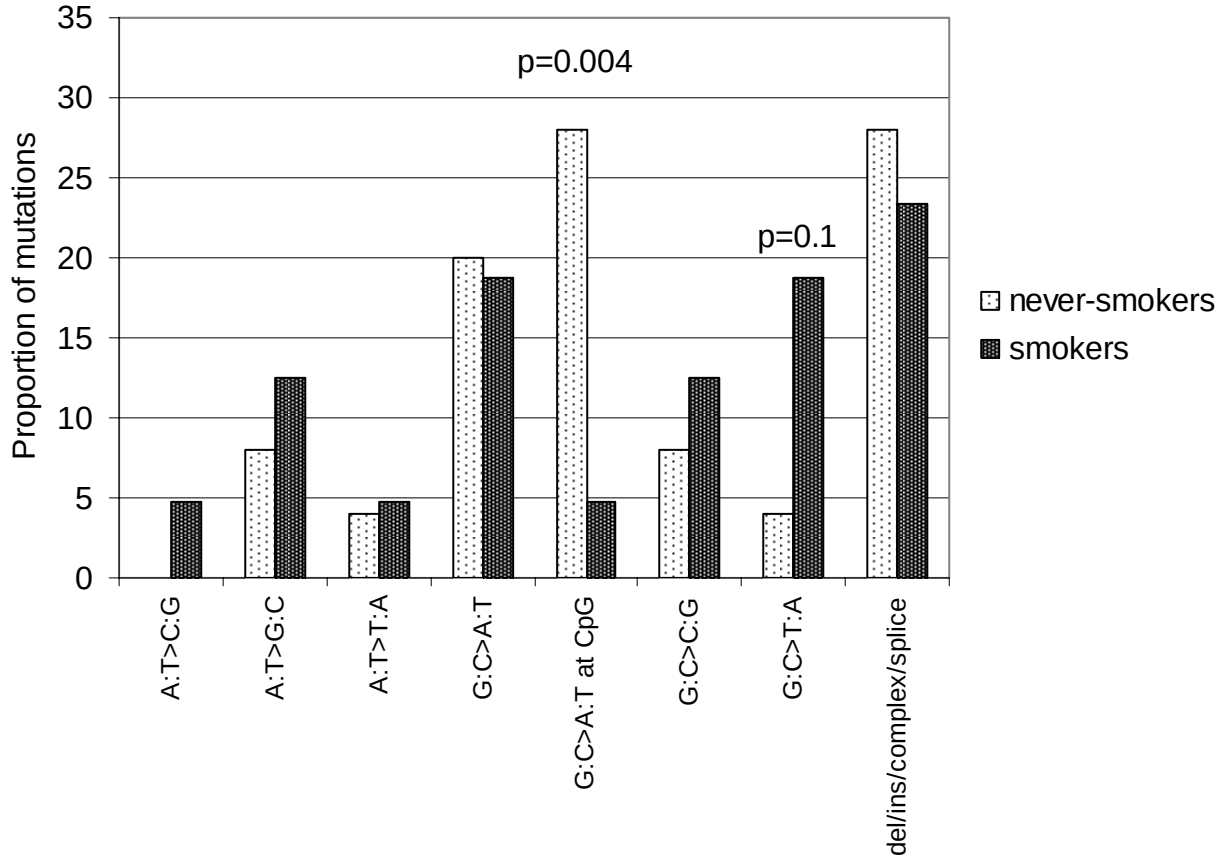


**Figure 4.** Types of *p53* mutations among 218 smokers and 189 never-smokers (overall p-value 0.08).

The codon distribution of the detected point mutations is shown in Figure 5 with codons 158, and 249 as most commonly mutated among smokers and codon 248 for never-smokers. However, this analysis is hampered by low numbers of mutations at each codon.

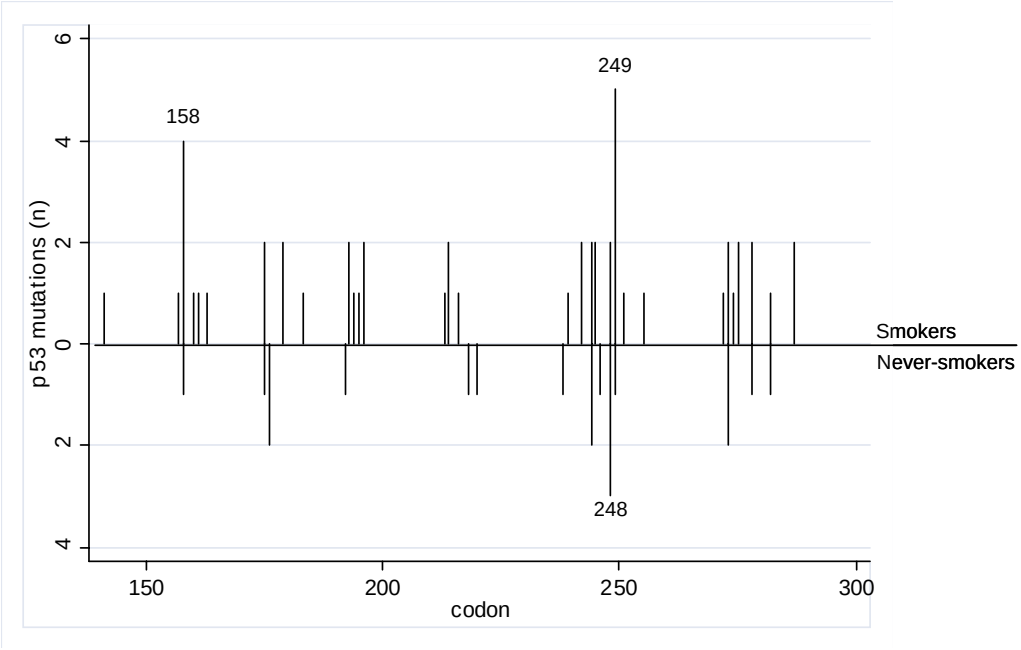


**Figure 5.** The codon distribution of base-changes detected in the *p53* gene among 218 smokers and 189 never-smokers.

## *p53* and residential radon

In paper I we detected 61 *p53* mutations in exons 5 to 8 in 58 different lung tumors among 243 sequenced. The overall mutation prevalence was 24%. Residential radon seemed to increase the *p53* mutation prevalence, at least at very high levels of exposure, OR 1.4 (95% CI 0.7-2.6) for those exposed to a time-weighted average level between 140 and 400 Bq/m$^3$ and OR 2.8 (95% CI 0.8-9.3) for those exposed to more than 400 Bq/m$^3$ compared to those exposed to less than 50 Bq/m$^3$. The suggested increase in *p53* mutation prevalence appeared more pronounced among never-smokers (OR 3.2, 95% CI 0.7-15.5). Among smokers, a negative interaction with radon was suggested for smokers of 10 cig/day or more (Table 4).

**Table 4.** Odds ratios and 95% confidence intervals[a] of presence of *p53* mutation in lung cancer cases diagnosed 1980-1984 in Sweden, according to time-weighted mean residential radon exposure since 1947 and smoking status.

| | Non-smoker | Current smoker <10 cig./day | Current smoker ≥10 cig./day |
|---|---|---|---|
| Low radon exposure ≤50 Bq/m$^3$ | 1 | 4.5 (1.0-19.6) | 4.2 (1.1-16.3) |
| High radon exposure >140 Bq/m$^3$ | 3.7 (0.8-16.8) | 11.2 (2.5-49.3) | 2.3 (0.5-9.8) |

[a]Adjusted for sex and age at diagnosis (3 categories)

Table 5 describes the different types of *p53* mutations. To be able to compare the results in Paper I and Paper IV with regard to radon exposure, the radon exposure period was marginally recalculated for cases who originated from Paper I.

**Table 5**. Detected *p53* mutations in relation to radon exposure among never-smoking and smoking lung cancer cases.

| | Never-smokers (Papers I and IV) | | Smokers (Paper I) | |
|---|---|---|---|---|
| Mutation | ≤50 Bq/m$^3$ | >50 Bq/m$^3$ | ≤50 Bq/m$^3$ | >50 Bq/m$^3$ |
| A:T>C:G | | | 1 | 2 |
| A:T>G:C | 1 | 1 | 4 | 2 |
| A:T>T:A | 1 | | 1 | 1 |
| G:C>T:A | 1 | | 7 | 3 |
| G:C>A:T (not at CpG) | 1 | 3 | 5 | 4 |
| G:C>A:T at CpG | 1 | 6 | 1 | 2 |
| G:C>C:G | 1 | 1 | 2 | 3 |
| Del/ins/splice/complex | 5 | 2 | 8 | 6 |
| Total | 11 | 13 | 29 | 23 |

When dividing the mutations into different types of mutations the numbers become very small. Clearly, we could not confirm the hot-spot mutation previously detected among uranium miners.[55] All 5 mutations in codon 249, of which one was a AGG to ATG transversion, occurred in cases not excessively exposed to residential radon. Among never-smokers G:C to A:T transitions were the most common base substitution and this type of mutation seemed to be more prevalent among those exposed to more than 50 Bq/m$^3$ (9 mutations out of 13) than among unexposed (2/11) and a majority occurred at CpG sites. Among smokers, we detected three identical splice mutations in intron 8, CGAGgt to CGAGtt, all among cases exposed to more than 140 Bq/m$^3$.

## *p53* and ETS

We detected 24 mutations in 181 never-smokers analyzed for *p53* mutations in exons 5-8, resulting in a mutation prevalence of 13%. Approximately 40% of the cases had been exposed to ETS and a tendency to a higher mutation prevalence among ETS exposed was suggested (OR 1.4, 95% CI 0.6-3.5), especially among those exposed during a period of at least 30 years (OR 2.4, 95% CI 0.8-7.3, compared to those without exposure to passive smoking).

The effect of ETS seemed to be limited to never-smokers also exposed to residential radon (Table 6). Those who had lived with a smoking spouse or cohabitant had an OR of 2.0 (95% CI 0.6-6.4) for radon exposure over 50 Bq/m$^3$ compared to exposure up to 50 Bq/m$^3$. In particular, long term

exposure to ETS (at least 30 years) in combination with exposure to residential radon showed an increase in the *p53* mutation prevalence (OR 4.9, 95% CI 1.2-21.1).

**Table 6.** Odds ratios and 95% confidence intervals of presence of *p53* mutation in lung cancer cases according to ETS- and residential radon exposure among never-smokers.

| Never-smokers | Radon ≤50 Bq/m$^3$<br>n *p53*+/tot n (%)<br>OR (95% CI) [a] | Radon >50 Bq/m$^3$<br>n *p53*+/tot n (%)<br>OR (95% CI) [a] |
|---|---|---|
| No ETS at home | 7/59 (12)<br>1 | 5/44 (11)<br>1.1 (0.3-3.8) |
| ETS at home | 4/36 (11)<br>1.0 (0.3-4.0) | 8/42 (19)<br>2.0 (0.6-6.4) |

[a]Adjusted for sex, age at diagnosis (3 categories) and method and time of analysis (3 categories)

The most prevalent mutation in Paper IV overall was G:C>A:T transversions and a majority of these occurred a CpG sites. The different types of mutations are described in Table 7 and smokers included in Paper I are included as comparison.

**Table 7.** Detected *p53* mutations in lung cancer cases stratified for tobacco exposure.

| Mutation | Never-smokers | | Smokers |
|---|---|---|---|
| | No ETS exposure | ETS exposure | |
| A:T>C:G | | | 3 |
| A:T>G:C | 1 | 1 | 6 |
| A:T>T:A | | 1 | 2 |
| G:C>T:A | 1 | | 10 |
| G:C>A:T (not at CpG) | 1 | 3 | 9 |
| G:C>A:T at CpG | 3 | 4 | 3 |
| G:C>C:G | 1 | 1 | 5 |
| Del/ins/splice/complex | 5 | 2 | 14 |
| Total | 12 | 12 | 52 |

Among never-smokers, we detected more than one mutation in codons 176, 244, 248 and 273. Codons 248 and 273 are considered mutational hot-spots and among those with ETS exposure in our studies all 4 mutations at these codons occurred at CpG dinucleotide sites. Only one mutation occurred at these codons in a case without ETS exposure and this was not at a CpG site.

Table 8 includes both smokers and never-smokers with regard to amount of smoking or ETS exposure, as well as residential radon exposure. The table illustrates that the suggested effect of residential radon among never-smokers seemed to be limited to those also exposed to ETS (Paper IV) and that smokers were indicated to have a different effect of radon on *p53* mutation prevalence due to amount smoked (Paper I).

**Table 8**. Odds ratios and 95% confidence intervals[a] of presence of *p53* mutation in lung cancer cases, according to time-weighted mean residential radon exposure, smoking consumption and ETS status.

| | Never-smokers without ETS | Never-smokers with ETS | Current smokers <10 cig./day | Current smokers ≥10 cig./day |
|---|---|---|---|---|
| Radon exposure ≤50 Bq/m$^3$ | 7/59<br>1 | 4/36<br>1.1 (0.3-4.2) | 11/38<br>3.0 (0.9-10.3) | 15/62<br>2.6 (0.8-8.2) |
| Radon exposure >50 Bq/m$^3$ | 5/44<br>1.1 (0.3-3.9) | 8/42<br>2.1 (0.7-6.5) | 15/31<br>7.1 (2.1-24.8) | 8/47<br>1.4 (0.4-5.0) |

[a]Adjusted for age, sex and batch of analysis

## *p53* and arsenic

In the study of *p53* mutations in lung tumors and arsenic exposure 115 tumors were successfully sequenced. Among those, we detected 27 mutations in 26 different tumors which gave a mutation prevalence of 23 % (26/115). Environmental exposure to arsenic seemed to decrease the *p53* mutational prevalence (OR 0.3, 95% CI 0.1-1.3) although this could only be assessed among smokers. The same tendency was indicated for occupational exposure to arsenic and we thus combined all arsenic exposed subjects into one group in the further analyses.

Among never-smokers, arsenic exposure was suggested to increase the *p53* mutation prevalence although the number of tumors is limited (Table 9). Among smokers, arsenic exposure seemed to affect the *p53* mutation prevalence in the opposite direction, with a suggested decrease in *p53* mutation prevalence. The lower *p53* mutation prevalence indicated among smokers occupationally or environmentally exposed to arsenic compared to smokers without arsenic exposure seemed particularly pronounced in smokers with SCLC (*p53* mutation prevalence 6% among exposed versus 54% among unexposed, OR 0.05, 95% CI 0.004-0.6).

**Table 9.** Odds ratios and 95% confidence intervals[a] of presence of *p53* mutation in male lung cancer cases according to smoking status and occupational or environmental exposure to arsenic.

| Smoking status | Unexposed to arsenic<br>n *p53*+/tot n (%)<br>OR (95% CI) | Environmental- or occupational exposure to arsenic<br>n *p53*+/tot n (%)<br>OR (95% CI) |
|---|---|---|
| Non-smoker | 1/8 (13)<br>1 | 2/6 (33)<br>4.0 (0.3-61.9) |
| Smoker | 12/40 (30)<br>3.9 (0.4-36.2) | 11/61 (18)<br>1.8 (0.2-16.5) |

[a] Adjusted for age at diagnosis (3 categories).

The most common type of *p53* mutation was G:C to T:A transversions, all 7 detected among smokers, and G:C to A:T transitions. The different types of mutations detected among smokers are shown in Figure 6. Smokers with arsenic exposure seemed to have fewer types of mutations than smokers without arsenic exposure.

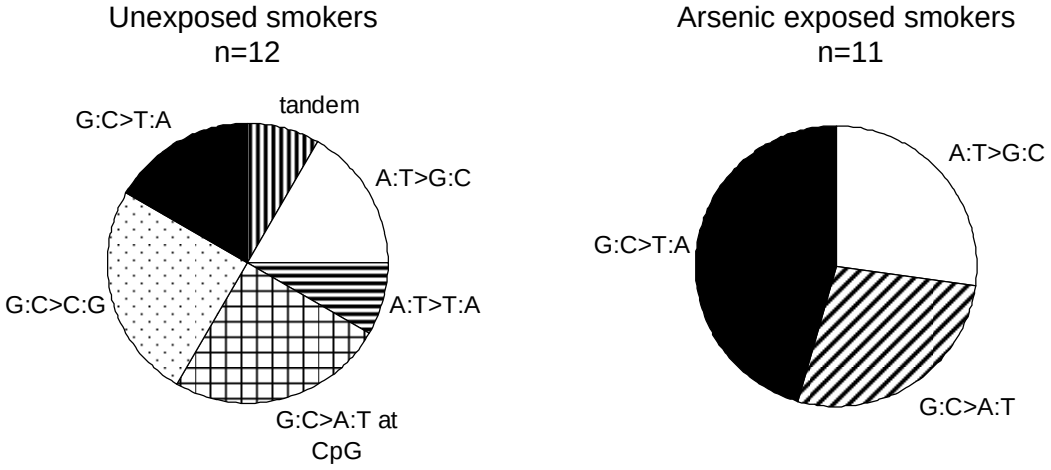


**Figure 6.** The proportion of different types of *p53* mutations among smokers with and without arsenic exposure.

## *Comparison of SSCP screening in combination with sequencing and direct solid-phase sequencing*

In Paper I the first 49 samples were analysed independently by two different laboratories, the first using SSCP screening in combination with DNA sequencing and the second using solid-phase sequencing. Three out of the 49 tumors differed with regard to *p53* status between the two laboratories. One transition was detected by solid–phase sequencing but not by the SSCP screening whereas one transition and one deletion was detected by SSCP screening but not by solid-phase sequencing. In total, 17 mutations were detected in the 49 tumors analysed independently with both methods. The concordance between the two methods was 82%.

## *Comparison of immunohistochemical analysis and DNA sequencing*

In the immunohistochemistry (IHC) analysis in Paper I, 100 tumors of the 243 analysed were classified as "reactive", of which we only could detect a *p53* mutation in 33.Twenty-five samples with a *p53* mutation detected in the SSCP analysis were not "reactive" in the IHC analysis. The concordance between the two methods was estimated to 62% and did not seem to be affected to any major extent by smoking or radon exposure.

## *Comparison of histological classification*

In Paper I all slides were stained with haematoxylin-eosin and classified according to histological type by one pathologist. The histological type had previously been classified according to pathology records and Table 10 shows the concordance between the two classifications.

**Table 10.** The concordance between the reviewed histological classification and the classification used from pathology records (n=243).[a]

| | Histology according to pathology records n(%) | | | |
|---|---|---|---|---|
| Histology according to the histological review | SCLC | SCC | AC | Other |
| SCLC | 67 (89) | 3 (4) | 2 (2) | 1 (14) |
| SCC | 3 (4) | 52 (73) | 3 (3) | 1 (14) |
| AC | 0 | 7 (10) | 77 (86) | 2 (29) |
| Other | 5 (7) | 9 (13) | 8 (9) | 3 (43) |
| Total | 75 (100) | 71 (100) | 90 (100) | 7 (100) |

[a] Among smokers only cases of the main histological subtypes according to pathology records were selected for inclusion in the study

The pathologist review and the classification according to pathology records agreed completely on main histological group for 82% of the tumor samples. Only 8 cases (3%) were reclassified between NSCLC (AC or SCC) and SCLC.

# DISCUSSION

## *Main findings*

### Lung cancer in the area surrounding Rönnskärsverken

Although a non significant increase in lung cancer risk was suggested among men who had been resident in the area closest to the Rönnskärsverken we could not observe any clear risk reduction for men deceased 1961-1979 compared to men deceased 1980-1990. This may be due to a long latency period for contracting lung cancer with environmental exposure to smelter emissions as one component cause. Another possible explanation is that our study population was too limited in size to capture moderate risk differentials with adequate statistical power. For both time periods the risk estimates have wide confidence intervals and the results were also compatible with no increase in lung cancer risk for living close to the smelter. An small true increase in risk is however the most likely interpretation, since occupational exposure to inorganic arsenic, at the Rönnskärsverken and other smelters, has been clearly associated with increased lung cancer risk.[21-25] Also environmental exposure has been associated with an excess lung cancer risk.[26-29] For workers at the Rönnskärsverken a decrease of the lung cancer risk over time with lowered exposure levels has been suggested.[73] The reductions in emissions from the smelter were made progressively over several decades, and uncertainties regarding possible relevant agents, actual exposure levels and latency periods make it difficult to perform dose-response analyses. Nonetheless, the risk tended to be higher among men exposed in the beginning of the operations and with exposure duration less than 20 years. An increase of the lung cancer risk with short duration of exposure has been noted in workers at Rönnskärsverken,[22] as well as in other occupational studies of cancers. This may be due to differences in personal habits,[74] confounding by other occupational exposures, misclassification of exposure as well as a consequence of the healthy worker effect.[75, 76]

Although the results for women are not supportive of the possibility that the environmental exposure is responsible for increased lung cancer risk, the wide confidence intervals are compatible with this hypothesis. Furthermore, the lack of a detectable risk increase among women may be due to the fact that most female cases occurred during the later period. Differential ages of smoking uptake and quitting between the genders may affect the latency to disease after environmental exposures. There may also be problems with misclassification of lung cancer among women, and this may be of most concern during the earlier part of the observation period when lung cancer was regarded as a disease chiefly among men.[77, 78] In two other studies of lung cancer risk among women living in smelter towns no increase in risk was observed.[79, 80] The arsenic exposure was, however, probably low in the investigated towns.

## *p53* mutations in lung cancer

The prevalence of *p53* mutations in our different studies varied between 13% and 21% among never-smokers and 23% and 28% among smokers. For never-smokers this is in accordance with what has been reported previously (range 10-26%),[46, 81-84] although a recent study observed a higher *p53* mutation prevalence among never-smokers.[43] For smokers, the mutation prevalence is lower than what has been observed earlier.[42, 43, 46, 85] This may be due to amount smoked since intensity and duration has been associated with *p53* mutation prevalence.[43, 85, 86] In Paper I, 66% of the smokers had smoked less than 30 pack-years whereas in two of the cited studies the smokers had smoked more, with only 50%[85] and 24%[43] of the smokers consuming less than 30 pack-years. Another explanation might be if the investigated exposures in combination with smoking decrease the *p53* mutation prevalence relative to smoking only, which our results suggested for arsenic exposure and, for heavy smokers, residential radon. The lower prevalence might also depend on an underestimation of the mutation prevalence due to the fact that only exons 5-8 were sequenced, which, however, is commonly done also in other studies, as well as a limited sensitivity of the mutation analyses. In addition, in Paper III not all of exons 5-8 were successfully sequenced for all subjects.

We observed a higher *p53* mutation prevalence and a higher proportion of the tobacco-specific G to T transversions among smokers. This has been shown earlier and PAHs, including B(a)P, in tobacco smoke have been shown to induce this type of mutation.[42, 44, 45] For the environmental exposures investigated, conclusions are more difficult to draw. Our results suggested that radon increases the *p53* mutation prevalence, at least among never-smokers also exposed to ETS as well as among smokers with light to moderate tobacco smoke exposure. This result may suggest that tobacco smoke carcinogens, at least when present at a moderate amount, and alpha radiation affect the same mechanistic pathway in lung cancer development involving the *p53* gene. This interpretation would provide some mechanistic support for the finding in a previous study which indicated a more harmful effect of residential radon among never-smokers also exposed to passive smoking. Why instead an antagonistic interaction between tobacco smoke and residential radon was suggested among heavy smokers we can only speculate. Possibly heavy smokers have a thicker mucous membrane preventing penetration of alpha-particles to target cells, or heavy smokers exposed to high levels of radon develop their lung cancer via other mechanisms not involving the *p53* gene.

In lung cancer, codons 157, 158, 245, 248, 249 and 273 are mutational hotspots[45] and several of these codons contained several mutations in our studies. Codons 157 and 158 are less frequently mutated in other cancer forms than lung cancer and are considered specific hotspots in lung cancer among smokers.[42] In agreement with the literature we only detected one mutation in these codons among never-smokers whereas they were commonly mutated among smokers. Codons 248 and 273 are instead commonly mutated in several types of cancer forms and we detected

several mutations in these codons both among never-smokers and smokers. In the comparison of *p53* mutations among never-smokers and smokers we excluded cases with arsenic exposure.

Among smokers, a possible negative interaction of exposure to arsenic (occupational or environmental) and tobacco smoking was suggested with regard to *p53* mutation prevalence and in SCLC the mutation prevalence was significantly lower among arsenic exposed smokers compared to unexposed smokers. A previous study has suggested a higher proportion of SCC and SCLC among never-smokers working at the Rönnskärsverken smelter compared to non-smoking subjects without occupational exposure to arsenic,[87] which might indicate that the occupational exposure to arsenic preferentially causes lung cancer of these smoking related histological types. SCC and SCLC have generally also been associated with the presence of *p53* mutations.[46, 65, 85, 88] Our result suggests in contrast that among cases with exposure to arsenic in combination with smoking, SCLC may more often develop via pathways unrelated to *p53* gene mutations. Tumors from smokers without arsenic exposure seemed to have a greater variety of base-changes than tumors from smokers with arsenic exposure, which may support the idea that arsenic exposure in combination with smoking can lead to a tumor development where *p53* mutations are of less importance.

## *Methodological considerations*

### Selection of study subjects

In Paper I and the major part of Paper IV, cases were selected from consecutive series of incident cases from well-characterised study bases which should minimise the risk of selection bias. However, in the studies including molecular analyses, only cases with available tumor samples containing sufficient untreated tumor material to analyze were possible to include. This may introduce some bias if timing of treatment or taking of biopsies, histopathological diagnosis, or tumor size are related to the presence of *p53* mutations.

In the study of arsenic and lung cancer risk in the area surrounding Rönnskärsverken only deceased subjects were included. Considering the very high lethality of lung cancer we believe that a high proportion of the lung cancer cases diagnosed in the area during the study period were included. This is also relevant for Paper III, which is based on male subjects from Paper II, as well as the female subjects included as a part of Paper IV.

In Paper II, we did not exclude any causes of death when controls were selected. If the controls would have included subjects who died from causes other than lung cancer that are associated with smelter emission this would tend to somewhat attenuate the estimated effect. This is unlikely to be an issue for bias since no pronounced difference in mortality rates have been found for other causes than lung cancer in the Rönnskärsverken area.[89] The method used for selection of controls

is another potential problem. Controls were matched on birth year and time period of death. For women, the time period is 30 years and for the 2 groups of men, 19 and 11 years, respectively. Cases who died at the end of a period force the selected controls to be born in the same year and to have died during the same period, implying that such controls will on average be younger than the cases. The opposite is true when the cases died at the beginning of the period. In the analysis we addressed this by adjusting for age differences within strata. However, individuals resident in Skellefteå municipality who survived past the end of the period were not available for selection despite being part of the theoretical study base. This could lead to underestimation of the risk if these individuals have a lower prevalence of risk factors compared to those included in our study. However, it is unlikely that air pollution exposure is strongly related to overall mortality.

## Exposure assessment

For most subjects the exposure information regarding tobacco consumption, exposure to ETS, residential addresses and working history was collected from next-of-kin. Earlier Swedish studies using similar data collection methods have shown that data concerning addresses and tobacco consumption from relatives to deceased subjects can be of high quality.[28, 90-92] The response rates were high, 82% for cases in Paper I, 94% in Paper II for both cases and controls and 82-94% for cases in the original studies on which Paper IV is based.

For ETS, we only estimated exposure at home and not at the workplace which may tend to dilute a true association due to non-differential misclassification as it is unlikely to be related to *p53* mutation status. In the assessment of exposure to residential radon there are substantial uncertainties; the measurements were performed retrospectively and there may have been changes in the houses affecting the radon level since the time the study person lived there. The relevant exposure window is not known and will probable differ between individuals. The fact that not all dwellings were available for measurements as well as technical measurement error are other potential sources of bias. If non-differential (unrelated to *p53* status), these uncertainties would also tend to introduce a dilution of the estimated risk. Overall, however, the radon measurements were very successful; in the original studies which form the basis for parts *i*-*iv* of Paper IV, and in the nationwide Swedish study (Paper I and part *v* of Paper IV), more than 78% and 72 % of the retrospective period intended for measurements was covered.

In Papers II and III, residential addresses were used to define if a subject was exposed or not (had lived or had not lived in the defined exposed area). For a subgroup of subjects the residential history was validated, and completed, using registry data. The information from the questionnaires and the parish data agreed well, although the parish data was generally only available from 1945 and onward. Classifying subjects as exposed or unexposed based on residence in the Rönnskärsverken area or not is a rather crude measure of exposure. The resulting misclassification of exposure is likely to be non-differential both with respect to case-control

status (Paper II) and *p53* mutation status (Paper III and part *iii* of Paper IV), and would thus tend to dilute a true association. We believe that the classification of occupational exposure to arsenic at the Rönnskärsverken is of high quality since the working reports are quite detailed and the estimation of occupational exposure partly was based on air measurements in different workplaces.[22] The occupational exposure was only assessed until 1967, which is a long time back in time for cases diagnosed in the end of 1980's. However, our exposure information concerned the time period when the highest emissions occurred. The latency time for smelter emissions and lung cancer is not well known. A previous study showed an excess of lung tumors within 20 years after first exposed to arsenic at a copper smelter although the excess risk was higher for exposure at least 20 years back in time.[21]

## Confounding

Gender and age might be considered as potential confounders and were adjusted for in the analyses as well as smoking, ETS or residential radon depending on study and analysis. In Paper IV we also adjusted for type of sequencing method since different methods were used. We did not adjust for histological subtype of lung cancer in any of the studies since the histological type of the lung tumor may be seen as a step on the pathway from exposure leading to a *p53*-positive (*p53*+) or a *p53*-negative (*p53*-) tumor, or the opposite, that the histological subtype may be influenced by presence of mutations in the *p53* gene.

In Paper II where conditional logistic regression was used we stratified for birth year, sex and recruitment period. We adjusted for occupational history and smoking as well as age differences within strata due to the method of control selection.

## Random error

In our studies, especially Papers I, III and IV including collection of tumor samples and molecular analysis, the number of cases is limited, which means that there is a large variability in the results that may be due to chance. The overall *p53* mutation rates were also lower than anticipated which adds to this problem. When interpreting the results the possibility of bias due to random error must therefore be considered. The limited number of *p53* mutations of different types in our studies likewise hampers comparisons of mutation pattern between exposure groups.

## Case only studies

Epidemiologic studies including only cases can be referred to as case-case studies, case-series studies or case-only studies.[93] In Papers I, III and IV we used the case-only design to evaluate the associations between the investigated exposures and presence of *p53* mutations. Differences in the strength of association between the exposure under study and cases with or without *p53* mutation may be due to different causal pathways or that the magnitude of effect via the same mechanistic pathway differs.[94] The selection of cases in a case-only study should follow the same

rules as in a case-control study. The odds ratio derived is the ratio of the relative risk for developing a *p53+* lung tumor to the relative risk of developing a *p53-* lung tumor.[94]

We illustrate the relationship between the case-only and the case-control designs using the control information from the nationwide study[16] and the results from Paper I regarding *p53* status among the cases. A similar comparison has been made in a published study on *p53* mutations, smoking and bladder cancer.[93] The number of cases and controls from the nationwide radon study is given in Table 11 and in Table 12 the odds ratios are calculated for the case-control and the case-only designs.

**Table 11.** Cases included in Paper I and controls from the nationwide radon study selected from the same categories of residential radon exposure as the cases. Only never-smokers and current smokers were included among both cases and controls.

| | Cases | | Controls |
|---|---|---|---|
| | *p53+* | *p53-* | |
| Radon exposure ≤50 Bq/m$^3$ | 29 | 110 | 673 |
| Radon exposure >140 Bq/m$^3$ | 29 | 75 | 338 |

**Table 12**. Odds ratio estimates in the case-control and the case-case analyses.

| Design | | OR with 95% CI (unadjusted) |
|---|---|---|
| Case-control | *p53+ vs*. controls | 1.99 (1.2-3.4) |
| | *p53- vs*. controls | 1.36 (1.0-1.9) |
| Case-only | *p53+ vs*. *p53-* cases | 1.47 (0.8-2.7) |

The estimate for the case-only design is the ratio of the odds ratio of residential radon in causing a *p53+* lung tumor to the odds ratio in causing a *p53-* lung tumor. This estimate can be calculated in a case-only study without inclusion of controls.

$OR_{cases} = OR_{p53+}/OR_{p53-} = 1.99/1.36 = 1.46$

However, the odds ratio obtained from the case-only design does not indicate the directions of the individual odds ratios for *p53+* and *p53-* lung tumors, and controls are therefore needed if one wishes to estimate the actual risk for a *p53*-defined subtype.[93]

## DNA sequencing

There are a number of different methods to detect *p53* mutations. We have used SSCP and DGGE screening in combination with sequencing as well as direct sequencing. Direct sequencing is usually considered the golden standard of molecular analysis.[37] This method is, however,

sensitive to the presence of normal cells in the tumor sample which can lead to an under-detection of the true number of mutations.[37] SSCP- and DGGE screenings allow a higher proportion of normal cell contamination.[37, 95, 96] In Papers III and IV we micro-dissected the tumor samples in order to obtain a high proportion of tumor cells. This was not done in Paper I, but in that study SSCP analysis was used as a screening method, so contamination with normal cells is of less importance.[37, 96]

Some underestimation of the mutation prevalence is likely since only exons 5-8 were sequenced. However, these exons are highly conserved through evolution, important for the function and have been shown to harbour a majority of the mutations, although exons 4, 9 and 10 have been found to contain approximately 15% of the reported *p53* mutations.[37] In Paper III some additional underestimation is likely due to the fact that not all exons were successfully sequenced for all samples. The samples with only one or two of the exons successfully sequenced were on average older but the success rate did not differ significantly between the different exposure groups. In Paper III all sequences were checked for *p53* mutations both visually and by using PolyPhred software. However, with this method deletions could easily be missed (personal communication Martti Tammi). There might also be false negative results due to the a poor sensitivity of the methods.[37] In the parallel analyses comparing SSCP analysis combined with DNA sequencing and direct solid phase sequence analysis one mutation was not detected in the SSCP analysis and two mutations were not detected in the direct sequencing, out of a total of 17 mutations detected by one or both methods.

The comparison between SSCP analysis and immunostaining indicated limited agreement. There might be several reasons for this, e.g. nonsense or frameshift mutations do not lead to accumulation of p53 protein,[37] the p53 protein might be accumulated depending on normal processes in the cell and there might be mutations outside exons 5-8 as mentioned above.

The majority of tumors included were primary tumors although metastases were also used. Since *p53* mutations seem to occur early in the lung cancer development,[35] the mutation prevalence in the metastases is probably a good estimate of the prevalence in the primary tumors.

Formalin fixation and paraffin embedding have been shown to affect the quality of the DNA which can lead to the induction of PCR artefacts that are misinterpreted as mutations.[97] We confirmed all mutations in a repeated analysis using a new PCR product to exclude possible artefacts. This was shown to be of great importance since almost half of the mutations detected by direct sequencing in Paper III were not confirmed in a second analysis and are therefore likely to be artefacts.

## *Summary*

Although some interesting and suggestive findings are made in our studies regarding the prevalence and spectrum of mutations in the *p53* gene in lung cancer patients with exposure to residential radon, ETS and environmental or occupational arsenic, the relationships have substantial statistical uncertainty. However, true hot-spot mutations very specific for the exposures investigated would have been detected in our studies. Thus, we may conclude that the information regarding mutations in the *p53* gene seems to be of limited use in risk assessment of these exposures at levels occurring in our studies. Possibly the combination of mutations in several genes may provide better resolution. Although, the *p53* gene did not turn out to be a useful marker in our studies, our results provide a substantial addition to the available *p53* data on never-smoking lung cancer cases and give some new evidence regarding mechanistic pathways in environmentally induced lung cancer.

# CONCLUSIONS

The main focus in this thesis was to assess the prevalence and spectrum of *p53* mutations in lung tumors in relation to residential radon, ETS and arsenic to evaluate if *p53* mutations may be of use in epidemiological studies. The following conclusions can be drawn:

- ✓ Smokers have a higher *p53* mutation prevalence, a higher proportion of G to T transversions and a lower proportion of G to A transitions at CpG sites in lung tumors as compared to never-smokers.

- ✓ Residential radon seems to increase the prevalence of *p53* mutations, especially among light-smokers and ETS exposed never-smokers.

- ✓ A possible antagonism was suggested for heavy smoking in combination with residential radon on the *p53* mutation prevalence.

- ✓ A small increase in *p53* mutation prevalence was suggested for exposure to ETS although this may be limited to subjects also exposed to residential radon.

- ✓ Arsenic exposure in combination with smoking seems to decrease the *p53* mutation prevalence, and fewer types of *p53* mutations were detected among cases with combined exposures, which may indicate a predominance of alternative pathways of carcinogenesis not involving *p53* mutations in arsenic-associated lung cancer.

- ✓ We could not detect any clear hot-spot mutations or exposure-specific patterns of *p53* mutations in lung tumors associated with exposure to ETS, arsenic or residential radon. Weak associations may have been missed, however.

- ✓ An increased lung cancer risk was suggested for men who had lived close to the Rönnskärsverken smelter in the beginning of operations when the emissions were substantial.

# SAMMANFATTNING (Summary in Swedish)

*p53*-genen är en tumörsuppressorgen och skyddar mot cancer genom att bl.a. reglera celldelning och celldöd, s.k. apoptos. *p53*-genen uppskattas vara muterad i ca. 40% av alla lungtumörer och förhoppningar har knutits till att kunna använda mutationer i *p53*-genen som en biomarkör för exponering eller subklassificering av sjukdom. En tidigare studie indikerade en förmodad radonspecifik mutationshotspot i *p53*-genen hos radonexponerade gruvarbetare med lungcancer och även för andra exponeringar har specifika mutationsmönster antytts. I den här avhandlingen undersökte vi sambanden mellan exponering för rökning, passiv rökning, bostadsradon samt arsenik och förekomsten och mönstret av *p53*-mutationer i lungtumörer. Vidare undersökte vi risken för lungcancer bland 316 fall och 727 kontroller knuten till boende i närheten av Rönnskärsverken, ett smältverk i norra Sverige, där stora utsläpp av bl.a. arsenik har förekommit. Vissa av lungcancerfallen i denna studie ingick därefter delvis i studierna av *p53*-mutationer.

Tumörmaterial efterfrågades från berörda patologavdelningar och exon 5-8 av *p53*-genen analyserades med en screeningmetod (SSCP eller DGGE) i kombination med DNA-sekvensering eller med direktsekvensering för totalt 479 lungcancerfall inklusive 196 icke-rökare. Information rörande rökvanor, yrkes- och boendehistorik insamlades genom intervjuer och/eller frågeformulär till studiepersonerna eller anhöriga. Radonexponering skattades med hjälp av mätningar med spårfilm i samtliga tillgängliga bostäder som lungcancerfallen bott i under en retrospektiv period av 32 år fram till 3 år före diagnos.

Resultaten från studien vid Rönnskärsverken antydde en något ökad lungcancerrisk för män boende i området närmast smältverket. Exponering under verkets första år, då utsläppen var som mest omfattande, i kombination med en boendeperiod i området kortare än 20 år tycktes vara av störst betydelse (oddskvot (OR) 2.5, 95% konfidensintervall (CI) 0.9-7.1, jämfört med oexponerade). Totalt fann vi 103 mutationer i 99 lungtumörer (mutationsprevalens 21%). Tobaksrökning var associerat med en ökad förekomst av *p53*-mutationer (OR 2.4, 95% CI 1.1-5.1) och en större andel G till T transversioner jämfört med icke-rökare, medan G till A transitioner vid s.k. CpG-sites var vanligare bland icke-rökarna. Även exponering för bostadsradon tycktes öka förekomsten av *p53*-mutationer, OR 2.8 (95% CI 0.8-9.3) för fall exponerade för minst 400 Bq/m$^3$ som tidsvägt medelvärde jämfört med dem som varit exponerade för mindre än 50 Bq/m$^3$, även om denna effekt möjligen sågs främst hos lungcancerfall som varit måttliga rökare eller varit icke-rökare men exponerade för passiv rökning. Lungcancerfall exponerade för både passiv rökning under en lång period (≥30 år) och radon i bostaden (>50 Bq/m$^3$) hade en klart ökad förekomst av *p53* mutationer jämfört med oexponerade (OR 4.9, 95% CI 1.2-21.1). För arsenikexponering verkade det finnas en negativ samverkan med rökning avseende *p53*-mutationsförekomst och tumörer från rökare som även exponerats för arsenik tycktes uppvisa färre typer av *p53*-mutationer än oexponerade rökare.

Inget tydligt exponeringsspecifikt mönster av *p53*-mutationstyper noterades vid exponering för ETS, arsenik eller bostadsradon. Svaga samband kan dock ha missats. Även om mutationer i *p53*-genen som biomarkör inte tycks vara en framkomlig väg att förbättra skärpan i cancerepidemiologiska studier så bidrar resultaten i avhandlingen till en ökad kunskap om förekomst av *p53*-mutationer bland icke-rökande lungcancerfall och nya rön rörande mekanismer för miljöinducerad lungcancer.

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# APPENDIX: DETECTED MUTATIONS

| Id. no. | Gender | Method of analyses | Smoking | Exon | Codon | Mutation | Amino acid exchange |
|---|---|---|---|---|---|---|---|
| 1 | F | SSCP+sequencing | ns | 7 | 248 | G:C>A:T at CpG | arg>gln |
| 2 | F | SSCP+sequencing | ns | 6 | 218 | A:T>T:A | val>glu |
| 3 | M | SSCP+sequencing | ns | 7 | 246 | A:T>G:C | met>val |
| 4 | F | SSCP+sequencing | ns | 7 | 260 | del CT | |
| 5 | M | SSCP+sequencing | ns | 7 | 248 | G:C>C:G | arg>gly |
| 6 | F | SSCP+sequencing | ns | 6 | 220 | A:T>G:C | tyr>cys |
| 7 | M | SSCP+sequencing | ns | 7 | 244 | GC:CG>AG:TC | gly>thr |
| 8 | F | SSCP+sequencing | ns | 5 | 158 | G:C>A:T at CpG | arg>cys |
| 9 | F | SSCP+sequencing | ns | 6 | 192 | G:C>A:T | gln>stop |
| 10 | M | SSCP+sequencing | ns | 6 | 210 | ins A | |
| 11 | F | SSCP+sequencing | ns | 7 | 248 | G:C>A:T at CpG | arg>trp |
| 12 | F | DGGE+sequencing | ns | 8 | 273 | G:C>A:T at CpG | arg>his |
| 13 | F | DGGE+sequencing | ns | 7 | 249 | G:C>T:A | arg>met |
| 14 | F | DGGE+sequencing | ns | 8 | 278 | G:C>A:T | pro>ser |
| 15 | F | DGGE+sequencing | ns | 5 | 176 | G:C>A:T | cys>tyr |
| 16 | M | direct sequencing | s | 6 | 214 | A:T>G:C | his>arg |
| 17 | M | direct sequencing | s | 7 | 242 | A:T>T:A | cys>ser |
| 18 | M | direct sequencing | ns | 5 | 175 | G:C>C:G | arg>gly |
| 19 | M | direct sequencing | ns | 8 | 271 | G:C>C:G | glu>gln |
| 19 | M | direct sequencing | ns | 8 | 285 | G:C>C:G | glu>gln |
| 20 | M | direct sequencing | s | 7 | 249 | G:C>T:A | arg>ser |
| 21 | M | direct sequencing | s | 7 | 248 | GG:CC>TT:AA | arg>leu |
| 22 | M | direct sequencing | s | 8 | 274 | G:C>C:G | val>leu |
| 23 | M | direct sequencing | s | 5 | 157 | G:C>T:A | val>phe |
| 24 | M | direct sequencing | s | 5 | 158 | G:C>T:A | arg>leu |
| 25 | M | direct sequencing | s | 5 | 163 | A:T>G:C | tyr>cys |
| 26 | M | direct sequencing | s | 7 | 239 | A:T>G:C | asn>asp |
| 27 | M | direct sequencing | s | 5 | 179 | A:T>G:C | his>arg |
| 28 | M | direct sequencing | s | 8 | 273 | G:C>T:A | arg>leu |
| 29 | M | direct sequencing | s | 8 | 282 | G:C>A:T | arg>trp |
| 30 | M | direct sequencing | s | 7 | 248 | G:C>C:G | arg>pro |
| 31 | M | direct sequencing | s | 7 | 249 | G:C>A:T | arg>lys |
| 32 | M | direct sequencing | s | 8 | 286 | G:C>T:A | glu>stop |
| 33 | M | direct sequencing | s | 6 | 196 | G:C>A:T | arg>stop |
| 34 | M | direct sequencing | ns | 7 | 244 | G:C>A:T | gly>ser |
| 35 | M | direct sequencing | s | 5 | 183 | G:C>C:G | ser>stop |
| 36 | M | direct sequencing | s | 5 | 161 | G:C>A:T | ala>thr |
| 37 | M | direct sequencing | unknown | 5 | 165 | G:C>A:T | gln>stop |
| 38 | M | direct sequencing | s | 7 | 246 | A:T>G:C | met>val |
| 39 | M | direct sequencing | s | 7 | 249 | G:C>T:A | arg>met |
| 40 | M | direct sequencing | s | 5 | 175 | G:C>A:T | arg>his |
| 41 | M | direct sequencing | s | 8 | 266 | G:C>T:A | gly>stop |
| 42 | F | SSCP+sequencing | s | 6 | 195 | A:T>T:A | ile>phe |
| 43 | M | SSCP+sequencing | s | 8 | 272 | G:C>A:T | silent |
| 44 | M | SSCP+sequencing | s | 5 | 179 | A:T>G:C | his>arg |
| 45 | F | SSCP+sequencing | s | 6 | 193 | G:C>A:T | his>tyr |
| 46 | F | SSCP+sequencing | s | 7 | 245 | G:C>T:A | gly>cys |
| 47 | M | SSCP+sequencing | s | 7 | 248 | G:C>T:A | arg>leu |
| 48 | M | SSCP+sequencing | s | 7 | 249 | G:C>C:G | arg>ser |
| 49 | M | SSCP+sequencing | ns | 8 | 282 | G:C>A:T at CpG | arg>trp |
| 50 | M | SSCP+sequencing | s | 8 | 287 | G:C>T:A | glu>stop |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 51 | M | SSCP+sequencing | s | 7 | 251 | A:T>C:G | ile>ser |
| 52 | F | SSCP+sequencing | ns | 7 | 238 | G:C>A:T | cys>tyr |
| 53 | F | SSCP+sequencing | s | 7 | 255-261+ int 7 | del 9 bp | |
| 54 | M | SSCP+sequencing | s | 5 | 158 | G:C>T:A | arg>leu |
| 55 | M | SSCP+sequencing | s | 7 | 255 | A:T>C:G | ile>ser |
| 55 | M | SSCP+sequencing | s | 6 | 196 | G:C>A:T at CpG | arg>stop |
| 56 | M | SSCP+sequencing | s | 7 | 255-261+ int 7 | del 73 bp | |
| 57 | M | SSCP+sequencing | s | 6 | 216 | G:C>A:T | val>met |
| 58 | M | SSCP+sequencing | ns | 5 | 175 | G:C>A:T at CpG | arg>his |
| 59 | F | SSCP+sequencing | s | 7 | 242 | A:T>T:A | cys>thr |
| 60 | M | SSCP+sequencing | s | 5 | 161 | G:C>T:A | ala>ser |
| 60 | M | SSCP+sequencing | s | 5 | 160 | G:C>A:T | met>ile |
| 61 | M | SSCP+sequencing | s | 8 | 294 | del G | |
| 62 | F | SSCP+sequencing | s | 5 | 157 | G:C>T:A | val>phe |
| 63 | M | SSCP+sequencing | s | 5 | 179 | G:C>A:T | his>tyr |
| 64 | F | SSCP+sequencing | s | 7 | 245 | G:C>T:A | gly>cys |
| 65 | F | SSCP+sequencing | s | 5 | 141 | A:T>C:G | cys>gly |
| 66 | F | SSCP+sequencing | s | 8 | 278 | G:C>A:T | pro>ser |
| 67 | M | SSCP+sequencing | s | 5 | 175 | G:C>A:T at CpG | arg>his |
| 68 | M | SSCP+sequencing | s | int 8 | | splice CGAGgt>CGAGtt | |
| 69 | M | SSCP+sequencing | s | 7 | 249 | A:T>G:C | arg>gly |
| 69 | M | SSCP+sequencing | s | 7 | 249 | G:C>A:T | silent |
| 70 | M | SSCP+sequencing | s | int 8 | | splice CGAGgt>CGAGtt | |
| 71 | M | SSCP+sequencing | s | 8 | 275 | G:C>A:T | cys>tyr |
| 72 | M | SSCP+sequencing | s | int 4/ex 5 | | splice ag>gg | |
| 73 | F | SSCP+sequencing | s | int 8 | | splice CGAGgt>CGAGtt | |
| 74 | M | SSCP+sequencing | s | 8 | 287 | G:C>T:A | glu>stop |
| 75 | M | SSCP+sequencing | s | 5 | 178 | del C | |
| 76 | M | SSCP+sequencing | s | 8 | 278 | G:C>C:G | pro>arg |
| 77 | F | SSCP+sequencing | s | 7 | 249 | A:T>G:C | arg>gly |
| 78 | M | SSCP+sequencing | s | 5 | 158 | G:C>T:A | arg>leu |
| 79 | F | SSCP+sequencing | ns | 7 | 255-261+int 7 | del 15 bp | |
| 80 | F | SSCP+sequencing | s | 8 | 273 | G:C>T:A | arg>leu |
| 81 | M | SSCP+sequencing | s | 5 | 158 | G:C>C:G | arg>pro |
| 82 | F | SSCP+sequencing | ns | 8 | 269-271 | del 8 bp | |
| 83 | M | SSCP+sequencing | s | 5 | 185-186 | del CG | |
| 84 | M | SSCP+sequencing | s | 6 | 193 | G:C>C:G | his>asp |
| 85 | M | SSCP+sequencing | s | 6 | 194 | A:T>G:C | leu>pro |
| 86 | F | SSCP+sequencing | ns | 6 | 209 | del AG | |
| 87 | M | SSCP+sequencing | s | 8 | 277-279 | del 7 bp | |
| 88 | M | SSCP+sequencing | s | 6 | 214 | A:T>G:C | his>arg |
| 89 | M | SSCP+sequencing | s | 5 | 135-137 | del 9 bp | |
| 90 | M | SSCP+sequencing | s | int 5/ex 6 | | splice gGT>aGT | |
| 91 | F | SSCP+sequencing | s | 5 | 163 | A:T>G:C | tyr>cys |
| 92 | M | SSCP+sequencing | s | 5 | 157-159 | del 6 bp | |
| 93 | F | SSCP+sequencing | s | 7 | 235-240 | del 15 bp | |
| 94 | M | SSCP+sequencing | s | 6 | 213 | G:C>A:T at CpG | arg>stop |
| 95 | M | SSCP+sequencing | s | 5 | 158 | G:C>C:G | arg>pro |
| 96 | F | SSCP+sequencing | ns | 8 | 273 | G:C>A:T at CpG | arg>his |
| 97 | F | SSCP+sequencing | ns | 6 | 222 | ins 9 bp | |
| 98 | M | SSCP+sequencing | ns | 5 | 176 | G:C>C:G | cys>trp |
| 99 | M | SSCP+sequencing | s | 8 | 275 | G:C>A:T | cys>tyr |

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