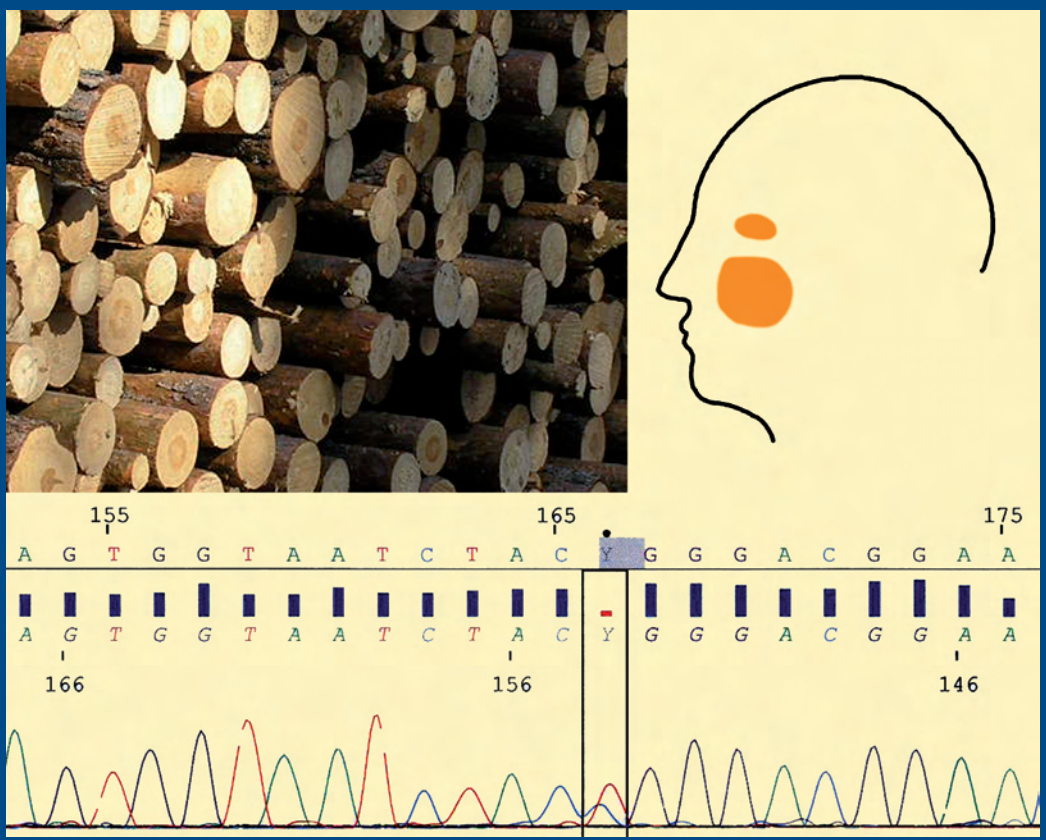
Reetta Holmila

Exposure-related human cancer:

Molecular changes in sinonasal cancer and lung cancer, with focus on *TP53* mutations



Finnish Institute of Occupational Health

People and Work
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TIIVISTELMÄ – ABSTRACT IN FINNISH

Syöpä on yksi yleisimmistä kuolinsyistä maailmanlaajuisesti ja sairastuneiden määrä on kasvussa. Monet näistä syövistä, esimerkiksi nenän alueen syöpä ja keuhkosyöpä, liittyvät erilaisiin ympäristötekijöihin, joten ne ovat mahdollisesti ehkäistävissä. Nenän alueen syöpä liittyy vahvasti työperäiseen puupölyaltistumiseen, kun taas keuhkosyövän tärkein riskitekijä on tupakointi. Keuhkosyövän kehittymiseen johtavia molekyylitason mekanismeja on tutkittu laajasti, mutta sen sijaan nenän alueen syöpään liittyvistä molekyylitason muutoksista on tähän mennessä tiedetty hyvin vähän. Tässä väitöskirjatyössä on tutkittu *TP53*-kasvurajoitegeenin mutaatioita nenän alueen syövässä ja keuhkosyövässä sekä tulehduksiin liittyvän proteiinin COX-2 ilmenemistä nenän alueen syövässä sekä näiden tekijöiden liittymistä altistumiseen riskitekijöille. Tulokset osoittavat, että *TP53*-geenin mutaatiot ovat yleisiä sekä nenän alueen syövässä että keuhkosyövässä ja näissä molemmissa muutokset liittyvät altistumiseen. Nenän alueen syövissä mutaatioita esiintyi enemmän tapauksilla, jotka olivat työssä altistuneet pitkään ja suhteellisen suurille pitoisuuksille puupölyä. Tupakointi ei vaikuttanut nenän alueen syövissä suorasti mutaatioiden esiintymiseen, vaan se liittyi useamman kuin yhden mutaation esiintymiseen samaan aikaan yksittäisessä syövässä. Lisäksi vaikuttaa siltä, että tulehdusvaikutukset ovat yksi puupölyaltistumiseen liittyvän nenän alueen syövän kehittymisen mekanismeista, sillä COX-2-proteiinin ilmeneminen liittyi erityisesti adenokarsinomasolutyyppiin, puupölyaltistumiseen ja tupakoimattomuuteen. Keuhkosyövässä *TP53*-mutaatioiden esiintyminen liittyi pitkään tupakointiaikaan, kasvaimen solutyyppiin ja sukupuoleen. Lisäksi osoitimme, että tupakoijille tyypillisen mutaatiotyypin (G>T) kantajilla oli normaalissa keuhkokudoksessa suuri määrä tupakointiin liittyviä DNA-vaurioita. Tämänkaltainen

tieto molekyylitason muutoksista ympäristöperäisissä syövissä täydentää epidemiologisista tutkimuksista saatua tietoa ja auttaa selventämään eri syytekijöiden osuutta ja niiden vaikutustapoja. Tämä puolestaan on tärkeää terveysriskien arvioimisessa ja ehkäisyssä.

ABSTRACT

Cancer is a leading cause of death worldwide and the total number of cancer cases continues to increase. Many cancers, for example sinonasal cancer and lung cancer, have clear external risk factors and so are potentially preventable. The occurrence of sinonasal cancer is strongly associated with wood dust exposure and the main risk factor for lung cancer is tobacco smoking. Although the molecular mechanisms involved in lung carcinogenesis have been widely studied, very little is known about the molecular changes leading to sinonasal cancer. In this work, mutations in the tumour suppressor *TP53* gene in cases of sinonasal cancer and lung cancer and the associations of these mutations with exposure factors were studied. In addition, another important mechanism in many cancers, inflammation, was explored by analyzing the expression of the inflammation related enzyme, COX-2, in sinonasal cancer. The results demonstrate that *TP53* mutations are frequent in sinonasal cancer and lung cancer and in both cancers they are associated with exposure. In sinonasal cancer, the occurrence of *TP53* mutation significantly increased in relation to long duration and high level of exposure to wood dust. Smoking was not associated with the overall occurrence of the *TP53* mutation in sinonasal cancer, but was associated with multiple *TP53* mutations. Furthermore, inflammation appears to play a part in sinonasal carcinogenesis as indicated by our results showing that the expression of COX-2 was associated with adenocarcinoma type of tumours, wood dust exposure and non-smoking. In lung cancer, we detected statistically significant associations between *TP53* mutations and duration of smoking, gender and histology. We also found that patients with a tumour carrying a G to T transversion, a mutation commonly found in association with tobacco smoking, had a high level of smoking-related

bulky DNA adducts in their non-tumorous lung tissue. Altogether, the information on molecular changes in exposure induced cancers adds to the observations from epidemiological studies and helps to understand the role and impact of different etiological factors, which in turn can be beneficial for risk assessment and prevention.

CONTENTS

ORIGINAL ARTICLES

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I–V):

I. Holmila R and Husgafvel-Pursiainen K. (2006). Analysis of *TP53* gene mutations in human lung cancer: Comparison of capillary electrophoresis single strand conformation polymorphism assay with denaturing gradient gel electrophoresis and direct sequencing. *Cancer Detect Prev,* **30**(1) 1–6.

II. Holmila R, Cyr D, Luce D, Heikkilä P, Dictor M, Steiniche T, Stjernvall T, Bornholdt J, Wallin H, Wolff H, Husgafvel-Pursiainen K. (2008) COX-2 and p53 in human sinonasal cancer: COX-2 expression is associated with adenocarcinoma histology and wood-dust exposure. *Int J Cancer,* **122**(9) 2154–2159.

III. Anna L*, Holmila R*, Kovács K, Gyorffy E, Gyori Z, Segesdi J, Minárovits J, Soltész I, Kostic S, Csekeo A, Husgafvel-Pursiainen K, Schoket B. (2009) Relationship between *TP53* tumour suppressor gene mutations and smoking-related bulky DNA adducts in a lung cancer study population from Hungary. *Mutagenesis* **24**(6) 475–480, *Equal contribution.

IV. Holmila R, Bornholdt J, Heikkilä P, Suitiala T, Fevotte J, Cyr D, Hansen J, Snellman S-M, Dictor M, Steiniche T, Sclünssen V, Schneider T, Pukkala E, Savolainen K, Wolff H, Wallin H, Luce D, Husgafvel-Pursiainen. (2009) Mutations in TP53 tumor suppressor gene in wood dust related sinonasal cancer. Int J Cancer, Nov 30. DOI:10.1002/ijc.25064

V. Holmila R, Bornholdt J, Suitiala T, Cyr D, Dictor M, Steiniche T, Wolff H, Wallin H, Luce D, Husgafvel-Pursiainen K. (2009) Profile of *TP53* gene mutations in sinonasal cancer. *Mutat Res – Fundam Molec Mech Mutagenesis*, **686**(1–2) 9–14.

ABBREVIATIONS

A	Adenine
APC	*Adenomatous polyposis coli*
ATM	*Ataxia telangiectasia mutated*
Bcl-2	*B-cell CLL/lymphoma 2*
BPDE	Benzo[a]pyrene diolepoxide
BRCA1	*Breast cancer 1, early onset*
BRAF1	*Murine sarcoma viral (v-raf) oncogene homolog B1*
CBP	CREB binding protein
C	Cytidine
CE	Capillary electrophoresis
CI	Confidence interval
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DGGE	Denaturing gradient gel electrophoresis
EGFR	*Epidermal growth factor receptor*
G	Guanine
HRAS	*v-Ha-ras Harvey rat sarcoma viral oncogene homolog*
Hupki	Human p53 Knock-in
IARC	International Agency for Research on Cancer
ICD	International Statistical Classification of Diseases and Related Health Problems
IL-1β	Interleukin-1β
KRAS	*v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog*
M	Mutant
MDM2	Murine double minute 2
MYC	*v-myc myelocytomatosis viral oncogene homolog*
NF1	*Neurofibromin 1*
NF-κB	Nuclear factor-κB
NKK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
NSCLC	Non small cell lung cancer
OR	Odds Ratio
p16/INK4a	CDKN2A, cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)

p21/WAF1/Cip1	*CDKN1A, cyclin-dependent kinase inhibitor 1A*
p300	E1A binding protein p300
p53	Tumour protein p53
p63	Tumour protein p63
p73	Tumour protein p73
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PET	Paraffin embedded tissue
PTEN	*Phosphatase and tensin homolog*
RAS	*Ras (rat sarcoma viral oncogene homolog) oncogene family*
RB	*Retinoblastoma*
RET	*Ret proto-oncogene*
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SH3	Src homology 3 domain (proline rich binding)
SCLC	Small cell lung cancer
SNP	Single-nucleotide polymorphism
SSCP	Single strand conformation polymorphism
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
SV40	Simian virus 40
T	Thymine
TNF-α	Tumour necrosis factor-α
TP53	*TP53 tumour suppressor gene*
UV	Ultraviolet
WHO	World Health Organization
WT	Wild type
WT1	*Wilms tumour 1*
XPA	*Xeroderma pigmentosum, complementation group A*

1 INTRODUCTION

Cancer is the leading cause of death worldwide and the total number of cancer continues to climb. This trend will most likely continue globally with the growth and ageing of the world's population [1]. Importantly, evidence from epidemiological studies suggests that a large proportion of human cancer can be attributed to environmental factors, if one defines the "environment" to include a wide range of exposures and lifestyle factors such as occupational exposure and dietary, social and cultural habits [2]. These potentially preventable cancers include a wide variety of human cancers, especially those associated with tobacco smoking and work-related exposures, exemplified by lung cancer and sinonasal cancer. Tobacco smoking and asbestos exposure are two of the most important causative factors of lung cancer [3–5], the most deadly of all human cancers with 1,3 million deaths per year globally [6], whereas sinonasal cancers represent a very rare form of cancer with a strong association to wood dust exposure at work [7–10].

Carcinogenesis is driven by changes in sequence and function of the genes that control cell proliferation, survival and other physiological processes know to be altered in malignant cell types [11–15]. Knowledge of these molecular mechanisms is important for the evaluation and prevention of health risks. Mechanistic information on cellular processes and changes in carcinogenesis complements the observations from epidemiological studies and helps to understand the role and effect of different etiological factors. This kind of data may also eventually open prospects for treatment. At present, molecular mechanisms in some cancers, such as lung cancer, have been widely studied, whereas for some others, for example sinonasal cancer, very little is known about the molecular changes involved in cancer development.

Mutations of the tumour suppressor *TP53* gene are an important feature in many human cancers, as the elimination of *TP53* pathway appears to be requisite at some point of carcinogenesis in most, if not all, human cancers [16]. Certain carcinogenic exposures have been shown to induce a typical and recognizable *TP53* mutation spectrum, which makes it a highly interesting gene in the search for molecular mechanisms of cancer in association to external exposures.

The studies in this PhD-thesis investigated molecular changes, with the emphasis on *TP53* mutations, in sinonasal cancer and in lung cancer and searched for their associations with exposure. Inflammation, which is another important mechanism involved in carcinogenesis in many types of human cancer, was investigated by analyzing the expression of the inflammation related enzyme, COX-2, in sinonasal cancer.

2 REVIEW OF THE LITERATURE

2.1 Cancer – an overview

Cancer is a group of diseases characterized by aberrant cellular growth. It affects many different tissues and types of cell, and is often defined by the tissue of origin. Cancer affects more people in our time than it did in the past due to the increase and aging of world population [2, 17]. The International Agency for Research on Cancer (IARC) has estimated that globally in the year 2008 12.4 million cases of cancer were diagnosed, 7.6 million patients died from cancer and 28 million persons were alive with cancer within five years from the initial diagnosis [2].

The most commonly diagnosed cancers (excluding all types of skin cancer) are those of the lung, colon and rectum and breast [17]. In men, lung cancer is the most common cause of death related to cancer, whereas in women breast cancer is the principal killer [2]. Nevertheless, the patterns of cancer trends, incidence, and projections vary greatly in the different parts of the world [17]. Many of the cancers could potentially be prevented as several risk factors are already known. In most cancers, environmental factors seem to play greater part in the acquisition of cancer than inherited susceptibility [1, 18, 19]. The known risk factors for cancer include certain lifestyle factors (tobacco and alcohol use, diet, obesity, physical inactivity), exposure to occupational or environmental carcinogens (for example, asbestos and wood dust), radiation (for example, ultraviolet and ionizing radiation) and some viral infections (for example, hepatitis B or human papilloma virus infection) [1]. These factors can affect carcinogenesis at different stages through both genetic and epigenetic mechanisms [6, 18].

Most, if not all, cancers start with alterations in one or a small number of cells [12, 20]. There is increasing evidence that these cells are likely to be cancer stem cells, i.e. cells that have the ability to proliferate and generate the vast array of different cells found in a tumour [11, 21, 22]. The development of cancer is a multistep process driven by genetic and epigenetic changes where cells undergo metabolic and behavioral changes, leading them to lose control of normal replication and growth [12, 17, 20]. As proposed by Hanahan and Weinberg [14], six essential cellular characteristics are shared by most, possibly all, types of human tumours : self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [14]. Inflammation might well be an equally important feature [23].

If a cell with alterations in critical cell functions is tolerated by the organism, it may accumulate even more modifications when proliferating and eventually become highly malignant [12, 20]. Somatic mutations that contribute to the acquisition of the essential tumour properties drive the transformation of normal human cells into malignant tumour cells while making this process irreversible [14, 15]. The order and way of acquiring the different malignant capacities varies widely, even among tumours of the same type and certainly between tumours of different types. Also, in certain tumours a specific genetic event may contribute only partially to the acquisition of a single capability, while in others the same event may result in simultaneous acquisition of several distinct capabilities [14].

Furthermore, tumours are complex tissues that depend on interaction between various cells found in the tumour stroma [24]. The stroma consists of non-malignant cells of a tumour, the vasculature and its cells, the activated fibroblasts, macrophages and other immune cells [24–26] and the cancer cells can alter the stroma to form a permissive and supportive environment for tumour progression [26]. The interactive signaling between stromal and tumour cells contributes to the formation of a multicellular tumour [26].

2.1.1 Genes involved in tumorigenesis

Approximately 23 000 genes are present in the human genome and several thousand (3000–5000) encode proteins involved in cellular processes

deregulated in cancer. Of these, about 300 different genes are mutated at some frequency in human cancers, however, large-scale re-sequencing studies on cancer genomes have detected only a relatively small number of genes as being commonly mutated in human cancers, whereas most of the mutations found in tumours are infrequent in general [12, 22, 27, 28]. Also, the genes mutated in cancer appear to be a part of a small number of pathways, like the 12 core pathways identified in pancreatic cancer [11, 29].

The genes involved in tumorigenesis include oncogenes like the *RAS* family genes, *β-Catenin, BRAF1*, tumour suppressor genes like *TP53*, *APC, p16/INK4a, PTEN*, and genomic stability genes like *BRCA1, ATM, XPA* [11, 15]. Proteins coded by oncogenes promote cell proliferation [30] and are activated by genetic alterations in cancer whereas tumour suppressor genes become deactivated. Under normal conditions, tumour suppressor genes function against clonal expansion and genomic mutability and are therefore able to inhibit uncontrolled growth and metastasis [31]. Stability genes, or caretaker genes, include the genes responsible for DNA repair and the control of mitotic recombination and chromosomal segregation. Their function is to keep genetic alterations to a minimum and if those genes become inactivated, the mutations will become more frequent [11]. Germline mutations in the genes involved in tumorigenesis lead to a predisposition to cancer, but other alterations in the cell's genome are still needed before the transformation of the cell into a cancer cell. Carriers of a germline mutation in a tumour predisposing gene often develop multiple cancers that occur at an earlier age as compared to individuals who acquire such mutations somatically [11, 13].

2.2 Mutations in cancer

Genetic alterations occur regularly in the DNA of all organisms, as cells are constantly subjected to endogenous and exogenous stress. These genetic alterations include many different kinds of structural changes in DNA resulting from various types of damage to the purine and pyrimidine bases, single- or double DNA-strand nicks and gaps, intrastrand or interstrand crosslinks and DNA-protein crosslinks [15]. Most of these alterations are identified and rectified by the cellular surveillance systems

that include DNA-repair mechanisms and cell cycle checkpoint control. However, some changes are likely to persist, especially if the mutation rate is high, in which case the alterations can be carried on to the next generation of cells [15, 32]. The number of spontaneous mutations in human cells has been estimated to be around 10^{-8}–10^{-10} per base pair per cell cycle, but in association with external exposure, the rate of mutations may rise by 10–1000 fold [32].

The sources of endogenous DNA damage include DNA-polymerase errors, oxidative stress from normal aerobic processes, and premutagenic DNA methylation [33–35]. External factors consist of different kinds of carcinogens and their active metabolites [15]. The mutations found in cancer vary from small changes in nucleotide sequences, such as point mutations, to changes in chromosome copy numbers or chromosomal alterations that affect long stretches of DNA such as translocations, deletions or amplifications [22]. In general, cancer cells are found to harbor a wide variety of alterations in their DNA [12, 27, 28].

Nucletiode changes in DNA sequence can be classified as deletions, insertions or base substitutions, classified either as transitions (change of a pyrimidine to another pyridimine or a purine to another purine) or transversions (change of a pyridimine to a purine or vice versa) [35]. Depending on the effect, a mutation can be further classified as a frameshift (deletion or insertion causing a shift in the translation reading frame), missense (a base substitution that changes the corresponding amino-acid to some other amino acid), nonsense (a base substitution that changes the amino-acid to a stop codon) or silent (a base substitution that does not change the amino acid) mutation.

In particular, C to T base transitions arise spontaneously, especially at genomic sites where cytosine residues are methylated and the hydrolytic deamination of the pyrimidine may occur [33]. Certain carcinogens appear to frequently cause specific kinds of mutations, which are then considered as a "carcinogen fingerprint" when studying the molecular etiology of cancer [15, 34]. Nevertheless, the detection of a mutation in a cancer cell does not necessarily mean that the mutation is the primary origin of carcinogenesis. In many cases, mutations are believed to arise as by-products of transformation or they may have occurred before the clonal expansion [15].

2.2.1 Analyzing mutations in cancer

Identification of sequence changes is becoming increasingly important in the diagnosis of different diseases as well as being useful in drug discovery and development. Traditional mutation detection methods analyse the DNA sequence either directly by techniques based on sequencing or probe hybridization, or indirectly by methods based on the analysis of sequence-dependent physico-chemical properties of DNA, like melting or folding [36]. Recently, the advances in technology have made it possible to study whole cancer genomes using large scale and high-throughput DNA sequencing. It is now possible to detect almost any genomic alteration in individual tumours and discover biologically significant changes. On the other hand, the amount of data generated is enormous and data processing and statistical analysis become extremely important in the evaluation and interpretation of the results [27, 37, 38].

Direct sequencing has long been considered as the "golden standard" for mutation analysis. It allows the identification of the exact location and type of sequence change, but at the same time it is both quite expensive and time consuming [36]. Various other methods traditionally based on gel electrophoresis have been used in mutation detection to separate the wildtype sequence from the mutated one. For example, denaturant gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) are two commonly used methods for mutation screening, both originally based on gel separation. SSCP and DGGE methods are especially useful when the location and type of alteration are not known, in other words, in screening for mutations.

In DGGE, a denaturing gradient is used to separate the homoduplexes (wild type-wild type or mutated-mutated) of DNA from heteroduplexes (wild type-mutated). Since the heteroduplexes are a combination of wild type and mutated DNA-strands, they have a lower stability than the homoduplexes. Due to the lower stability, they denature in a lower percentage of denaturant and can be separated from homoduplexes by electrophoresis [39, 40]. In SSCP, the separation is based on different electrophoretic mobility of a sequence specific conformation of the single stranded DNA fragment [40, 41]. These methods are simple to run and do not typically require large investments in equipment, but on the other hand they are not suitable for analyzing large numbers of samples, as

they are not easily automated. With the application of the capillary electrophoresis (CE) technique, SSCP has become more readily applicable for investigating large collections of DNA samples [42]. Ultimately, with mutation screening methods, the DNA fragments with alterations must be sequenced before the exact type and location of the altered sequence can be identified.

Analyzing paraffin embedded tissue (PET) samples has proven to be challenging, as fixing the DNA by formaldehyde or some other agent causes damage that can impair PCR or result in polymerase errors [43]. *Taq* polymerase has a tendency to insert adenosines when no template base is present, causing alterations, artifacts, that may get amplified in the subsequent rounds of PCR [44]. However, finding the exact same artifact in a second independent PCR is very improbable [44]. Furthermore, the sensitivity of sequencing has been shown to be lower as compared to mutation screening methods [45–48]. Careful planning of the mutation analysis strategy is therefore extremely important while studying PET samples to ensure the validity of results.

2.3 *TP53* tumour suppressor gene and p53 protein

The protein encoded by *TP53* tumour suppressor gene, the p53 tumour suppressor, was first identified in 1979 as a protein that was bound by SV40 T-antigen in SV40 induced tumours [49]. Early observations led to the belief that p53 might function as an oncogene; however, in the late 1980s the normal function of p53 was clarified as being anti-oncogenic [50].

The *TP53* is commonly mutated in human cancer; somatic *TP53* mutations are found in about 50 % of human cancers [51, 52], and they are often associated with a loss of the second allele of the gene [34]. It is generally believed that also in tumours that carry a wild-type *TP53*, the *TP53* pathway is inactivated by some other mechanism [52]. The elimination of p53 function is generally required in order to achieve resistance to apoptosis which is an important feature of most, if not all, types of cancer [14].

A germline mutation of the *TP53* gene is the most common reason for Li-Fraumeni syndrome, a familial cancer syndrome where the carriers of *TP53* germline mutation are predisposed to a variety of tumours, and

90 % of the carriers are diagnosed with cancer by the age of 60 years [16, 53].

2.3.1 Structure

The tumour suppressor *TP53* gene is a single copy gene situated in human chromosome 17p13.1 [34, 54]. It is 20 kb long and contains 11 exons, with the first exon and part of exon 11 being non-coding (Figure 1) [54]. The *TP53* DNA-sequence is highly conserved in vertebrates and it is small enough to be relatively easily analyzed [32, 34]. *TP53* belongs to a small family of related genes that includes two other members, *p63* and *p73*. Although they all are structurally and functionally related; *p63* and *p73* have roles in normal development, whereas the main role of *TP53* seems to be the prevention of malignant growth [55]. Nevertheless, p53 also seems to play a role in the developmental process [56]. Recently different isoforms of p53 have been found. Some of them have been shown to be aberrantly expressed in tumours, however, the function and significance of different p53 isoforms remains still somewhat unclear [57].

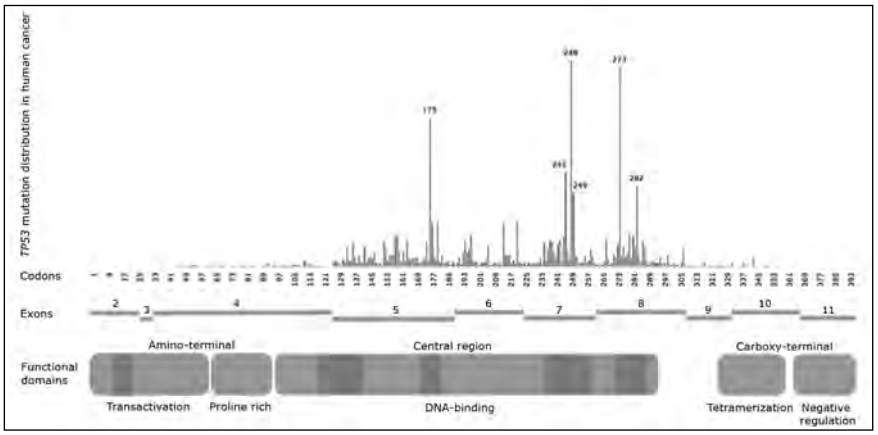

Figure 1. The *TP53* gene and protein. *TP53* mutation distribution in human cancer (as described in the IARC database [58]) and mutation hot spots are shown in relation to the exons and functional domains. Highly conserved regions are marked with darker shading.

TP53 encodes for a 53 kDa phosphoprotein that is expressed at very low levels in the nucleus of normal cells [34, 54]; it is active as a homotetramer [59, 60]. The human p53 has 393 residues [59] and contains five widely conserved areas (Figure 1) [54]. The protein can be divided into three different parts: the amino-terminal containing transactivation domain and regulatory domains, the central region containing sequence-specific DNA-binding domain, and the multifunctional carboxy-terminal (Figure 1) [50].

The amino-terminal (N-terminal) houses the transactivation domain (residues 1-63) that interacts with a number of regulatory proteins such as MDM2 and p300/CBP followed by the proline-rich region (residues 64-92), which contains SH3-domain binding motifs and is thought to have a regulatory role (Figure 1) [59, 61].

The central region (residues 94-292) is responsible for specific DNA-binding, and it contains the most evolutionary conserved sequences of the protein (Figure 1) [52, 59, 61]. In the homotetramer, p53 central regions form strong interactions with each other and co-operatively bind DNA. These interactions facilitate DNA bending as well as looping, which might be needed when binding to promoters where the p53-binding sites are not next to each other, for example, in the p53 target genes *p21/WAF1/Cip1* and *cyclin G* [50]. The central region is also an important domain for specific protein-protein interactions [62].

Finally, the carboxy-terminal (C-terminal) includes the tetramerization domain (residues 325-355), which regulates the oligomerization of p53 and the negative autoregulatory domain of the extreme carboxy-terminus (363-393). The latter contains acetylation and phosphorylation sites and regulates the DNA-binding activity of p53 (Figure 1) [59, 61]. The carboxyl terminal can also bind nonspecifically to different kinds of DNA, including damaged DNA and reannealing complementary single strands of DNA or RNA [50].

2.3.2 Function

The tumour suppressor protein, p53, plays a central role in a cell's response to different kinds of stress situations that include many of the events associated with cancer initiation and progression. The p53 induction is extremely sensitive to DNA damages (genotoxic stress),

such as those caused by cellular processes, exposure to different kinds of chemical carcinogens or radiation. Many other signals also activate p53, including oncogene activation and various cellular stresses, such as the changes in temperature or the redox-equilibrium, telomere shortening, hypoxia and deficiency of ribonucleotides [56, 63, 64]. Generally, when activated, p53 mediates a broad spectrum of processes that suppress growth [64]. The diversity of cancer related signals that trigger the p53 response explains, at least in part, why the p53 pathway is inactivated in almost all types of cancer [63].

In normal non-stressed cells, p53 is maintained at very low steady-state levels. The relatively few p53 molecules that exist appear to be rather ineffective as transcription factors, even though they contribute to the maintenance of basal expression levels of several p53 target genes [63]. Activation of p53 in response to stress involves a clear increase in the number of p53 molecules within the cell nucleus. This is achieved by post-translational changes to protect p53 from degradation and thus enable the transcriptional activation of target genes. The activity of p53 can also be controlled by the subcellular localization of p53 and other pathway components [55].

It is known that p53 acts as a sequence-specific DNA-binding transcription factor that can activate or repress the transcription of a large number of target genes [55, 60]. The DNA sequence found in the various p53 response elements contains a consensus sequence with similar elements, but it is not exactly the same for different targets genes, and as a result p53 binds to different sites with highly heterogeneous affinities [52]. In addition to activation of genes with p53-binding sites, p53 is also capable of strongly inhibiting transcription of certain genes lacking p53-binding sites. Several genes, many with anti-apoptotic functions, have been identified as *in vivo* targets of p53 repression [50, 55, 60]. Transcription independent activities of p53 that trigger apoptosis have also been described [55, 56]. These mechanisms might involve direct binding of the Bcl-2 protein family members at the mitochondria and most likely, both transcription-dependent and -independent pathway cooperate and complement each other in the induction of apoptosis [65].

Several cellular responses can be induced by p53, most importantly cell cycle arrest and apoptosis. There is also evidence that p53 plays a role in the regulation of glycolysis, autophagy, cell survival and oxidative stress, and is involved in cellular senescence, angiogenesis, differentiation,

repair of DNA-damage, invasion and motility, and bone remodelling. The outcome of p53 activation depends on many factors that are both intrinsic and extrinsic to the cell [55, 56, 63]. The elimination of damaged, stressed or abnormally proliferating cells by p53 has been considered to be the principal means by which p53 inhibits tumour growth [66], but p53 has also another important mechanism of reducing tumours. When dealing with low levels of damage that are encountered during normal cellular life, p53 acts to reduce the levels of ROS and promotes DNA repair and survival of the slightly damaged cell to allow the repair to proceed [56, 62.] On the other hand, inappropriate or prolonged activation of p53 in normal tissues can lead to tissue damage and it has been associated with multiple sclerosis, neurodegenerative disorders and exacerbation of ischemic damage from stroke or cardiac arrest [66].

2.3.3 *TP53* mutations in human cancer

TP53 mutations are found in most human cancers arising from a variety of tissues, whereas other important tumour suppressor genes (for example *APC*, *WT1*, or *NF1*) seem to have a more limited tissue variation [18, 34] In addition, *TP53* mutations are found in significant frequency in human cancer, although the frequency varies from one cancer type to the next [34, 55, 67]. Information about *TP53* mutations in human cancer has been collected in databases [58, 68], which contain thousands of mutations. In certain cases the *TP53* mutation spectrum appears to reflect the DNA damage caused by a particular carcinogen, and these data may be useful in defining the molecular mechanisms responsible for carcinogenesis [32, 51]. The frequency and type of *TP53* mutations have also been suggested to help to evaluate the type and level of carcinogen exposure [32]. Overall, the *TP53* gene has many useful properties when studying carcinogenesis making the *TP53* one of the most extensively studied genes in cancer research.

The accumulation of p53 proteins altered by mutation in tumour cells can in principle have two consequences in carcinogenesis: i) a dominant negative role of the mutated allele by hetero-oligomerization with wild-type p53 expressed by the second allele, or ii) a specific gain of function of mutant p53 [62, 69–71]. In most cancer cells, *TP53* mutations are present on only one allele, the other being either wild-type or lost,

indicating that the efficiency of dominant-negative inhibition might not be complete and probably depends on the type and location of the initial mutation [15, 34, 55, 69]. All *TP53* mutations are not equivalent and they display a marked variation in structure as well as in loss of function. While the mutations most often found in human cancers (the "hot spot" mutants, Figure 1) show total loss of transactivating capacity, other mutants may retain at least partial activity and still transactivate a subset of target genes, leading to a wide range of possible mutant activities [52]. However, rare mutants seem to have still most, if not all, wild type *TP53* activity [72]. The *TP53* mutants with only a partial loss of activity might require a second mutation in order to fully inactivate the protein. Since p53 functions as a tetramer, two weak mutations in two different alleles could potentially lead to a fully inactive protein [72].

The mutations selected in carcinogenesis affect the properties of p53 that, when alterated, lead to increased tumorigenic potential of the cell [15, 50]. Some *TP53* mutants can be even considered oncogenes [55, 69]. On the other hand, the biological effect of a total absence of *TP53* due to a nonsense or frameshift mutation is most likely very different [15, 34, 55, 69]. Mouse models support the claims for the heterogeneity in response to different kinds of *TP53* mutations. Knock-out mice without *TP53* display a different spectrum of tumours than the knock-in mice with various *TP53* hot spot mutants. In contrast to knock-out animals, the knock-in mice exhibit a higher frequency of solid tumours with a high potential for metastasis, as observed also in mice expressing one mutant allele in a *TP53* null background. This is one of the strongest arguments for a gain of function of mutant *TP53* [52, 62].

In addition, numerous single nucleotide polymorphisms (SNPs) and other sequence variations are present at the *TP53* locus. Most of these variations are found in introns and probably do not have any significant role in carcinogenesis. So far, the majority of *TP53* polymorphisms has not been assessed for altered function or increased susceptibility to cancer. Only for two of these SNPs (P47S and R72P) is there sufficient molecular evidence to suggest a functional change in the p53 pathway attributable to the polymorphism [73]. However, there are no studies with large enough populations to report clearly significant associations between altered cancer risk and *TP53* polymorphisms and the molecular models have been based mainly on in vitro studies with cell lines [73].

2.3.3.1 *TP53* mutation profile

The mutations in cancer are scattered along the *TP53* gene (Figure 1), and occur also at the splice junctions [15, 34]. Three hundred of the 393 codons in the *TP53* gene have been reported to be mutated in human cancer [34]. Nonetheless, according to the available data, only about 5 % of *TP53* mutations appear to be in the regulatory domains (amino terminal and carboxyl terminal), whereas most of the *TP53* missense mutations are clustered in the central part, particularly within four highly conserved regions (Figure 1) [50, 55]. Each codon in the central region has been reported as being mutated, while the mutation rates range from two cases for infrequent mutations to more than 1000 for hot spot mutations, which occur in codons 175, 245, 248, 249, 273 and 282 (Figure 1) [15, 52, 55, 62]. These hot spot codons contain about 30 % of all reported mutations [15, 55]. The distribution of mutations cannot be explained only by factors related to acquisition of mutations; hence, other mechanisms, in particular preferential repair or choice of inactivating or gain-of-function mutants, may influence the selection of mutants in cancer tissue. It is commonly assumed that inactivation of the apoptotic activity is the main target of *TP53* gene mutations [62].

Unlike most other tumour suppressor genes (such as *RB* or *APC* gene) that are inactivated by frameshift or nonsense mutations leading to the formation of unstable or truncated protein, the most common *TP53* mutation type found in tumours is a missense mutation caused by a single amino-acid substitution. About 80 % of the *TP53* mutations described so far are of this type (Figure 2) [15, 31, 32, 55, 62, 69, 70]. Most *TP53* missense mutations lead to a synthesis of stable protein that has lost totally or partially its the transcription capabilities and accumulates in the nucleus of tumour cells [62, 69]. As common a predominance of missense mutations occurs in oncogenes such as *KRAS* [15]. In general, the missense mutations seem to be the most frequent genetic alterations in the coding sequence of human cancer genome (excluding large genomic rearrangements) and the distribution of *TP53* mutation types in cancer closely resembles this overall distribution of mutation types in cancer in all genes [28, 62, 74]. Consequently, the distribution of *TP53* mutation types does not necessarily reveal any specific biological function, but reflects the importance of the p53 central region structure for DNA-binding; even small modifications in this region can lead to a loss of p53 transcriptional activity [62].

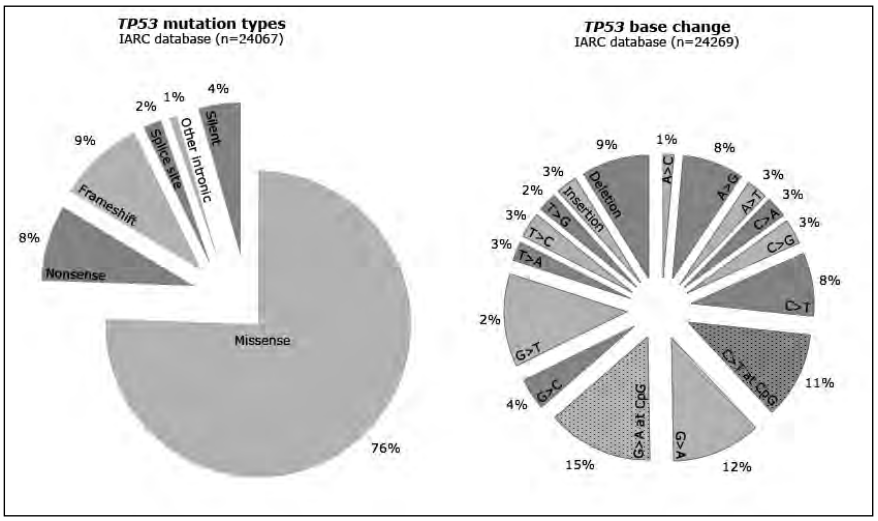

Figure 2. The general *TP53* mutation profile by type of mutation and base substitution in human cancer according to the IARC database R13 [58].

Transitions are more frequent than transversions among *TP53* missense mutations and they occur particularly often at CpG sites, (Figure 2). The frequent methylation of the C residues, which can potentially lead to deamination of 5-methylcytosine to thymine (T), is assumed to be the reason for a C to T transition in these sites [75–77]. Although the majority of *TP53* alterations are missense mutations, there are also 8 % of nonsense mutations and 9 % of small deletions or insertions (Figure 2) [32, 62]. It is noteworthy that these types of mutations occur more frequently outside the central region (they represent 54 % of the mutations in exons 2–4 and 77 % of those in exons 9–11 [32]) as compared to the central region (they represent 20 % of the mutations found in exons 5–8) [32, 62]. This might be explained with the observation from functional analysis of missense mutations outside the central region showing that the transcriptional activity of p53 is not severely impaired by one missense mutation and that at least two independent point mutations are required to inactivate the transcriptional activity [15, 62]. In addition to the other mutation types, about 4 % of *TP53* mutations are silent (Figure

2). These silent mutations may be passenger mutations co-selected with another mutation in *TP53* [62].

A number of specific *TP53* mutational events have been recognized in different types of human cancer. The best known examples of molecular linkages between exposure to carcinogens and *TP53* mutations in cancer are the correlation of exposure to UV light with tandem double CC>TT transition mutations at dipyrimidine sites in skin cancer, the correlation of dietary aflatoxin B1 exposure with G to T transversion at codon 249 of *TP53* in hepatocellular carcinoma and the correlation of exposure to cigarette smoke with G to T transversion in lung carcinomas [15, 32, 51, 69, 78].

2.4 Inflammation and cancer

Inflammation is a well-coordinated physiological process that occurs after various kinds of cellular and tissue damage. It can be caused by microbial pathogen infection, chemical irritation, wounding, and/or endogenous injury [79], and it is normally self-limiting. Inflammation may be related to tumorigenesis in three ways: (i) acute inflammation can contribute to the regression of cancer [79–82], (ii) certain chronic inflammatory conditions, such as inflammatory bowel disease, ulcerative colitis, haemochromatosis and viral hepatitis [23, 81, 83, 84] can increase cancer risk, and (iii) oncogenic mutations, for example mutations in *RAS*, *MYC* and *RET*, may evoke inflammation since various types of oncogenes promote cellular inflammatory transcriptional programs, for example the induction of angiogenesis [23, 81].

During the injury associated with tissue wounding, cell proliferation is enhanced while the tissue regenerates, but both proliferation and inflammation should cease after the repair is completed [85]. If the control of inflammatory components fails, acute inflammation can convert to chronic inflammation, and generate a pathologically conducive microenvironment as the inflammatory cells produce a great amount of growth factors, cytokines, and reactive oxygen and nitrogen species (ROS and RNS, respectively) [79, 80, 83]. In chronic inflammation, continued tissue damage is sustained, which in turn induces cell proliferation [79, 80]. This is likely not a direct cause of cancer, but incessant cell proliferation in

an environment filled with inflammatory cells, growth factors, activated stroma, and DNA-damage promoting agents can predispose to carcinogenesis [79, 85]. The continual tissue damage and regeneration of tissue in the presence of highly reactive nitrogen and oxygen species can result in permanent genomic alterations, when these free radicals interact with the DNA of proliferating cells [85]. In line with this, patients with chronic inflammatory diseases have been shown to exhibit alterations in cancer-related genes and proteins [83]. For example, in rheumatoid arthritis, mutations in the tumour suppressor gene *TP53* are seen at frequencies similar to those occurring in tumours [86]. In addition, irrespective of the cause of the cancer initiation, inflammatory cells and mediators are present in the microenvironment of most, if not all, tumours [81]. It has been stated that "in a sense, tumours act as wounds that fail to heal" [85, 87].

In epidemiological studies a link has been demonstrated between chronic inflammation and various types of cancer, including bladder, cervical, gastric, intestinal, lung, oesophageal, ovarian, prostate and thyroid cancer [79, 81, 88]. It has been estimated that underlying infections and inflammatory mechanisms play a role in about 15–20 % of all global cancer deaths [81, 85]. Furthermore, treatment with non-steroidal anti-inflammatory drugs, such as acetylsalicylic acid (aspirin), that inhibit cyclooxygenase-enzymes (COX-1 and COX-2) has been shown to decrease the incidence and mortality of several tumour types [81]. In addition, targeting of inflammatory cells, the mediators of inflammation (chemokines and cytokines, for example TNF-α and IL-1β) or the key transcription factors involved in inflammation (for example NF-κB and STAT3), decreases the incidence and spread of cancer [81].

2.4.1 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) enzymes catalyze the conversion of arachidonic acid to thromboxanes and prostaglandins, the key mediators of inflammation [79, 89, 90]. There are two isoforms, COX-1 and COX-2, which share about 60 % amino acid identity [90, 91]. Although there are some exceptions, in general COX-1 is constitutively expressed and responsible for the basal prostaglandin synthesis [89, 91]. In contrast, COX-2 is rarely expressed in normal tissues but its expression can be induced by many physiological and stress-related signals including growth factors, cytokines and other

mediators of inflammation, tumour promoters, oxidizing agents and DNA damaging agents [89, 92]. The distinct prostaglandin(s) synthesized as the result of COX-2 induction depends on the tissue, availability of substrates and the specific synthase enzymes present in the cell [89]. The regulation of COX-2 gene takes place mainly at the transcription level and several inflammatory factors regulate COX-2 by directly activating its promoter [84, 91]. Many of the signals that activate COX-2 also induce tumour suppressor p53 [84].

There is considerable evidence from molecular, pharmacological and clinical studies to link COX-2 with the development of cancer. Increased amounts of COX-2 are found commonly in both premalignant and malignant tissues and the elevated COX-2 expression has been reported in many cancers, for example in lung cancer [93], colon cancer [94], pancreatic cancer [95] and stomach cancer [96]. In general, COX-2 expression is higher in well-to-moderately differentiated tumours and in metastasis compared to poorly differentiated tumours [84]. Inhibitors of COX, such as acetylsalicylic acid or other non-steroidal anti-inflammatory drugs, have been shown to reduce the incidence of different malignancies [84, 93, 97–99]. An inverse relationship between COX-2 overexpression and survival of cancer patients has also been reported in retrospective studies [84, 100, 101]. Overall, the COX-2 expression may not be the driving force for the carcinogenesis but rather it appears to play a role in enhancing cancer development in the overall context of chronic inflammation [79].

Interactions between COX-2 and tumour suppressor p53 have been shown *in vitro* and *in vivo*, but the results have been contradictory. It has been shown that p53 can upregulate COX-2 [92, 102, 103], but it can also suppress the transcription of COX-2 [98, 104]. Additionally, COX-2 has been observed to exhibit strong inhibitory effects on p53 transcriptional activity [102, 103]. As proposed in the recent publication of de Moraes et al [84], these different results might be explained by two different mechanistic scenarios, where the variable patterns of interaction between COX-2 and p53 depend largely on the inflammatory context. In inflammation-derived tumours, NF-κB and p53 can co-operate to induce COX-2, whereas in non-inflammatory tumours, p53 does not appear to participate in the activation of COX-2 and in those cases, *TP53* gene mutations would act as an independent factor in the tumorigenesis 84. In addition, a correlation between COX-2 expression and *TP53*

wild type status has been found in adenocarcinoma but not in SCC of Barrett's esophagus [92]. This suggests that the participation of p53 in the regulation of COX-2 expression may also be tissue specific.

2.5 Sinonasal cancer

Cancer of the nose and paranasal sinuses (ICD-7 code 160, except 160.1, corresponding to ICD-10 code C30.0, C31) is a rare form of cancer, with an incidence of 0.5 to 1.5 new cases per year per 100 000 in men and 0.1 to 0.6 per 100 000 in women [9, 105]. The incidence of sinonasal cancer varies markedly between different countries and even within the same country. This variation has not been attributed to individual susceptibility, such as genetic differences, but is most likely due to differences in exposure [9, 105].

The main histological types of sinonasal cancer are adenocarcinoma and squamous cell carcinoma (SCC) [106]. The most common form is the squamous cell carcinoma; only 4–20 % of sinonasal tumours are adenocarcinomas, though adenocarcinoma is the histological type more strongly associated with wood dust exposure [107]. Sinonasal adenocarcinomas are well characterized morphologically by the presence of neoplastic glandular structures that arise in the nasal cavity and paranasal sinuses and they are located mainly in the ethmoid sinus and the upper part of the nasal cavity. Local recurrence is the most common cause of death among patients with sinonasal adenocarcioma; the average 5-year survival is 20–50 % [108]. Distant and lymph node metastases are rare (5–10 % of all cases) [107]. A fraction of sinonasal adenocarcinomas are classified as the intestinal type adenocarcinoma, based on their close histopathological resemblence to adenocarcinoma of the colon [109].

2.5.1 Risk factors

The first evidence for the extremely high risk ratios associating wood-dust exposure and sinonasal cancer came from the UK, and subsequently numerous epidemiological studies have confirmed this association [9]. Very high relative risks have been invariably found and 10–45-fold risks have been indicated for the adenocarcinoma cell type in association to occu-

pational exposure to hardwood dust. The risk related to softwood dust exposure is less clear [8–10, 110, 111]. Another risk factor for sinonasal cancer suggested by the epidemiological studies is cigarette smoking, with a two- or threefold increased risk of nasal cancer observed among smokers and a reduction in risk among long-term quitters. This effect may, however, be limited to the squamous cell carcinoma rather than adenocarcinoma [112, 113]. Other risk factors for sinonasal cancer include occupational exposure to textile or leather dust, to chromium (VI) compounds, to nickel and its organic compounds and possibly to formaldehyde, but so far the published studies on these exposures are partially conflicting [114].

2.5.1.1 Occupational wood dust exposure

Exposure to wood dust in the occupational setting is a common occurrence. It has been estimated that in the years 2000–2003 about 3.6 million workers (approximately 2.0 % of the working population) were occupationally exposed to inhalable wood dust in the European Union [115] and over half a million of these workers were estimated to be exposed to high levels (exceeding 5 mg/m^3) of wood dust [115]. Roughly two-thirds of the wood used commercially is softwood, but the wood species used vary regionally and also depending of the type of product being manufactured. In general, the terms "hardwood" and "softwood" refer to the taxonomic categorization of trees and not necessarily to the hardness of the wood. Gymnosperms or conifers are referred to as hardwoods while angiosperms or deciduous trees represent softwoods [9]. While hardwoods are generally denser than softwoods, the density varies considerably within each family. Mixed exposure to dusts from more than one species of wood and to dusts from wooden boards typically occurs in most branches of the wood-working industries [9, 115].

Wood dust is a complex mixture of compounds including a wide variety of biologically active substances, also genotoxic and carcinogenic compounds [9, 110]. Chemically, it is a mixture of organic and inorganic components while the composition varies according to tree species [9, 110]. The particulate nature of the wood-dust exposure also plays a role in generating ROS within cells and inducing DNA damage and evoking an inflammatory response [9, 110, 116–119]. Asthma has been linked with occupational wood dust exposure by epidemiological studies [120], further

demonstrating the connection between inflammation and wood dust exposure.

Sinonasal cancer, especially adenocarcinoma, is strongly associated with occupational wood-dust exposure, as reported in many epidemiological studies [9]. Furthermore, some, though not all, studies have found elevated risks in association with wood dust exposure for other types of cancer, including lung cancer [9, 121–124]. Consequently, wood dusts, in particular those originating from a variety of hardwood species, are classified as carcinogenic to humans [9, 110, 124].

2.5.2 Molecular mechanisms

Multiple mechanisms of carcinogenesis have been proposed to be involved in the development of sinonasal cancer related to wood-dust exposure, but there is very little experimental or human data in the literature. The published findings have been based on a relatively limited number of cases, mostly involving adenocarcinomas. In these studies, high frequencies of DNA copy number changes as detected by comparative genomic hybridization have been detected [125, 126], while the mutation rates reported for the *KRAS* gene [127–132] and the *TP53* tumour suppressor gene have been lower [131, 133–135]. Initially, *KRAS* and *HRAS* mutations were found to be quite frequent in sinonasal cancer, with implications for histogenetic and prognostic significance [128–132], but recent results show that tumours with *KRAS* mutations might represent only a small proportion of all sinonasal cancers [127].

Inflammation has also been proposed to play a significant role in sinonasal carcinogenesis. Epidemiologic studies have suggested that environmental factors or inflammation, for example chronic sinusitis or human papilloma virus infection, may represent etiologic factors in the induction of maxillary sinus carcinoma [133]. There are also consistent reports of impaired mucociliary clearance and mucosal alterations encountered during chronic wood-dust exposure [9]. Mucosal alterations include dysplasia and metaplasia of the columnar epithelium, and to a lesser extent, changes in the squamous epithelium [9, 120].

2.5.3 *TP53* mutations in sinonasal cancer

At present, there are only a few studies that have studied *TP53* mutations in sinonasal cancer (Table 1). Most of them have concentrated on cancers of the intestinal type of adenocarcinoma, and the numbers of cases studied have been rather low. In these studies, a variable occurrence of *TP53* mutations has been reported (18–60%) [131, 133–135]. Some of the studies have also examined the accumulation of p53 in the cell nucleus in adenocarcinoma type of sinonasal cancer (Table 1). The accumulation of p53 may reflect a *TP53* mutation, but other reasons for p53 accumulation are also known; furthermore, not all mutations induce nuclear accumulation of p53. The results reported indicate that p53 accumulation is a common feature in adenocarcinomas, with immunopositivity ranging between 20–80% [132, 135, 136].

Table 1. Earlier studies on *TP53* alterations in sinonasal cancer found in literature.

Study	Number of cases	Tumour histology[1]	Tumour origins	Exposure information	Technique used to analyse TP53[2]	TP53/p53 results[1]	Other alterations/markers studied
Wu TT, Barnes L, et al. (1996) [131]	12	Intestinal type of adenocarcinoma	Nasal cavity, ethmoid sinus and sphenoid sinus	No	Immunohistochemistry and sequencing	Expression was seen in 10/12 of cases and mutations in 2/12 of cases	*KRAS* mutations
Caruana SM, Zwiebel N, et al. (1997) [137]	24	Benign, nondysplastic IP, IP with dysplasia, IP containing SCC, SCC	Maxillary sinus	Smoking and alcohol use	Immunohistochemistry, SSCP and sequencing	Mutations were found in 1/5 of SCC cases, 1/4 of IP+SCC cases (one with double mutation), 3/7 of IP+dysplasia cases and 0/7 of benign cases. All the mutations were located in exon 7 or in exon 8. Expression was seen in 0/5 of SCC cases, 3/4 of IP+SCC cases, 2/7 of IP+dysplasia cases and 0/7 of benign cases.	Human papilloma virus DNA
Bandoh N, Hayashi T, et al. (2002) [133]	70	SCC	Maxillary sinus	No	Immunohistochemistry and sequencing	Expression was seen in 39/70 (56%) of cases and mutations in 20/70 (29%) of cases	Fas, bax and bcl-2 expressions and apoptosis
Bashir AA, Robinson RA, et al. (2003) [136]	11	Adenocarcinoma	Maxillary sinus, ethmoid sinus and nasal cavity	Various occupations listed	Immunohistochemistry	Expression was seen in 8/10 of cases	CK7, CK20, AE 1/3, CAM 5.2, smooth muscle-specific actin, muscle specific actin, desmin, S-100, carcinoembryonic antigen and HER-2/neu expressions
Perrone F, Oggionni M, et al. (2003) [135]	21	Intestinal type of adenocarcinoma	Nasal cavity and paranasal sinuses	Occupational exposure, including wood dust for 10 cases	Double gradient DGGE and sequencing	Mutations were found in 8/20 (40%) of cases	Deregulation of $p14^{ARF}$ and $p16^{INK4a}$ and *HRAS* mutations

Licitra L, Suardi S, et al. (2004) [134]	30	Intestinal type of adenocarcinoma	Ethmoid sinus	Occupational exposure, not specified	Double gradient DGGE and sequencing	Mutations were found in 18/30 (60%) of cases	None
Valente G, Ferrari L, et al. (2004) [138]	125[3]	Intestinal type of adenocarcinoma	Nasal cavity and paranasal sinuses	Wood dust exposure and smoking	Immunohistochemistry	The mean percentage of p53-positive cells found in the metaplastic epithelium was 28.6% in woodworkers and 7.97% in controls. In the tumour cases, the number of p53-positive cells of normal epithelium was higher in woodworkers than in controls, 12.3 and 1.2 %, respectively.	None
El-Mofty SK and Lu DW. (2005) [139]	39	SCC, non-keratinizing carcinoma, undifferentiated carcinoma	Nose and paranasal sinuses	No	Immunohistochemistry	Expression was seen in 8/21 of SCC cases, 3/8 of non-keratinizing carcinoma cases and 3/10 undifferentiated carcinoma cases	Human papilloma virus DNA using PCR, p16 and Ki-67 expressions
Yom SS, Rashid A, et al. (2005) [132]	15	Seromucinous adenocarcinomas and enteric-type adenocarcinoma	Ethmoid sinus, maxillary sinus and nasal cavity	Smoking and dust exposure	Immunohistochemistry	Expression was seen in 3/15 of cases	Mutations in *KRAS*, *APC* and *beta-catenin* genes, microsatellite analysis of markers hMLH1 and hMSH2

1) IP: inverted papilloma, SCC: squamous cell carcinoma
2) SSCP: single strand conformation polymorphism, DGGE: denaturing gradient gel electrophoresis
3) 60 wood workers with minimum exposure of 10 years, 50 functional and/or esthetic nasal surgery patients (controls) and 15 patients with intestinal type adenocarcinoma

2.6 Lung cancer

Lung cancer is the leading cause of cancer death with over 1 million deaths each year worldwide [5, 17, 140]. It is the primary preventable type of cancer since tobacco smoking is the cause for most lung cancers [5, 141, 142]. The association between smoking and lung cancer is well established with a 10- to 20-fold increased occurrence of lung cancer in smokers compared to never smokers [5, 112, 143]. Duration of smoking and number of cigarettes smoked are the strongest determinants of the lung cancer occurrence in smokers, both showing positive proportionality [88]. Nevertheless, it has been estimated that about 10–15 % of all lung cancers in men and about 50 % in women are not attributable to active tobacco smoking, which overall accounts for 25 % of all lung cancers worldwide [5]. The known environmental risk factors for lung cancer are second hand tobacco smoke, asbestos, radon, chromium, arsenic, cadmium, silica and nickel, as well as outdoor air pollutants. In addition, previous lung disease and genetic, hormonal, viral and dietary factors have been implicated [5, 140, 143]. In Western countries (North America, Western and Northern Europe, Australia), the lung cancer incidence has stabilized over the past decades, especially among men [5, 112, 140]. In contrast, the lung cancer incidence among women and in the developing countries is still increasing [5, 112].

Lung cancers are classified into four major histological types; additionally multiple minor or rare forms exist. The main types are the small cell lung cancer (SCLC) and the three non-small-cell lung cancers (NSCLCs): squamous cell carcinoma (SCC), adenocarcinoma and large cell carcinoma [5, 140]. Tobacco smoking is associated with all histological types of lung cancer with varying strength of association and there is good scientific evidence indicating multiple smoking-related mechanisms of lung carcinogenesis [5, 88, 112, 144]. Small cell lung carcinoma has the strongest association with smoking [5, 141, 144]. With respect to non-small cell lung carcinomas, smokers have most commonly had squamous cell carcinomas. However, over time, there has been a shift in the distribution of the major histological types observed in lung cancer. Globally, the incidence of squamous cell carcinoma is decreasing while the incidence of adenocarcinoma is increasing. This is most likely a result of changes in cigarette design and composition [5, 88, 140]. The benzo[a]pyrene content

of cigarettes has decreased by about 60 % whereas the formation of nitrosamines during tobacco storage, processing and smoking has increased following the changes in the nitrate content in blends of tobacco [5, 88, 140]. Nitrosamines, such as nicotine-derived nitrosaminoketone (NKK), are potent lung carcinogens that induce adenocarcinomas in experimental animals [5, 88, 140, 145, 146]. In addition, the lower nicotine content may cause deeper inhalation when smoking to compensate for the reduced nicotine content and the smoke inhaled deeper may penetrate to more peripheral parts of the bronchi [88]. For never smokers, adenocarcinoma is the most common histological type [5, 141, 144].

Common genetic changes in all histological types of lung cancer include mutations in *TP53*, defects in the $p16^{INK4}$/*RB* pathway, loss of heterozygosity at alleles on chromosome 3p [15, 144] and inactication of *LKB1* [147]. Mutually exclusive mutations at codon 12 in *KRAS* or in the tyrosine kinase domain of the *EGFR* have been found in about 30 % of adenocarcinomas but are rare in other histological types [15, 144]. There are also indications that cigarette smoking evokes chronic inflammation that is associated with the development of lung cancer [148].

2.6.1 Tobacco smoking

Smoking is a major cause of disease and mortality that could be prevented [88, 112]. There are about 1.3 billion smokers globally and approximately 150 million deaths related to tobacco use are projected for the years 2000–2024 if there is no change in current smoking patterns. In the more developed countries, smoking is involved in over 30 % of all cancer deaths [15, 88, 149]. Cigarette smoke contains several thousand chemicals including over 60 identified as carcinogens by IARC [5, 145, 149, 150]. These include potent carcinogens like the polycyclic aromatic hydrocarbons (PAHs) and the nicotine-derived nitrosamine ketones (NKK and NNN) but also aldehydes and other volatile organic compounds such as benzene and butadiene are present [5, 88, 112, 145, 149]. Metabolites of the tobacco smoke carcinogens are able to bind covalently to DNA, often at certain specific sites, and form bulky adducts. These bulky adducts can be removed by DNA repair such as the nucleotide excision repair system, of they may lead to apoptosis. If they persist, bulky adducts can result in mutations that potentially could initiate or promote carcinogenesis. Since the ad-

duct formation and persistence plays a important role in carcinogenesis, the balance between activation, detoxification and DNA repair of these lesions is a significant factor in determining individual susceptibility to cancer [5, 149].

As mentioned, smoking displays a well-established causal relationship with lung cancer and also involuntary or secondhand smoke is a risk factor [5, 88, 112, 151]. In addition to lung cancer, tobacco smoking is an important causal factor for oral, otopharyngeal, hypopharyngeal, laryngeal, and oesophageal cancers as well as those of pancreas, bladder, and renal pelvis. Smoking has also been associated with the cancers of the nose, stomach, liver, colon, renal body, and cervix and with myeloid leukaemia [112, 149, 152]. The increased cancer risk induced by smoking rapidly declines after quitting. The benefit of stopping smoking is evident within five years and it progressively becomes more marked with time, the risk remaining, however, higher than among non-smokers [2, 153, 154]. In addition to cancer, tobacco use contributes also to other serious disorders: cardiovascular disease, cerebrovascular disease, peripheral vascular disorders, abdominal aortic aneurysm, chronic obstructive pulmonary diseases and fetal disorders [88, 154].

2.6.2 *TP53* mutations in lung cancer

Lung cancer is known to frequently harbour *TP53* mutations, with an overall frequency of about 50 %. They are more common in small cell and squamous cell carcinomas than in adenocarcinomas [140]. The frequency of mutations is higher in smokers (50–80 %) than in non-smokers (10–28%) [15, 144, 150] and the occurrence of *TP53* mutations in lung cancer from smokers has been shown to be associated with lifetime cigarette consumption and duration of smoking [150].

The *TP53* mutation spectrum is different between smokers and non-smokers [150, 155]. An overrepresentation of G to T transversion mutations in lung tumours in smokers has been observed in many studies [15, 51, 150]. These transversions often occur at *TP53* codons 157, 158, 245, 248 and 273 in smokers while underpresented in non-smokers [15, 51, 144, 155]. Mutations at codon 157 are also detectable in histologically normal lung tissue adjacent to cancer growth in smokers as well as in the lungs of smokers without cancer [51, 144]. *In vitro* studies have demonstrated that

BPDE, the metabolically activated form of benzo[a]pyrene, and some other carcinogenic PAHs from cigarette smoke bind to guanosine residues at these hot spot codons [51, 144, 150]. There is a concordance between the distribution of *TP53* mutations in lung cancer in smokers and the experimental spectrum of DNA-adducts induced by PAH metabolites in the *TP53* [15]. A higher proportion of G to T transversions (30 %) is also a characteristic difference in the mutation spectra between lung cancer and most other types of human cancers (frequency of G to T transversions about 10 %) [15, 51, 144, 150].

3 AIMS OF THE STUDY

The focus of this PhD-study was on *TP53* mutations in sinonasal and lung cancer and their associations with the major causative exposures in these two cancer types, i.e. work-related wood dust exposure and tobacco smoking, respectively. An increased knowledge on the molecular mechanisms in cancer associated with carcinogenic exposures is needed for risk assessment and prevention, especially with respect to sinonasal cancer related to wood-dust exposure, for which the current data are sparse.

The specific aims were:

- To compare CE-SSCP, DGGE and direct sequencing to find out the benefits and relative sensitivities of the three methods for detection of somatic mutations of the *TP53* gene in larger series of human tumour samples.
- To study the frequency of *TP53* mutations in sinonasal cancer and lung cancer populations with detailed exposure data available for molecular epidemiology analysis.
- To study association between the *TP53* mutation profile and tobacco smoke-induced primary DNA damage in a lung cancer population of moderate to heavy smokers.
- To analyze the *TP53* mutation profile in sinonasal cancer and to examine the relations between exposures and the discovered mutation profile. In addition, to compare the mutation profile detected in sinonasal cancer to known data from other cancers available in large international databases [58, 68].
- To explore cancer-related inflammation by analyzing the expression of an inflammation-related enzyme, COX-2, in sinonasal cancer.

4 MATERIALS AND METHODS

4.1 Patients and samples

4.1.1 Comparison of mutation detection methods (I)

Lung cancer cases (n = 20) that were a subset of an earlier study [156] were analyzed for *TP53* mutations. In this study, new DNA-samples were extracted from paraffin-embedded tissue (PET) samples of lung cancer, in order to ensure that all the samples were treated the same way and that there was enough DNA for the analysis. This was not possible for few cases (T174, T182, T213, T246, and T316), for which DNA but no tumour specimens were available. In these cases, the DNA extracted in the earlier study was used. Samples were analyzed under dummy codes so that the *TP53* mutation status was not known.

4.1.2 Molecular changes in sinonasal cancer (II, IV, V)

Paraffin-embedded tissue samples of sinonasal cancer tumours were collected from three European countries (Denmark, Finland and France) (Figure 3) (II, IV). All incident cases of the cancer of the nose and paranasal sinuses (ICD-7 code 160, except 160.1, corresponding to ICD-10 code C30.0, C31) were identified from the nationwide cancer registries in Denmark for the years 1991–2001, as described elsewhere [127], and in Finland for 1989–2002 (II, IV). In Finland, every other case of squamous cell carcinoma (SCC) was randomly included. Informed consent was obtained for patients still living. In the case of the patient was deceased, the permission was received from the appropriate national

authorities (II, IV). The archival formalin fixed paraffin embedded tissue samples were collected from pathology laboratories. In France, cases were identified in the regional cancer registries of Isère, Somme and Doubs for the years 1990–2002. The tissue fixation medium used in pathology laboratories was often other than formalin (Bouin, which makes DNA-based molecular analyses difficult to perform), resulting in a more restricted collection of paraffin embedded tissue samples (II, IV). The study was approved by the appropriate national and institutional Ethics Review Boards in Finland, Denmark and France.

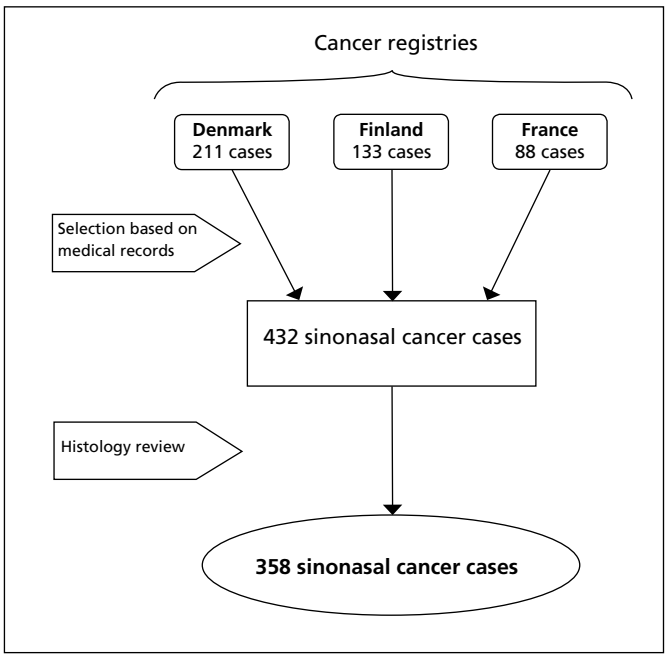

Figure 3. Tumour collection in the sinonasal cancer study. Initially cancer registries in Denmark, Finland and France were contacted to acquire information on sinonasal cancer patients. After the selection based on the medical records, the tumour sections from eligible cases were reviewed by a pathology panel, which resulted in the final collection of 358 histologically verified sinonasal cancers.

A panel of three pathologists reviewed tumour sections for the entire tumour collection (432 cases, Figure 3) without reference to the clinical details, exposure background or mutation status. Common criteria for inclusion and exclusion were established as presented earlier [127] (II, IV). In brief, tumours were included that corresponded to one of the main histopathologic categories of cancer of the nose and paranasal cavities as indicated in the WHO classification of tumours [127, 157]. Benign tumours, metastatic lesions and malignant tumours outside of these categories were excluded, as were cases that were miscoded in the registries. The latter included, for example, intranasal melanomas, skin cancers, olfactory neuroblastomas and adenoid cystic carcinomas. When appropriate, immunohistochemistry was used to confirm the tumour type in the excluded cases. A common reason for exclusion was location of the tumour in the vestibulum nasi or in the nasopharynx. In addition, some cases were not included in the study due to missing pathology reports, inadequate hospital records, poor condition of tissue sample or poor yield of DNA from paraffin-embedded tissue. The final collection for the molecular analysis consisted of 358 sinonasal cancer cases (170 cases from Denmark, 109 cases from Finland, and 79 cases from France), each case with a consensus histology diagnosis of adenocarcinoma, squamous cell carcinoma, or carcinoma not otherwise specified (NOS) (II, IV). Demographic and other data for the cases included are reported in IV, Table 1. In the series under study, squamous cell carcinoma was the most common tumour histology (59 %, 213/358), followed by adenocarcinoma (34 %, 122/358) and carcinoma NOS (6 %, 23/358). With respect to the adenocarcinomas, 88 out of 122 (72 %) fulfilled the WHO criteria [157] for intestinal type of adenocarcinoma and 92 % (81/88) of this type of tumour were in males (II, IV).

4.1.3 Lung cancer in smokers (III)

A series of cases with adenocarcinoma (n = 67) and squamous cell carcinoma (SCC; n = 37), the two major histological types of lung cancer, were selected from a Hungarian study population comprising various histological types of lung cancer. The sample set consisted of 104 cases, 42 females and 62 males, who underwent lung resection for primary lung cancer (III). After the selection for histology, there was no additional

selection for smoking status. The samples of lung tumour and histologically non-tumorous peripheral lung tissue were gathered after obtaining informed consent. Information on smoking history was provided by the patients via self-reporting. Three major smoking categories were defined as follows: (i) smokers, that included current smokers who smoked up to the time of surgery, and those patients who had quit smoking within one year before the surgery; (ii) former smokers who had given up smoking more than one year before the surgery, and (iii) never smokers (III).

4.2 Exposure background in sinonasal cancer (II, IV, V)

4.2.1 Work history

In all study centers, patients or next-of-kin (preferably the last spouse or a child) of the deceased patients were interviewed by telephone in Denmark and Finland. In France, personal interviews were done and both techniques utilized a structured questionnaire designed for this study. The interviews provided data on demographics, tobacco use, and employment histories, including occupational exposure to wood dust, formaldehyde, chromium (VI) compounds, nickel and its organic compounds, and textile and leather dust. Interview data was received in 84 (49 %) of the Danish cases, 69 (63 %) of the Finnish cases and 51 (65 %) of the French cases. In Denmark and Finland, the information obtained via the interviews on lifelong employment history was supplemented with data from additional sources. First, employer records were retrieved from the Danish National Pension Fund (Danish cases) and the Finnish Centre for Pensions (Finnish cases). These registries record employment histories for all companies; if a person has been employed, the working history and period of employment can be retrieved back to 1964 in Denmark and to 1963 in Finland. In Denmark, the commercial classification of each company was obtained from Statistics Denmark, and in Finland, the companies were classified by an industrial hygienist according to an extended version of International Standard Industrial Classification of all economic activities in 1990. Finally, job titles of the subjects were extracted from the Central Person Registry in Denmark

(data available since 1968) and from Statistics Finland (data available at five-year intervals; retrieved for 1970, 1975, 1980, 1985 and 1990). In all, data on wood dust exposure was available for 79% (283/358) of the cases, on other occupational exposures, including formaldehyde, chromium compounds, nickel and its organic compounds, and textile and leather dust (with some overlap in exposures), for 59 % (210/238) of the cases, and on smoking for 56 % (201/358) of the cases (II, IV, V).

4.2.2 Exposure assessment

For each case, exposure to wood dust and to other risk factors of sinonasal cancers as listed above was assessed based on the entire work history by an experienced industrial hygienist in each study center. The criteria and procedure for exposure assessment were harmonized between the study centers in a common meeting where each rater assessed exposures of five cases, in order to minimize variation of exposure estimates between the assessors. There was also a discussion about differences in exposure characteristics between the countries and these were taken into account in the deliberation. Wood dust exposure estimates were subdivided by wood species and products used (soft wood, hard wood, wooden boards). The industrial hygienists involved in the exposure assessment were blinded to the results of the mutation analysis (II, IV, V).

In Finland and in France, for each job held by a patient, the concentration of exposure to wood dust was assessed by the industrial hygienists into six categories: unexposed, very low (<0.3 mg/m^3), low (0.3–<1 mg/m^3), medium 1 (1–<2 mg/m^3), medium 2 (2–<5), or high (≥5 mg/m^3). Quantitative values (midpoints of the intervals) were assigned to each category of concentration. The frequency of exposure (proportion of working time during which exposure occurred) was assessed separately. A level of exposure was then calculated for each job by multiplying the concentration by the frequency. The classification of exposure levels was based on data available in Finland and France [158, 159] and on exposure estimates generated for the EU member states in another arm of the larger study program to which this study also belonged [115]. In Denmark, exposure levels were estimated by measurements of inhalable wood dust concentrations conducted in different Danish woodprocessing industries in the 1990's. A 6 % yearly decline as compared to the level in 1990

was assumed for the years 1975–1990 and exposures were assumed to be constant before the year 1975. This assumption was based on a large number of random measurements in the Danish wood industry taken from 1985 to 2003 [160, 161]. These showed a quite consistent a 6–7 % yearly decline. In line with this, a 7 % yearly decline has been reported from 1978 to 1997 for the US wood industry [162]. Exposure level estimates were also adjusted based on the average working distance from the exposure source (II, IV, V).

4.3 Molecular analysis of *TP53* mutations (I, III, IV, V)

4.3.1 DNA extraction and PCR

DNA was extracted from frozen or paraffin embedded tissue (PET) samples using a standard phenol-chloroform extraction protocol. Extracted DNA was stored in aliquots of 100 µm/ml concentration at -20°C. For PCR amplification of the gene sequences under study, 100 ng of genomic DNA was used in a 25 µl reaction, with the reaction carried out according to the instructions of the manufacturer of the enzyme (TaqGold, Applied Biosystems) (I, IV, V).

4.3.2 Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP)

Samples were amplified by PCR, exon by exon, using primers labeled with fluorescent dyes (6-carboxyfluorescein, 6-FAM, for the forward primer and 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein, HEX, for the reverse primer). The CE-SSCP analysis was performed as described earlier [163]. Briefly, after the PCR-amplification, the samples were diluted 1:20 and 1µl of the diluted sample was mixed with 10,5 µl deionized formamide, 0,5 µl NaOH (0,3 M) and 0,5 µl GeneScan 350 TAMRA size standard (Applied Biosystems). The samples were denatured at 95 °C for 5 min, and put directly onto ice. The CE-SSCP analysis of *TP53* exons 5–9 was performed in ABI PRISM 310 capillary sequencer or in ABI PRISM 3100-avant capillary sequencer (Applied Biosystems) at 30 °C.

In some cases with unclear results, additional running temperatures were used. Mutations were detected as mobility shifts and/or changes in the electrophoregrams (I, III, IV).

4.3.3 Denaturing Gradient Gel Electrophoresis (DGGE)

After PCR-amplification of DNA, the DGGE-analysis was run as described earlier [156]. Briefly, 1-mm thick gels containing 7 % acrylamide (Bio-Rad) and 0,3 % bis-acrylamide (Bio-Rad) in 1x Tris-acetate-EDTA (TAE) buffer were used. Formamide (Merck) and urea (Merck) were used as denaturing agents at different concentrations, 100 % denaturant corresponding to 9 M formamide and 7 M urea. The gel polymerization was activated using ammonium persulfate (Bio-Rad) and TEMED N,N,N',N'-tetramethylethylenediamide (Bio-Rad). The samples were run in perpendicular gels in 1x TAE buffer at 60 °C in DCode Universal Mutation Detection System apparatus (Bio-Rad). The electrophoresis time was 4 h using 150 V electrophoresis voltage. The gels were stained with ethidium bromide (Aldrich-Chemie) and photographed with a UV transilluminator (Stratagene Eagle Eye II) (I).

4.3.4 Direct Sequencing

DNA was first amplified by PCR and the PCR-products were purified with QIAquick PCR purification Kit (Qiagen Nordic) or by using ExoSAP-IT (USB). The sequencing reaction was prepared with BigDye Terminator v3.0 Cycle Sequencing ready Reaction kit (Applied Biosystems, California, USA) and sequencing was performed with an ABI Prism 310 capillary sequencer (Applied Biosystems) or an ABI Prism 3100-avant capillary sequencer (Applied Biosystems). Mutations were only accepted if the alteration was detected both in forward and reverse strands. Mutations that could not be identified by direct sequencing, probably due to the quality of DNA from the PET samples, were confirmed by at least one independent CE-SSCP from a new PCR reaction (I, III, IV, V).

4.4 Determination of bulky DNA adducts in lung tissue by ^{32}P-postlabelling (III)

Bulky DNA adducts were determined in non-tumorous peripheral lung DNA by the ^{32}P-postlabelling method essentially as described before [164]. Briefly, DNA (4 µg) was digested overnight with micrococcal nuclease (Sigma) and spleen phosphodiesterase (ICN and MP Biomedicals). Adduct enrichment was made with nuclease P1 (Sigma) digestion of the normal mononucleotides. Radiolabelling occurred with 50 µCi carrier-free [γ-^{32}P]ATP (end-labelling grade, ICN and MP Biomedicals) and 6 U of T4 polynucleotide kinase (USB Corporation and Fermentas). Multidirectional thin-layer chromatography of the radiolabelled DNA digests was performed on 10 × 10 cm poly(ethyleneimine) cellulose sheets (Macherey-Nagel) as detailed earlier [164]. Radioactivity patterns were detected by Cerenkov counting and electronic autoradiography (InstantImager, Packard). Background radioactivity of the blank area, corrected for the size of the adduct areas was subtracted from the radioactivity of the adduct areas. DNA adduct levels were calculated by normalisation for an in vitro-modified benzo[a]pyrene diol-epoxide-DNA standard [110 adducts/10^8 nucleotides] as described earlier [164]. Two to four replicate analyses were performed with each human DNA sample in separate assays (III).

4.5 Immunohistochemistry analysis of COX-2 and p53 (II)

COX-2 expression. Sections (4 µm) cut from paraffin tissue blocks on coated slides were incubated overnight at 37 ºC, deparaffinized and then microwaved for 4 x 5 min in 0.01 Mmm Na-citrate buffer (pH 6.0). In order to block endogenous peroxidase activity, the slides were immersed in 1.6 % hydrogen peroxide in methanol for 30 min and then in blocking solution for unspecific binding sites (0.5 % BSA and 1.5 % normal serum in PBS). Immunostaining was performed following a protocol described earlier [165] with monoclonal IgG against human COX-2 protein peptide (Cayman Chemical Co.) at a dilution of 1:100 overnight at room temperature. After this, the slides were treated with

biotinylated secondary antibody (anti-mouse IgG) at a dilution of 1:250 (Vectastain Elite ABC kit PK-6102, Vector Laboratories) in a solution of 0.5 % BSA in PBS for 30 min. Antibody binding sites were visualized with avidin-biotin peroxidase complex solution (Vectastain Elite kit PK-6102, Vector Laboratories), after treatment for 30 min, and AEC (3-amino-9-ethylcarbazole) liquid for 15 min. Counterstaining was done with Mayer hematoxylin. The COX-2 index was calculated by multiplying the percentage of COX-2 positive cells by the intensity (1–5) of the COX-2 staining. Cases with intensity 1 or a COX-2 index less than 40 were considered as negative (-), indexes 41–200 were scored as weak (+), 201–300 as moderate (++) and over 300 as strong (+++) (II).

p53 accumulation. Sections (4 µm) cut from paraffined tissue blocks on coated slides were incubated overnight at 37 °C, deparaffinized and then microwaved for 4 x 5 min in 0.01 mM Na-citrate buffer (pH 6.0). Immunohistochemistry (IHC) staining was performed using the Ventana Benchmark automated immunostainer (Tucson Medical Systems) following the protocols provided by the manufacturer. The antibody used was DO-7 for wild type p53. The p53 nuclear accumulation was scored as follows: if nuclear staining was seen in only 0–1 % of the cells, the case was considered as negative (-), in 2–33 % as weak (+), in 33–79 % as moderate (++) and over 80 % as strong (+++) (II).

4.6 COX-2 expression by real time quantitative PCR (II)

To further confirm the COX-2 expression detected by IHC, we performed a real-time quantitative PCR analysis. RNA was successfully extracted from 10 adenocarcinomas by using High Pure RNA Paraffin kit (Roche) according to manufacturer's instructions. The RNA extraction was successful for a subset of adenocarcinomas; for the remainder, the tissue samples were either too small or possibly too old to yield good enough quality RNA for the analysis. The cDNA synthesis and real-time quantitative PCR (Taqman assay) with an AbiPrism 7700 Sequence Detector System (Applied Biosystems) were performed as described earlier [166]. PCR primers and probes designed by Applied Biosystems and purchased from the company were used. The results were expressed

as relative units (RU), which were calculated by the comparative CT method [166] (II).

4.7 Statistical analysis

4.7.1 Sinonasal cancer (II, IV, V)

Several exposure variables were used to summarize the lifetime exposure to wood dust: ever-exposed *versus* never-exposed, total duration of exposure, cumulative level of exposure (calculated by summing the total work history plus the job-specific products of level and duration), and average level of exposure (calculated as the cumulative level divided by total duration) (II, IV, V).

The average daily consumption of cigarettes was calculated by dividing the total lifetime number of cigarettes by the total duration of smoking. Cumulative tobacco consumption was expressed as pack-years (pack-years = total duration x average number of packs smoked per day; 1 pack = 20 cigarettes) (II, IV, V).

Odds ratios (ORs) for *TP53* mutations and the corresponding 95 % confidence intervals (CI) were calculated by unconditional logistic regression. All ORs were adjusted for age, sex and country. Quantitative variables related to wood dust exposure and smoking were categorized in three classes according to the distribution among all exposed subjects (unexposed, $<50^{th}$ percentile, $\geq 50^{th}$ percentile). Other occupational exposures were coded into two categories (unexposed, exposed). Comparisons of proportions were performed using χ^2 or Fisher exact tests (II, IV, V).

For analysis of COX-2 expression, a score ++ or +++ was considered positive. Fisher's exact test (two-sided) was used to compare proportions of positive cases (II).

All analyses were performed using the STATA statistical package (Stata statistical software: Release 9. Statacorp LP 2005)

4.7.2 Lung cancer (III)

The statistical analyses were performed with GraphPad Prism 4.0 software, using Fisher's exact test and Mann-Whitney U-test. Two-sided P values are given (III).

5 RESULTS

5.1 Comparison of mutation analysis methods (I)

In the search for an efficient screening method, a series of 20 lung tumour DNA samples were analyzed by three different methods: CE-SSCP, DGGE and direct sequencing. DGGE and direct sequencing had been used in the laboratory earlier, while the CE-SSCP was set-up according to literature and evaluated against the other two methods.

Altogether 17 out of the 20 samples analyzed were found to be carrying a mutation in the *TP53* gene; 15 (88 %) of them were detected by DGGE, 16 (94 %) by CE-SSCP and 12 (71 %) by direct sequencing. Thus, DGGE detected one mutation less than CE-SSCP, but this mutation resided in the intron outside the area efficiently screened with the DGGE assay due to the primer design. Therefore those two methods were considered to be equally sensitive (94 %). Direct sequencing, with a sensitivity of only 71 %, was clearly less sensitive than the other two methods. In the laboratory, CE-SSCP was the easier method to use than DGGE. In addition, it was more easily automated and displayed good repeatability and documentation of data.

5.2 Sinonasal cancer: tumour histology and exposure (IV)

A statistically significant association was found between wood-dust exposure and tumour histology. The occurrence of adenocarcinoma was significantly elevated (OR 8.8, CI 95 % 4.2–18.3, adjusted for age, sex

and study center) among wood-dust exposed cases when compared to squamous cell carcinoma (IV, Figure 2 and Table 2). The pathology panel classified 72 % (88/122) of the adenocarcinomas as the intestinal type [109]. Most cases of the intestinal type of adenocarcinoma were encountered in wood-dust exposed individuals (64 %, 25/39).

The wood dust exposed cases of sinonasal cancer had mostly been exposed to both hardwood and softwood dusts, but the histologic type found almost exclusively among cases exposed mainly to hardwood dust was adenocarcinoma. The cases mostly exposed to softwood dust exhibited more often squamous cell carcinoma than adenocarcinoma (the difference in the distribution of histologies by the type of wood dust exposure was statistically significant, $p < 0.001$; IV: Figure 2).

The majority of patients with squamous cell carcinoma and adenocarcinoma were smokers, with similar proportions in both histologies. When adjusted for age, sex and study center, smoking was associated with a non-significantly decreased risk of having an adenocarcinoma (IV: Table 2). None of the 15 wood-dust exposed cases that were non-smokers presented with squamous cell carcinoma (13 adenocarcinomas and 2 carcinoma NOS).

The association between wood-dust exposure and histology remained significant when further adjusting for smoking (OR for adenocarcinoma: 12.6, 95 % CI 5.0–31.6). After additional adjustment for wood dust exposure, smoking was associated with a significantly decreased OR of having an adenocarcinoma (OR = 0.4, 95 % CI 0.2–0.96). There were also cases exposed to formaldehyde (n = 59), many of them had also a history of wood dust exposure. The majority of cases exposed to formaldehyde but without wood dust exposure (n = 19) carried squamous cell carcinomas (n = 15) (IV).

5.3 *TP53* in sinonasal cancer (II, IV, V)

5.3.1 p53 immunohistochemistry in sinonasal cancer (II)

Overall, about half of the 41 cases that underwent p53 immunohistochemistry analysis expressed p53. No statistically significant associations

between the exposures and p53 accumulation were found; however, the sinonasal cases with wood-dust exposure more often exhibited intense staining for p53 as compared with the non-exposed cased (p = 0.062). In the adenocarcinoma type of tumours, all the more strongly positive p53 accumulations were found in non-smokers (p = 0.061) and wood dust -exposed cases (p = 0.15). Among cases with the squamous cell carcinoma type of tumours, smokers tended to show more p53 accumulation (from cases with higher p53 accumulation levels and data available for smoking history, 7/7 were smokers.) (II).

5.3.2 *TP53* mutations and their association with tumour histology and exposure (IV)

A high overall frequency of *TP53* gene mutation positive cases (77 %, 277/358) was found in a series of histologically confirmed cases of sinonasal cancer. *TP53* mutations were observed in all tumour cell types, but they were most common in adenocarcinoma (OR 2.0, 95 % CI 1.1–3.7; compared to squamous cell carcinoma). No difference in the *TP53* mutation frequency was found between intestinal type of adenocarcinoma (86 % 76/88) and adenocarcinoma of non-intestinal type (85 %, 29/34). About half of the mutated cases (51 %, 131/268) and both histologal types displayed concurrent mutations in the *TP53* gene (IV).

TP53 mutations showed an overall, non-significant association to wood dust exposure (OR 1.6, 95 % CI 0.8–3.1). The occurrence of *TP53* mutation was significantly elevated in association with duration (≥24 years, OR 5.1, 95 % CI 1.5–17.1), average level (>2 mg/m^3; OR 3.6, 95 % CI 1.2–10.8), and cumulative level (≥30 mg/m^3 x years; OR 3.5, 95 % CI 1.2–10.7) of wood dust exposure. The association between mutation and a long duration of wood dust exposure remained when analyzing the histological types separately, even though the increased odds ratios for *TP53* mutation were statistically insignificant. Adjustment for smoking or formaldehyde affected the risk estimate for *TP53* mutation associated with wood-dust exposure only marginally (IV).

Smoking was not associated with the overall risk of having a *TP53* mutation, but smokers were found to exhibit significantly more frequently multiple mutations than non-smokers (OR 2.7, 95 % 1.1–6.5; IV: Table 4), this was seen in both wood-dust exposed and non-exposed

groups (IV: Figure 4). Multiple mutations were also less common among cases exposed to wood dust (OR 0.5 95 % CI 0.3–1.1).

Finally, 81 % (29/36) of the cases with exposure in the past to one or several other risk factors (chromium, nickel, leather or textile dust) carried a mutation. However, the occurrence of *TP53* mutation was not significantly associated with any of these exposures (IV).

5.3.3 *TP53* mutation profile (V)

Direct sequencing was successful in identifying the type and location of 159 *TP53* mutations of the 477 sequence alterations detected in the 277 mutation positive sinonasal tumours. More than half of the mutations (60 %, 95/159) were missense mutations; there were also 28 (18 %) frameshift or nonsense mutations, and 36 (23 %) intronic or silent mutations. 86% (19/22) of the silent mutations detected were in tumours with multiple mutations (V: Figure 2). The distribution of mutation types differed significantly between the different histologies (V: Figure 2), exposure groups (Figure 4) and tumours with single or multiple mutations (V: Figure 4).

The mutations were scattered over a large number of codons (IV: Figure 3), with codon 248 was the most frequently mutated codon with 8 mutations (5 C**G**G to C**A**G and 3 **C**GG to **T**GG). It was followed in frequency by codon 135 (7 mutations; 5 T**G**C to T**T**C and 2 T**G**C to T**A**C), codon 175 (5 mutations; all C**G**C to C**A**C), and codon 179 (5 mutations; 2 C**A**T to C**T**T, 1 C**A**T to C**G**T and 1 **C**AT to **T**AT). The most common base change detected was a C to T transition (43/125, 34 % of base changes in the coding region; V: Figure 3). In particular, C to T transitions were frequent among the silent mutations (16/22, 73 %), and they were most often found in non-wood dust exposed sinonasal cancer cases (V: Figure 3). The G to A transition was the second most common base change with 27 mutations (22 % of base changes in the coding region) found mainly in smokers (V: Figure 3). G to T transversions occurred at a frequency of 10% (of mutations in the coding region, 12/125) and they were detected predominantly in smokers (V: Figure 3) and resulted in missense mutations. Frameshift mutations were also common, they represented the third most frequent type of mutation (20/159, 13 % of all mutations).

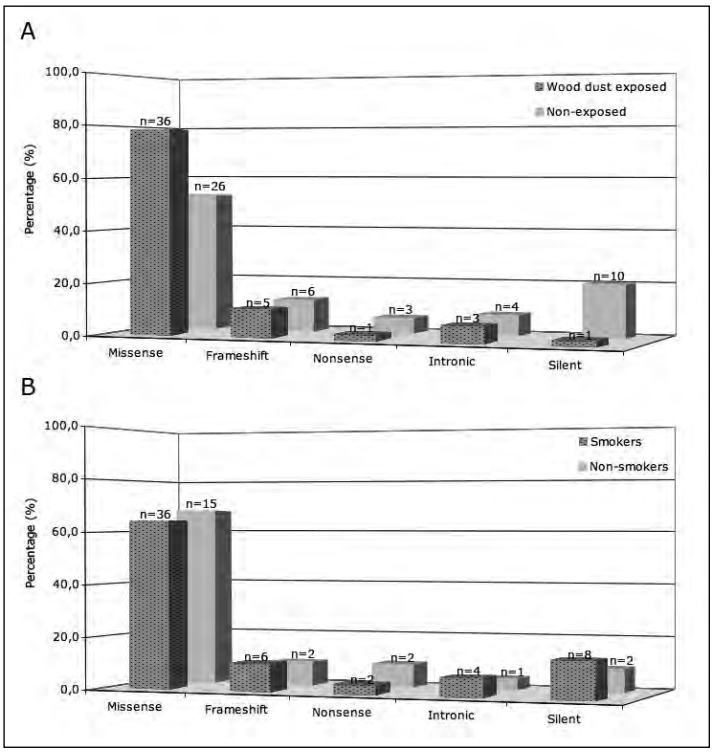

Figure 4. Distribution of *TP53* mutation types of in sinonasal cancer subdivided according to different exposures as identified in V. A) Mutation profiles of wood dust exposed and non-exposed, B) Mutation profiles of smokers and non-smokers.

5.4 COX-2 expression relative to exposure and *TP53* in sinonasal cancer (II)

A total of 48 sinonasal cancer tumours were analysed for COX-2 expression by immunohistochemistry. COX-2 was expressed at high levels in adenocarcinoma as compared to squamous cell carcinoma ($p < 0{,}001$; II: Table 1). COX-2 expression showed significant association with occupational exposure to wood dust ($p = 0.024$, II: Table 1), and with non-smoking status ($p = 0.001$, II: Table 1). All but one (92 %; 12/13) of the adenocarcinoma tumours exhibiting moderate or strong COX-2 expression were adenocarcinomas of intestinal type. No associa-

tion between COX-2 expression and tumour site or tumour grade was observed. The association between p53 accumulation as detected by immunohistochemistry and COX-2 expression was close to statistical significance in adenocarcinomas (p = 0.054) but not in squamous cell carcinomas (II).

When looking at the relation of COX-2 expression and *TP53* mutation by different histological cell types or by wood dust exposure (Figure 5), it seemed that COX-2 was mainly expressed in tumours with *TP53* mutation and adenocarcinoma histology and/or wood-dust exposure.

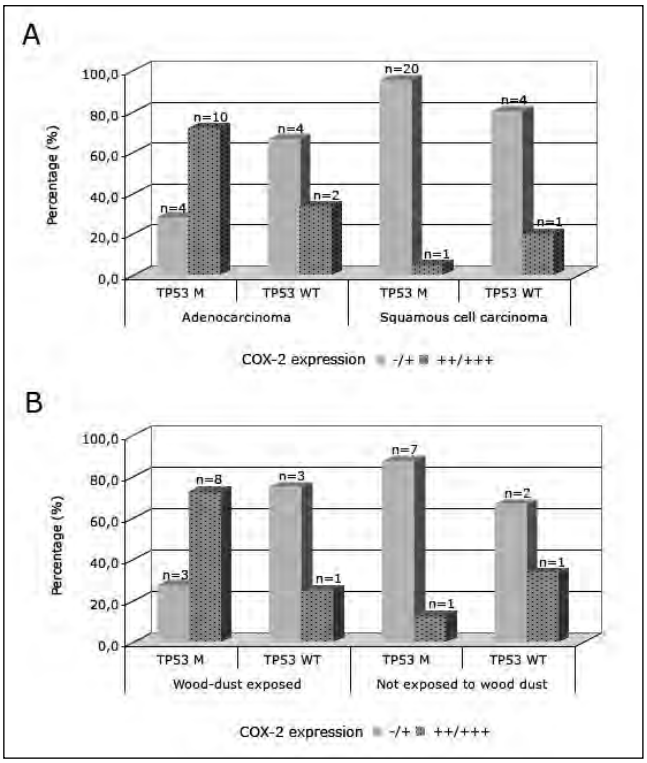

Figure 5. The relationship between COX-2 expression and *TP53* mutations in sinonasal cancer subdivided according to: A) histology and B) wood-dust exposure. (-/+): weak or no expression of COX-2; (++/+++): moderate or strong expression of COX-2; TP53 M: mutated *TP53* gene; TP53 WT: wild-type *TP53* gene.

COX-2 expression could also be detected at the mRNA level. This analysis was performed only on a subset of adenocarcinomas (10 cases) due to limitations with tissue material available. The levels of COX-2 mRNA expression detected by real time quantitative PCR were in most cases comparable with the expression determined by immunohistochemistry (Pearson correlation coefficient between the immunohistochemistry index and the relative unit of real time quantitative PCR was 0.63) (II).

5.5 *TP53* mutations and DNA adducts in lung cancer (III)

In total, 45 % (47/104) of the cases, 55 % (34/62) of men and 31 % (13/42) of women, carried a *TP53* mutation. In all, 51 *TP53* mutations were detected; 48 cases had a single mutation, two cases carried a double mutation, and one case displayed three different mutations. The prevalence of *TP53* mutations was statistically significantly associated with duration of smoking (55 % in those who had smoked more than 20 years *vs.* 14 % in those who had smoked less, p = 0.002), tumour histology (70 % in SCC vs. 31 % in adenocarcinoma, p = 0.0002) and gender (55 % in men *vs.* 31 % in women, p = 0.045) 9 (III).

Smokers (combined group of current and short-term former smokers who quit less than year before surgery) had approximately twice as high a level of bulky adducts as the combined group of former and never smokers (10.9 ± 6.5 vs 5.5 ± 3.4 adducts /10^8 nucleotides, p<0.0001). Among former and never smokers, higher levels of bulky DNA adducts were detected in non-tumorous lung tissue from *TP53* mutation carriers as compared to non-carriers (P = 0.076) (III) (Figure 6). In the group of current smokers, the carriers of wild type *TP53* gene had a slightly higher level of bulky DNA adducts, whereas in the group of short-term former smokers the *TP53* mutation carriers tended also to have higher levels of bulky DNA adducts (Figure 6).

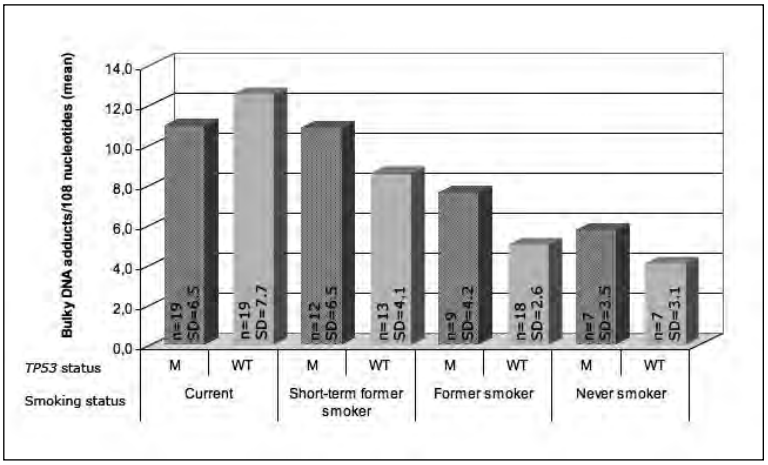

Figure 6. Bulky DNA adducts in non-tumorous lung tissue from lung cancer patients and their relation with *TP53* gene mutation and smoking status. Short-term former smokers gave up smoking less than one year before the surgery and former smokers more than a year before surgery. SD = standard deviation, M = mutant, WT = wild type.

The overall codon distribution of the mutations followed the pattern reported in the IARC database for lung cancer. The most commonly mutated *TP53* codon was codon 175 (3 mutations), followed by codons 146, 157, 248 and 266, each with two mutations. All but one of the mutated codons in tumours from never smokers were different from those mutated in smokers. The most common sequence change detected was a G to A transition (8/43, 19 %, in addition there was one intronic g to a change), followed by G to T transversion (8/43, 19 %), frameshift mutation (7/43, 16 %) and G to C transversion 7/43, 16 %) (III: Figure 2). G to A transitions were more commonly seen among former smokers and never smokers than among smokers (III: Figure 2). A G to T transversion was detected exclusively in smokers and their carriers were those found with a high burden of bulky DNA adducts in their lungs (III: Figure 2). The majority of G to C transversions was also detected in smokers (III: Figure 2). Frameshift mutations were frequently seen in smokers (4/12, 33 %), who had smoked for more than 35 years; none was detected in smokers who had smoked for 20 or fewer years (III).

6 DISCUSSION

Tumour suppressor *TP53* gene mutations are an important feature of many kinds of human cancer, as the aberration of p53 function is required at some point of carcinogenesis in most, if not all, human cancers [52]. The *TP53* gene encodes a transcription factor that among its other functions is known to induce DNA repair or trigger apoptosis in response to cellular DNA damage [64], features that are both important in inhibiting tumour initiation and growth. Certain carcinogenic exposures have been shown to induce a typical and recognizable *TP53* mutation spectrum [15, 51, 78], which makes *TP53* an interesting gene for studies with a molecular epidemiology approach. This work studied *TP53* mutations in sinonasal cancer and lung cancer, two exposure-related human cancers.

Both sinonasal and lung cancer have clear exposure-related risk factors. With respect to lung cancer, the primary risk factor is tobacco smoking [5, 141, 142], with 10- to 20-fold increased risk of lung cancer for smokers compared to never smokers [5, 112, 143]. Smoking is associated with all the histological types of lung cancer [5, 88, 112, 144]. Sino-nasal cancer is strongly associated with wood dust exposure, especially the adenocarcinoma histological type [7–10], as was also found in this study (I). The odds ratios have been particularly high among cases with history of occupational exposure to hardwood dust and tumour of intestinal-type adenocarcinoma histology that originates from the ethmoidal sinus [7–10]. In addition to wood dust, also smoking has been reported to increase the risk of sinonasal cancer, with a two- to threefold increase in risk among smokers and a reduction in risk among long-term quitters [112, 113], but this association has been suggested to be limited to squamous cell carcinoma and no clear association has been observed for the adenocarcinoma histological type of sinonasal cancer [112, 113].

Earlier, *TP53* mutations in sinonasal cancer have been studied in only a few relatively small studies [131, 133–135], whereas the *TP53* mutation profile in lung cancer has been established by numerous studies and is well described in the *TP53* mutation databases [68, 167]. The current studies showed that *TP53* mutations were frequent in both sinonasal cancer (IV, 77 % overall *TP53* mutation frequency) and lung cancer (III, 45 % overall *TP53* mutation frequency). In both cancers, *TP53* mutations were observed to be associated with exposure. In sinonasal cancer, the odds ratios for *TP53* mutation were significantly increased with the duration and average and cumulative level of wood dust exposure (IV) well in line with epidemiologic data demonstrating an association between duration and level of wood dust exposure and elevated risk of sinonasal adenocarcinoma [9, 10]. Smoking did not influence the overall risk of having *TP53* mutations in sinonasal cancer (IV). In lung cancer, it was noted that the current smokers carried more often mutations than former smokers and the frequency of mutations increased with increasing duration of smoking (III), which is in accordance with earlier studies and the epidemiological evidence of associating heavy and persistent smoking with high risk of lung cancer [112, 144, 168].

The highest frequency (86 %) of *TP53* mutations in sinonasal cancer in the present study was found in adenocarcinoma type of tumours (IV), however, there was no difference between the intestinal type of adenocarcinomas and the non-intestinal type of sinonasal cancer (IV). Squamous cell carcinomas exhibited also a high frequency of mutations (74 %). Nevertheless, the adenocarcinoma cases had a statistically significant, two-fold increased occurrence of *TP53* mutations in comparison to squamous cell carcinoma (IV). In lung cancer, the prevalence of *TP53* mutations was particularly high (70 %) in squamous cell carcinoma histological type, higher than reported for the same histological type in the IARC database (49 % [58]). This may reflect the effect of having a relatively high proportion of heavy smokers included in the series of lung cancer patients (III). On the other hand, the mutation frequency detected in the adenocarcinoma type of lung cancer was very close to that reported in the database (III).

Interestingly, about half of the sinonasal tumours with *TP53* mutations carried a mutation not only in one but in two or more of the exons, (IV); we also found a few similar cases in lung cancer (III). In sinonasal

cancer, the occurrence of multiple mutations was significantly associated with smoking; this could be seen in both wood-dust exposed and non-exposed cases (IV). Earlier, multiple mutations have been reported also in squamous cell carcinoma of the head neck [169, 170], for which smoking and alcohol consumption are known to be the most important risk factors [171].

The codon distribution of mutations in sinonasal cancer and lung cancer in the present studies followed overall the general pattern found in human cancer [58] and mutations were scattered over a large number of codons (IV, V and III). Both cancers had mutations in the hotspot codons 175 and 248, but also some differences were found. In lung cancer (III), one of the most mutated codons was the codon 157, which is frequently mutated in smoking-associated lung cancer but not found in non-smokers [51, 144, 155]. It has been experimentally identified as a site of adduct formation by benzo[a]pyrene [172, 173]. Accordingly, we found both of the codon 157 mutations in smokers. In sinonasal cancer, the codon 135 was one of the most commonly mutated codons (V). Generally, codon 135 mutations are not considered to be hot spots in human cancer, even though spontaneous codon 135 mutations have been found in untreated human p53 knock-in (Hupki) murine embryonic fibroblast cells. Interestingly, the types of mutation observed in codon 135 code for proteins with highly impaired transactivation capabilities [174]. The codon 135 mutations in Hupki cell line have been proposed to be due to oxygen radicals in cell culture incubators [175]. As the generation of ROS is likely to be one of the cellular consequences of wood dust exposure and the subsequent inflammatory process [116], a ROS -related induction of the codon 135 mutations would seem plausible.

When examining the mutation profile, there were differences between the distribution of the mutation types in lung cancer (III) and in sinonasal cancer (V). In lung cancer, it was noted that the most common base change was G to T (19 % of mutations), found exclusively in smokers. This is in line with the *TP53* mutation databases [58, 68], where the *TP53* mutation profile in lung cancer contains about 30 % of G to T transversions, seen often in hot spot codons [176, 177]. G to T transversions have also been found in non-tumorous lung tissue from smoking lung cancer patients [51]. This specificity of mutation type is compatible with the evidence associating DNA adducts with the types of mutations en-

countered after exposure to benzo[a]pyrene and other PAH compounds [176, 178, 179]. The PAH-related bulky DNA adducts have been shown to be present in the non-tumorous lung tissue from smokers and cause G to T transversion in vitro [51, 180]. For the first time, the present study was able to demonstrate that the majority of the lung cancer cases exhibiting a G to T transversion were indeed those with high levels of smoking-related bulky adducts detected in the adjacent non-tumorous lung tissue (III). These findings strongly support the proposal that the specific mutation spectrum of smokers originates from continued exposure to a complex mixture of carcinogenic and mutagenic PAHs [176, 179, 181, 182]. Other factors, such as selective DNA repair, are likely to participate to some extent in the process [172, 176, 181–183], a hypothesis supported by our results that among former and never smokers, higher levels of bulky DNA adducts were detected in non-tumorous lung tissue from *TP53* mutation carriers as compared to non-carriers, whereas smokers had approximately twice as high bulky adduct level as the combined group of former and never smokers (III). In addition, it is probable that other carcinogens present in tobacco smoke also contribute to the mutation profile [140, 145, 176].

Unlike the G to T transversion in lung cancer, it was not possible to detect any specific type of mutation for sinonasal cancer (V). The most common base change detected was C to T transition, which was mainly found in silent mutations and in tumours from non-wood dust exposed patients. G to T transversions were also in sinonasal cancer detected predominantly in smokers, they represented the fourth most common mutation type with 10 % of the mutations in the coding region. The low prevalence of G to T transversions is in good agreement with the finding showing that smoking was not significantly associated with the overall risk of having a *TP53* mutation in sinonasal cancer (IV). The frequency of missense base substitutions (59 %) detected in sinonasal cancer (V) was somewhat lower in comparison to the overall *TP53* mutation profile in human cancer (about 80 %), and, accordingly, the prevalences of frameshifts (13 %) and silent mutations (14 %) were higher than those generally found among mutations in the *TP53* gene (9 % and 4 %, respectively [58]). However, the frequencies found here, with the exception of the number of silent mutations, were not different from data on 600 mutations reported in the database for the larger anatomical entity, head and neck cancer [58].

There were significant differences in mutation profiles of sinonasal tumour with single or multiple mutations (V). The prevalence of silent mutations in tumours with a single mutation in our data (4 %) corresponded to that generally reported for *TP53*, while almost all (86 %) of the silent mutations had occurred in tumours exhibiting multiple mutations. Overall, silent and non-splice site intronic *TP53* mutations represented 33 % of all mutations detected in sinonasal tumours carrying multiple mutations. The higher frequency of silent mutations might be explained by their predominant occurrence concurrently with another mutation. Tumours with multiple mutations may reflect a process in which more mutations have been able to accumulate in the tumour with the mutagenic pressure of tumour progression, while the *TP53* mutation may not have been the initiation or driving force in the tumorigenesis.

In addition to silent mutations, frameshift mutations were more common in the sinonasal tumours with multiple mutations than in the single mutation tumours, but overall the frequency resembled the data from head and neck cancers, in which frameshift mutations have been reported to occur more commonly than in other cancer types [58, 171]. In head and neck squamous cell carcinoma, frameshift mutations are often found in the subgroup of patients using both tobacco and alcohol [171]. It can be speculated that similarly in sinonasal cancer, the regular, continued presence of two mutagenic and carcinogenic exposures, wood dust and tobacco smoking, may generate a continual high genotoxic stress in the target cells, resulting in an excess of frameshift mutations. In lung cancer (III), we found frameshift mutations frequently in smokers, who had smoked for more than 35 years (33 % of them carried a frameshift mutation); none of these types of mutation was detected in smokers who had smoked for 20 or fewer years. Typically, frameshift mutations represent about 11 % of the mutations found in lung cancer of smokers [58].

Multifactorial pathways are most likely involved in the development of cancer. One mechanism proposed for both sinonasal cancer and lung cancer involves inflammation. In sinonasal cancer, we found that COX-2 overexpression was associated with wood dust exposure and adenocarcinoma histological type (II). In the cases with adenocarcinoma or history of wood dust exposure, COX-2 was detected in tumours with *TP53* mutation. Wood dust has been shown to induce an inflammatory response as well as genotoxicity in experimental studies [116–119]. DNA-

damage may, at least partially, be due to production of ROS and it seems to be independent of the cytokine-induced inflammation [116, 117].

Interestingly, COX-2 expression was also associated with non-smoking (II) in sinonasal cancer, whereas in lung cancer, cigarette smoke has been shown to cause proinflammatory changes [184] and COX-2 overexpression has been detected in lung tumours [89]. Also, increased levels COX-2 have been detected in the exhaled breath condensate of non-small cell lung cancer patients compared to the control group without cancer [185]. Among cancer patients, increased levels of COX-2 were observed in smokers and ex-smokers compared to non-smokers and the exhaled COX-2 correlated with smoking as measured in pack-years [185]. Furthermore, it was shown recently that inflammatory mechanisms account for the tumour-promoting effect of exposure to tobacco smoke on lung cancer in mice [186]. Altogether, these results support the hypothesis that inflammation related to smoking participates in the development of lung cancer [4].

Technological advances and the sequencing of human genome have made it possible to analyze whole cancer genomes [22, 38]. This has revealed that relatively few gene alterations are constantly implicated in the development of a tumour [22]. On average, there are about 80 amino-acid altering mutations in a typical cancer but only a subset, less than 15 mutations, are likely to contribute significantly to the initiation or progression of the tumour [28]. These genes include well-known oncogenes and tumour-suppressor genes like *KRAS* and *TP53*, but new genes have also been identified [22, 27, 28, 74]. Importantly, the data from cancer genome analyses have confirmed the cancer-specific information obtained earlier by studying *TP53* mutations [27, 62]. As the *TP53* mutations have been shown to associate with exposure-related DNA damage, they have been suggested to be useful as biomarkers in molecular epidemiology and to help to dissect the role of carcinogenic exposures in tumorigenesis [22, 27]. This type of information is important for risk assessment and prevention.

7 CONCLUSIONS

The present study showed that CE-SSCP is a sensitive and reproducible assay for screening of unknown *TP53* mutations in human tumour samples. The sensitivity of mutation detection in CE-SSCP was higher than in direct sequencing, and it was less laborious than DGGE. Ultimately, though, the DNA alterations found with CE-SSCP must be sequenced for the identification of the exact type and location of the altered sequence.

Tumour suppressor *TP53* gene mutations are frequently encountered in sinonasal cancer and they are associated with wood dust exposure at work. The occurrence of *TP53* mutations increased significantly in association to a long duration and a high level of occupational exposure to wood dust. Smoking was not observed to influence the overall risk of having a *TP53* mutation in sinonasal cancer, but was associated with multiple *TP53* mutations. The highest frequency of *TP53* mutations was found in adenocarcinoma type of tumours, but no difference was found between the intestinal type of adenocarcinomas and non-intestinal type of sinonasal cancer. The data, together with earlier studies, suggest that mutational mechanisms, especially the mutations of *TP53* are important in sinonasal carcinogenesis related to wood dust exposure.

The overall codon distribution of mutations in sinonasal cancer in the present study followed the general pattern found in human cancer with the mutations scattered over a large number of codons. A specific feature in the *TP53* mutation distribution in sinonasal cancer was that the codon 135 was one of the most commonly mutated codons. Generally, this codon is more rarely mutated in human cancer. Based on some earlier experimental data, it is possible that mutations of this codon in sinonasal cancer are related to wood-dust induced inflammation and the

subsequent ROS production. Sinonasal tumours from individuals with a history of wood dust exposure exhibited predominantly missense mutations, whereas tumours from smokers contained an excess of frameshift and silent mutations in *TP53*. The mutation profile of *TP53* in sinonasal cancer further points towards high genotoxic stress generated by regular exposure to carcinogenic substances such as wood dust.

Inflammation appears to play a role in sinonasal carcinogenesis as suggested by the results that COX-2 expression was associated with adenocarcinoma type of tumours, wood dust exposure, and non-smoking. Among the adenocarcinomas or cases with wood dust exposure history, COX-2 expression appeared to occur in tumours carrying also a *TP53* mutation. In all, taking into account the mutation profile in sinonasal cancer, together with the high overall frequency of *TP53* mutations, sinonasal carcinogenesis related to wood-dust exposure appears to be a process where both high genotoxic stress and inflammation, play a role.

In lung cancer, a statistically significant association between *TP53* mutations and duration of smoking, gender, and tumour histology was found. Furthermore, the patients with a tumour carrying a G to T transversion, a mutation commonly found in association with tobacco smoking, had a high level of smoking-related bulky DNA adducts in their non-tumorous lung tissue. Among former and never smokers, higher levels of bulky DNA adducts were detected in non-tumorous lung tissue from *TP53* mutation carriers as compared to non-carriers. These results underline the distinctive role of the mutagenic and carcinogenic tobacco smoke components as the origin of specific mutation spectrum typically found in smokers.

Sinonasal and lung cancer are cancers related to exposures to agents with a DNA damaging capacity. In both cancers, the mutation of *TP53* gene appears to be a key element, and it shows association with the exposure. These exposures associated with the mutation load are typically regular and take place over a relatively long period of time. The information on molecular alterations in exposure-related human cancers adds to the observations from epidemiological studies and helps to understand the role and effect of different etiological factors, which in turn can be beneficial for risk assessment and prevention. For example, the data associating wood-dust induced inflammation not only with asthma and asthma-like symptoms but also with molecular pathways of sinonasal

cancer could well provide further motivation to reduce wood-dust exposure in the occupational setting.

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ORIGINAL ARTICLES I–V

I

Analysis of *TP53* gene mutations in human lung cancer: Comparison of capillary electrophoresis single strand conformation polymorphism assay with denaturing gradient gel electrophoresis and direct sequencing

by

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Analysis of *TP53* gene mutations in human lung cancer: Comparison of capillary electrophoresis single strand conformation polymorphism assay with denaturing gradient gel electrophoresis and direct sequencing

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Abstract

Introduction: Automated capillary electrophoresis single strand conformation polymorphism (CE-SSCP), denaturant gradient gel electrophoresis (DGGE), and direct sequencing were compared to investigate the benefits and sensitivity of each of the methods for detection of unknown *TP53* mutations in human lung cancer. *Methods:* Twenty previously analyzed DNA samples from lung tumors were examined under dummy laboratory codes for occurrence of mutations of the *TP53* gene. *Results:* Mutations were found in 17 samples; 15 (88%) of them were detected by DGGE, 16 (94%) by CE-SSCP and 12 (71%) by direct sequencing. One of the two mutations that remained undetected in DGGE was in fact outside the sequence area covered by DGGE screening, thus rendering DGGE and CE-SSCP equally efficient in mutation detection. Direct sequencing performed less well in finding mutations than the two other assays, as also shown previously. *Conclusion:* The study showed that CE-SSCP is a fast and highly reproducible method, which is considerably less laborious compared to DGGE, for screening of unknown *TP53* mutations.

Keywords: Capillary electrophoresis; SSCP; DGGE; *TP53* gene; Mutation; Human lung cancer; Single strand conformation polymorphism; DNA extraction; Direct sequencing; Detection methods; Sensitivity; Sample preparation

1. Introduction

The tumor suppressor gene *TP53* is one the most studied genes in cancer research. The p53 protein mediates many different cellular processes that control cell growth, including effects on cell cycle, DNA repair, apoptosis, differentiation and angiogenesis [1]. It plays a central role in the cell's response to various kinds of stress situations, including damage to DNA. Mutation of the *TP53*-gene is the most common genetic alteration in the human cancers [2,3]. *TP53* is a fairly large gene, and mutations discovered in human cancer are scattered all over the 10 encoding exons. Still, majority of the mutations occur in exons 5–8, which is the conserved area of the gene. Mutations of the *TP53* gene are typically of diverse types, including base substitutions, small deletions, and insertions [4]. Therefore, investigation of human tumor DNAs for presence of unknown mutations must rely on an efficient screening system with high sensitivity.

Various methods based on gel electrophoresis have traditionally been used in mutation detection to separate the wildtype sequence from the mutated ones. The methods based on gel separation are simple to run and do not require large investments in equipment. Denaturant gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP) are both commonly used methods for mutation screening. They enable rather quick and reliable detection of variations in DNA sequence, but neither of them provides information about the type or exact location of the variation. Generally, DGGE is considered to be more

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sensitive than the standard SSCP assay [5], whereas the latter is considered simpler to run. These methods are, however, typically time consuming and they cannot be easily automated. Application of the capillary electrophoresis (CE) technique, among other fairly recently developed methods such as denaturing high performance liquid chromatography (DHPLC), has made it possible to achieve faster analysis and greater automation [6,7]. With the use of CE technique, SSCP has become more readily applicable for investigating large collections of DNA samples [8]. Ultimately, for identification of the alterations detected in screening, the DNA fragments must be sequenced.

In the present study, capillary electrophoresis SSCP (CE-SSCP) was compared with the traditional slab gel DGGE, and direct sequencing to find out the benefits and relative sensitivities of the three methods for detection of somatic mutations of the *TP53* gene in human tumor samples.

2. Materials and methods

2.1. Tumor samples and DNA extraction

Twenty lung cancer samples were analysed for *TP53* mutations. DNA samples were prepared from fresh lung tumor specimens from which non-tumor tissue was carefully excluded macroscopically by pathologist before storage at −80 °C, as described earlier [9]. For the present work, new DNAs were prepared from samples cut from the same tumor tissue specimens, except for the cases for which DNA samples but no tumor specimens were available (samples # T174, T182, T213, T246, and T316). The DNA was purified from the tissue by phenol–chloroform extraction. First, the samples were incubated 2 h in lysis buffer (Applied Biosystems) at 55 °C. Proteinase K (Merck) was added and the samples were incubated overnight at 55 °C. The DNA extraction was done twice with phenol/water/chloroform mix (Applied Biosystems) and once with chloroform (Merck). DNA was precipitated with ethanol and glycogen (Merck) and diluted in water. The quality of DNA was determined on 1% agarose gel. The samples were analysed under dummy laboratory codes so that the analyzer did not know the mutation status of the samples.

2.2. PCR

For PCR, 200 ng of genomic DNA was used in a 50 μl reaction. The reaction mix contained 22.5 μM of each dNTP (Pharmacia Biotech), 1 μM of each sense and antisense primers (Sigma Genosys), 0.0125 U/μl of HotStarTaq

Table 1
Comparison of analysis results from the three detection methods evaluated in the study (denaturing gradient gel electrophoresis, DGGE; capillary electrophoresis single strand comformation polymorphism, CE-SSCP, and direct sequencing)

TP53 exon/sample	Method DGGE[a]	CE-SSCP[a]	Sequencing[b]		
			Codon	Base change	Amino acid change
Exon 5					
T25	−	−	−	−	−
T107	−	+	Between 138 and 139	Insertion 8 bp (AAGACCTG)	Frameshift
T117	+	+	157	G → T	Val → Phe
T137	+	+	−	−	−
T246	+	+	175	G → A	Arg → His
T251	+	+	161	C → A	Ala → Asp
T182	−	−	−	−	−
T306	+	+	172	Deletion 1 bp (T)	Frameshift
Exon 6					
T174	+	+	194	T → G	Leu → Arg
T240	+	+	−	−	−
T316	+	+	209	Deletion 2 bp (AG/GA)	Frameshift
Exon 7					
T159	+	+	−	−	−
T160	+	+	248	C → T	Arg → Trp
T243	+	+	−	−	−
T291	+	+	237	G → T	Met → Ile
Exon 8					
T156	−	−	−	−	−
T262	+	+	282	C → T	Arg → Trp
T197	+	−	−	−	−
Exon 9					
T99	+	+	314–319	Deletion 17 bp (CCTCTCCCCAGCCAAAG)	Frameshift
T213	−[c]	+	Mutation in intron[c]		

[a] +, Mutation found; −, mutation not found.
[b] −, Mutation not found, otherwise the mutated codon indicated.
[c] Mutation was outside the efficient screening area for DGGE.

enzyme in 1× Qiagen buffer. In DNA amplification for DGGE, one primer of each primer pair had a 40 bp GC rich extension (GC-clamp) and for CE-SSCP both primers were labeled at 5′-end with different fluorescent dye (6-carboxyfluorescein, 6-FAM, and 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein, HEX). The primer sequences were the same for CE-SSCP and DGGE analysis and are given elsewhere [9]. PCR was performed with a thermocycler (Applied Biosystems) for 35 cycles: denaturation at 94 °C for 30 s, primer annealing at 55–60 °C for 30 s and primer extension at 74 °C for 1 min.

2.3. DGGE

As described earlier [9], 1 mm thick gels containing 7% acrylamide (Bio-Rad) and 0.3% bis-acrylamide (Bio-Rad) in 1× Tris-acetate-EDTA (TAE) buffer were used. Formamide (Merck) and urea (Merck) were used as denaturing agents in different concentrations, 100% denaturant corresponding to 9 M formamide and 7 M urea. The gel polymerization was activated using ammonium persulfate (Bio-Rad) and TEMED *N*,*N*,*N*′,*N*′-tetramethylethylenediamide (Bio-Rad). The samples were run in perpendicular gels in 1× TAE buffer at 60 °C in DCode Universal Mutation Detection System apparatus (Bio-Rad). The electrophoresis time was 4 h using 150 V electrophoresis voltage. The gels were stained with ethidium bromide (Aldrich-Chemie) and photographed by a UV transilluminator (Stratagene Eagle Eye II).

2.4. CE-SSCP

After the PCR-amplification, samples were diluted 1:20, and 1 μl of the diluted sample was mixed with 10.5 μl deionized formamide, 0.5 μl NaOH (0.3 M) and 0.5 μl GeneScan 350 TAMRA size standard (Applied Biosystems). Samples were denatured at 95 °C for 5 min, and put directly on ice. The CE-SSCP analysis was performed using ABI PRISM 310 capillary sequencer (Applied Biosystems) at 30 °C, and with ABI PRISM 3100 Avant capillary sequencer (Applied Biosystems) at five different analysis temperatures (18, 25, 30, 35 and 40 °C). Electrophoresis time was 30 min and electrophoresis field 13 kV. The separation media was 5% GeneScan Polymer (Applied Biosystems) in 1× Tris-Borate-EDTA (TBE) buffer containing 10% glycerol, and the electrophoresis buffer was TBE buffer containing 10% glycerol.

2.5. Direct sequencing

Locations and types of the mutations found in DGGE and/or CE-SSCP analysis were determined by direct sequencing. DNA was amplified by PCR and the products were purified with QIAquick PCR purification Kit (Qiagen). The sequencing reaction was done with BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems), and the sequencing was performed in ABI Prism 310 capillary sequencer (Applied Biosystems).

3. Results

In the present analysis, altogether 17 out of 20 samples of the lung tumor DNA analyzed were found to carry a mutation. For three tumors, the mutation discovered in the previous analyses was not detected by any of the assays used. Since new DNA needed to be extracted from the tissue specimens for the present work, it may be that the newly cut tissue sections did not contain large enough proportions of the mutated cells to appear positive in the analysis. Of the total of 17 mutations, 15 were detected with the DGGE assay, 16 with the CE-SSCP assay, and 12 with direct sequencing. Detailed comparison of the results from DGGE, CE-SSCP, and sequencing is shown in Table 1.

In the CE-SSCP assay, mutations are discovered as shifts in the mobility through the capillary. Fig. 1 illustrates the electropherograms of the wildtype for *TP53* exons 5 and 6, and two different mutations. Different alleles are marked with different colors. The shape of the electropherogram depends on the type of the alteration present in the DNA sequence under study, as well as on the running temperature. Sometimes the mobility shift is observable in one of the

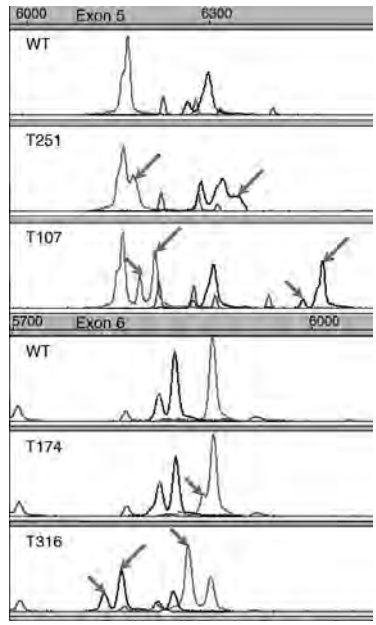

Fig. 1. Examples of CE-SSCP electropherograms of *TP53* exon 5 and exon 6. For both of the exons, the wildtype (WT) and two mutated tumors are shown. The peaks resulting from mutated sequence are marked with arrows.

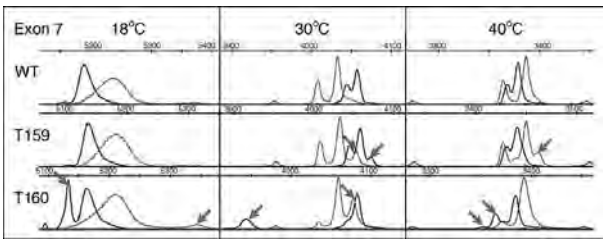

Fig. 2. Examples of temperature changes on CE-SSCP electropherograms. Wildtype sample (WT) and two mutations of *TP53* exon 7 are shown at three different temperatures (18, 30 and 40 °C). The peaks resulting from mutated sequence are marked with arrows. It can be noted that the change for sample # T159 cannot be seen at 18 °C.

strands only (sense or antisense). It is not possible to predict the pattern of an unknown mutation in a CE-SSCP run.

In Fig. 2, the effects of changes in the analysis temperature on the patterns of CE-SSCP electropherograms are demonstrated. Wildtype sequence, and two exon 7 mutations of the *TP53* gene are shown after running them in three different temperatures (18, 30, and 40 °C). In one case (# T159), no change in the electrophoresis pattern indicative of an altered sequence was seen at 18 °C, whereas the mutation was clearly detectable in the two higher temperatures (Fig. 2).

In DGGE, mutations are detected as changes in electrophoresis mobility patterns in acryl amide gel, as shown in Fig. 3. In our study, DGGE detected one mutation less than CE-SSCP, but this mutation resided in intron, outside the area efficiently screened with the DGGE assay

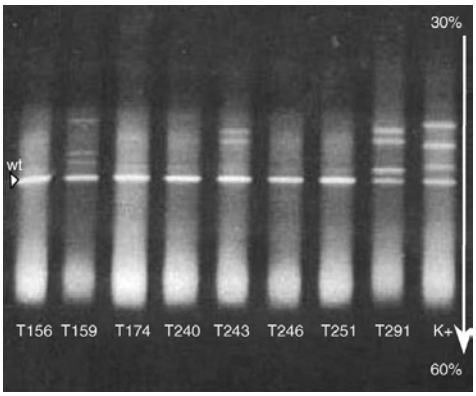

Fig. 3. Example of a DGGE analysis of *TP53* gene exon 7. The denaturant gradient is 30–60% to the direction of the arrow. The wildtype homoduplexes are marked with "wt". C+ stands for positive control, which contains a known mutation of exon 7. The samples # T156, T174, T240, T246, and T251 all contain a wildtype sequence, whereas the samples # T159, T243, and T291 contain mutated sequence. For each mutated sample, the two wildtype−mutamutation heteroduplexes, and the mutation−mutation homoduplex, respectively, appear on the top of the wildtype homoduplex ("wt".).

(sample # T213). Even though the primer sequences were the same for both methods, the mutation situated so near the artificial high melting domain (GC-clamp), that it could not be detected in DGGE. The two methods can be considered equally sensitive (94%). Direct sequencing, with sensitivity of 71%, was clearly less sensitive than the other two methods.

4. Discussion

In the present study, all the mutation detection methods used were able to recognize the majority of the pre-known *TP53* mutations present in the lung tumors examined. CE-SSCP assay had a high efficacy in detecting mutations (16 out of 17, 94%), DGGE assay detected one mutation less than CE-SSCP, but the mutation was situated in the intronic area and was thus not properly within the area efficiently screened with DGGE. Even though the sequences of the primers were the same for both of the methods, the intronic change probably resided too close to the high melting GC-rich area to be detected in DGGE. It can therefore be concluded that, in this study, DGGE and CE-SSCP showed both very high, almost equal sensitivity. Apart from automation, there were some additional differences between the assays that were in favor of CE-SSCP. For example, in DGGE the analysis of exon 5 was more problematic than the other *TP53* exons, because the sequence is rich in GC-pairs [10], causing an unclear melting profile in the assay. In CE-SSCP, exon 5 performed excellently.

There are previous reports indicating that the sensitivity of CE-SSCP can be improved using different analysis temperatures [11,12]. In our study, all mutations were detectable in 30 °C. However, the running temperature clearly affected the electrophoresis patterns, as can be seen in the Fig. 2. As the electropherograms in the figure also point out, not all mutations were necessarily seen in all temperatures (for example, sample # T159), and the change in temperature affected different mutations differently. A pattern change that is almost undetectable in one temperature might be clearly seen in another. Using

different running temperatures routinely in CE-SSCP diminishes the chance that a sequence alteration would be left undetected in the assay.

The efficacy of direct sequencing in detecting mutations was found to be lower than that of either of the two other screening methods. According to previous studies, a DNA sample should contain at least 12–50% of mutated DNA to be detected by automated sequencing, whereas the detection limit for DGGE is 3–6% of the mutated sequence [13]. In an earlier report, the detection limit for CE-SSCP was reported to be close to that of DGGE, with a detection limit of about 5% of mutated sequence [14]. Another application of DGGE, namely DHPLC, was recently considered as sensitive as the standard DGGE, and clearly more sensitive than direct sequencing [15]. Furthermore, it was reported that sequencing was sometimes successful only after the mutated DNA was separated from the wildtype sequence by running the sample first in standard DGGE and then extracting the corresponding homoduplex band from the gel for further analysis [15]. Another investigation using DHPLC [16], noted that diluting a mutated sample with wildtype sample (in dilutions 1:8 and greater) makes the mutations undetectable by direct sequencing. Such an effect was not seen in DHPLC but the mutation remained detectable [16].

In the laboratory, CE-SSCP is technically simpler to carry out than DGGE. The benefits include automated injection of samples, no need for laborious handling of the gels, and generation of raw data that can be more easily further analyzed and documented. In addition, less handling of hazardous chemicals (e.g. polyacryl amide) is needed. Variation between the runs is smaller when using the automated capillary sequencer as compared to standard DGGE where every gel needs to be prepared and cast separately. In the automated CE-SSCP, the samples can easily be re-run several times using e.g. different temperatures, or other analysis conditions that can be changed very quickly. In particular, it is very beneficial that only a tiny amount of DNA is needed for each analysis (e.g. initial PCR reaction with 50 ng of template yields $\geq$50 CE-SSCP runs). This allows repeated analyses as well as saves the template DNA for sequence identification, or new sequences/genes can be investigated from each sample. The consumable (capillaries, size standard etc) and instrument (purchase and maintenance) costs are higher for CE-SSCP than for DGGE, but, on the other hand, CE-SSCP requires a lot less manual work. In addition, CE-SSCP can be run using the same instrument that is used for sequencing, after changing a different polymer and running conditions, opposite to e.g. DHPLC. Optimally however, the sequencer should have a cooling device for maintaining the accurate, pre-determined running temperature during the analysis. The advantages, including the high sensitivity and reproducibility, became evident when we recently applied CE-SSCP for analysis of sequence variations in a newly identified gene [17].

In summary, this study demonstrates that the benefits of CE-SSCP compared to DGGE in detection of unknown mutations are (i) simple procedure for sample preparation, (ii) automation, (iii) fast and easy repeatability, and (iv) good documentation of data facilitating further analysis of the results. In our hands, CE-SSCP was a highly sensitive and reproducible assay for screening of *TP53* mutations in human tumor samples.

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II

COX-2 and p53 in human sinonasal cancer: COX-2 expression is associated with adenocarcinoma histology and wood-dust exposure

by

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SHORT REPORT

COX-2 and p53 in human sinonasal cancer: COX-2 expression is associated with adenocarcinoma histology and wood-dust exposure

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The causal role of wood-dust exposure in sinonasal cancer (SNC) has been established in epidemiological studies, but the mechanisms of SNC carcinogenesis are still largely unknown. Increased amounts of COX-2 are found in both premalignant and malignant tissues, and experimental evidence link COX-2 to development of cancer. Many signals that activate COX-2 also induce tumor suppressor p53, a transcription factor central in cellular stress response. We investigated COX-2 and p53 expressions by immunohistochemistry in 50 SNCs (23 adenocarcinomas, and 27 squamous cell carcinomas (SCC); 48 analyzed for COX-2; 41 for p53). Occupational histories and smoking habits were available for majority of the cases. Most of the adenocarcinoma cases with exposure history data had been exposed to wood dust at work in the past (88%, 14/16). For smokers, 63% (12/19) presented with SSC, whereas 64% (7/11) of nonsmokers displayed adenocarcinoma. COX-2 was expressed at higher levels in adenocarcinoma as compared to SSC ($p < 0.001$). COX-2 expression showed significant association with occupational exposure to wood dust ($p = 0.024$), and with nonsmoking status ($p = 0.001$). No statistically significant associations between the exposures and p53 accumulation were found; however, the p53 accumulation pattern ($p = 0.062$ for wood dust exposure) resembled that of COX-2 expression. In summary, our findings show increased COX-2 expression in SNC adenocarcinoma with wood dust exposure, suggesting a role for inflammatory components in the carcinogenesis process. In contrast, SCCs predominated among smokers and expressed COX-2 rarely; this may suggest at least partially different molecular mechanisms.

Key words: sinonasal cancer; COX-2 expression; p53 expression; wood dust exposure; smoking

Cancer of the nose and paranasal sinuses is a rare form of cancer, with an incidence of 0.5–1.5 new cases per year per 100,000 in men and 0.1–0.6 per 100,000 in women. However, the incidence of this cancer varies markedly from one country to the next, and even between different parts of the same country. This variation has not been attributed to individual susceptibility, such as genetic differences, but rather to differences in exposure.[1,2] Numerous epidemiological studies have established a causal role of exposure to wood dust in the development of sinonasal cancer, with high relative risks, up to 10–30-fold, indicated for the sinonasal adenocarcinoma cell type.[2,3]

Multiple mechanisms of carcinogenesis have been proposed to be involved in the development of SNC, but with very little experimental or human data. Genotoxicity has been attributed to the chemical components of wood as well as to the physicomechanical properties of wood-dust particles, but the data are in general sparse.[2] Also, inflammation has been suggested to play a significant role in SNC carcinogenesis. In addition to experimental studies where wood dust has been shown to elicit an inflammatory response,[4–6] there are epidemiological studies revealing that occupational exposure to dusts from various wood species is associated with asthma.[7] There are also consistent reports of impaired mucociliary clearance and mucosal alterations during chronic exposure.[2] Mucosal alterations include dysplasia and metaplasia of the columnar epithelium, and, to a lesser extent, changes in the squamous epithelium.[2,7]

There is considerable evidence from genetic, pharmacological and clinical studies to link COX-2 to the development of cancer. Increased amounts of COX-2, in comparison to normal tissues, are found commonly in both premalignant and malignant tissues, and inhibitors of COX, such as aspirin or other nonsteroidal anti-inflammatory drugs, have been shown to reduce the incidence of different malignancies.[8–11] Expression of COX-2 is induced by many physiological and stress signals including growth factors, cytokines and other mediators of inflammation, tumor promoters, oxidizing agents, and DNA damaging agents.[12] Many of the signals that activate COX-2 also induce tumor suppressor p53. The p53 is a transcription factor that induces antiproliferative responses such as cell cycle arrest, DNA repair, or apoptosis in response to DNA damage.[12] The p53 pathway is disturbed in practically all main types of human cancer.[13]

The present study was designed to investigate the expression of COX-2 in a series of sinonasal carcinomas. We sought to determine whether the pattern of COX-2 expression showed an association with tumor histology, wood-dust exposure, or smoking. In addition, we investigated p53 expression in the tumor tissue to analyze possible parallel patterns of exposure between COX-2 and p53. The overall goal of the study was to examine the involvement of inflammation in the tumorigenesis process of SNC. To our knowledge, COX-2 expression has not been studied before in sinonasal carcinoma.

Material and methods

Patients and samples

The sample set consisted of 50 sinonasal cancer (ICD-10: C30.0, C31; ICD-9: 160 except 160.1) cases from Finland and France. COX-2 staining was performed for 48 samples and p53 for 41 samples. Archival tumor specimens were collected in collaboration with the Finnish cancer registry and 3 French cancer registries (in the areas of Isère, Somme and Doubs) as part of a

Henrik Wolff and Kirsti Husgafvel-Pursiainen contributed equally to this work

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TABLE I – COX-2 AND p53 EXPRESSIONS (IMMUNOHISTOCHEMISTRY) IN SINONASAL CANCER

Tumor/patient characteristic	COX-2 (−/+)[1]	COX-2 (++/+++)[1]	*p* (Fisher's exact test)	p53 (−/+)[1]	p53 (++/+++)[1]	*p* (Fisher's exact test)
Histology						
Adenocarcinoma	9	13	<0.001	9	10	
Squamous cell carcinoma	24	2		11	11	1.00
Tumor site						
Ethmoid	6	7		4	9	
Nasal Cavity	16	6		9	8	
Maxillary sinus	11	2	0.13	7	4	0.30
Smoking						
Ever	16	2		8	7	1.00
Never	3	8	0.001	5	5	
Wood dust exposure						
Yes	7	10		5	9	
No	12	2	0.02	9	5	0.06

[1] + = weak expression; ++ = moderate expression; +++ = strong expression; − = no expression.

larger multicentre study investigating the biological effects of wood-dust exposure. All SNC cases included were of Caucasian origin, with 19 cases collected in the Finnish study centre (Finnish Institute of Occupational Health, Helsinki) and 31 in the French counterpart (Inserm, St. Maurice). Of the tumors, 13 were situated in ethmoid sinus, 12 in maxillary sinus, 22 in nasal cavity and 3 in an unspecified sinus.

A panel of 3 pathologists (MD, TS, HW) reviewed the histopathological diagnoses of the tumor sections, in order to achieve consistent diagnostic criteria throughout the series. The tumor histologies included in the study were adenocarcinoma ($n = 23$) and squamous cell carcinoma (SCC, $n = 27$) cell types. Adenocarcinomas were further subclassified for intestinal type adenocarcinoma (ITAC) or non-ITAC. ITAC was composed of intestinal-type epithelium with or without mucin production in contrast to the restrictive WHO definition, where only adenocarcinomas with *both* intestinal-type epithelium and mucin production are considered to be ITAC.[14] Of the total of 23 adenocarcinomas studied, 12 were ITAC according to the WHO classification, whereas 22 represented the intestinal subtype according to our classification. Altogether 12 adenocarcinomas were of ethmoidal origin.

Patients or next-of-kins for deceased patients were interviewed to collect information on smoking and occupational exposure to wood dust; for the Finnish cases, this information was supplemented with data from the Finnish Centre for Pensions. Wood dust exposure at work was assessed by an industrial hygienist (PH), with consultations with 2 other industrial hygienists experienced in wood processing industry. There were 17 wood dust-exposed cases and 15 nonexposed; information was unavailable for 18 cases. In all, 19 cases were smokers and 11 nonsmokers, with information missing in 20 cases. The study was approved by the appropriate national Ethical Review Boards in Finland and France.

Immunohistochemistry analysis

COX-2 expression. Sections (4 μm) cut from paraffined tissue blocks on coated slides were incubated overnight at 37°C, deparaffinized and then microwaved for 4 × 5 min in 0.01 mM Na-citrate buffer (pH 6.0). To block endogenous peroxidase activity, the slides were immersed in 1.6% hydrogen peroxide in methanol for 30 min and then in blocking solution for unspecific binding sites (0.5% BSA and 1.5% normal serum in PBS). Immunostaining was performed by a similar protocol as described earlier,[15] with monoclonal IgG against human COX-2 protein peptide (Cayman Chemical Co.) at a dilution of 1:100 overnight at room temperature. After this, the slides were treated with biotinylated secondary antibody (anti-mouse IgG) at a dilution of 1:250 (Vectastain Elite ABC kit PK-6102, Vector Laboratories) in solution of 0.5% BSA in PBS for 30 min. Antibody binding sites were visualized with avidin-biotin peroxidase complex solution (Vectastain Elite kit PK-6102, Vector Laboratories), after treatment for 30 min, and AEC (3-amino-9-ethylcarbazole) liquid for 15 min. Counterstaining was done by Mayer hematoxylin. The COX-2 index was calculated by multiplying the percentage of COX-2 positive cells by the intensity (1–5) of the COX-2 staining. Cases with intensity 1 or COX-2 index less than 40 were considered as negative (−), indexes 41–200 were scored as weak (+), 201–300 as moderate (++) and over 300 as strong (+++).

p53 accumulation. Sections (4 μm) cut from paraffined tissue blocks on coated slides were incubated overnight at 37°C, deparaffinized and then microwaved 4 × 5 min in 0.01 mM Na-citrate buffer (pH 6.0). Immunohistochemistry (IHC) staining was performed using the Ventana Benchmark automated immunostainer (Tucson Medical Systems) following the protocols provided by the manufacturer. The antibody used was DO-7 for wild type p53. The p53 nuclear accumulation was scored as follows: if nuclear staining was seen in only 0–1% of the cells, the case was considered as negative (−), in 2–33% as weak (+), in 33–79% as moderate (++) and over 80% as strong (+++).

COX-2 expression by real-time quantitative PCR

To further confirm the COX-2 expression detected by IHC, we performed a real-time quantitative PCR analysis. RNA was successfully extracted from 10 adenocarcinomas using High Pure RNA Paraffin kit (Roche) according to manufacturer's instructions. The RNA extraction was successful for a subset of adenocarcinomas; the rest of the tissue samples were either too small or possibly too old to yield RNA of acceptable quality. The cDNA synthesis and real-time quantitative PCR (Taqman assay) with an AbiPrism 7700 Sequence Detector System (Applied Biosystems) were performed as described earlier.[16] PCR primers and probes designed by Applied Biosystems and purchased from the company were used. The results were expressed as relative units (RU), which were calculated by the comparative CT method.[16]

Statistical analysis

Statistical analyses were performed using the Stata software (Stata statistical software: Release 9. College Station, TX: Statacorp LP 2005), A score ++ or +++ was considered positive. Fisher's exact test (2-sided) was used to compare proportions of positive cases.

Results

COX-2

Altogether 48 SNC tumors were analyzed for COX-2 expression by immunohistochemistry. A summary of IHC results on COX-2 and p53 is shown in Table I, and representative examples of COX-2 expression in sinonasal cancer are illustrated in Figure 1. We found that expression of COX-2 was statistically significantly associated with the adenocarcinoma histology ($p < 0.001$, Table I; Fig. 2). In SCCs, COX-2 was poorly or not at all expressed, whereas in adenocarcinomas, a strong COX-2 expression was seen. All but one (92%; 12/13) of the adenocarcinoma tumors exhibiting moderate or strong COX-2 expression were

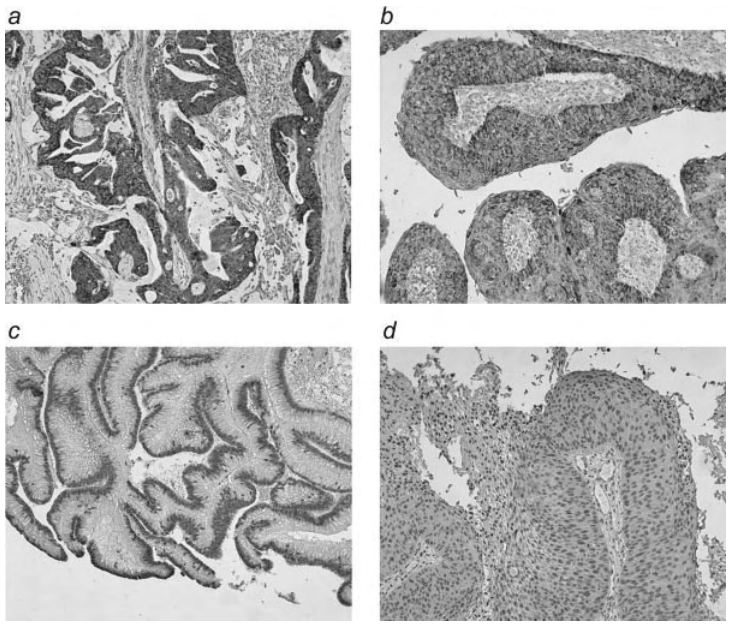

Figure 1 – Representative examples of COX-2 expression in sinonasal cancer: (*a*) strong expression of COX-2 (+++) in ethmoidal adenocarcinoma; (*b*) moderate expression of COX-2 (++) in SCC from nasal septum; (*c*) no expression of COX-2 (−) in ethmoidal adenocarcinoma; (*d*) no expression in SCC from nasal cavity. The original magnification was ×100 in each example (*a–d*).

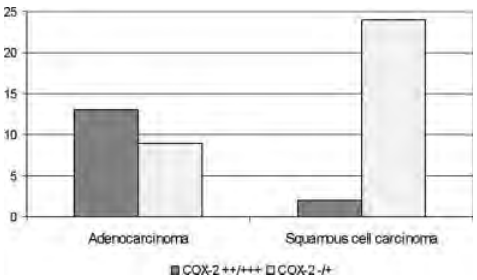

Figure 2 – Distribution of COX-2 expression according to tumor histology in sinonasal cancer. (−/+): weak or no expression of COX-2; (++/+++): moderate or strong expression of COX-2.

ITAC. Interestingly, COX-2 expression was stronger in nonsmokers ($p = 0.001$) and in the wood dust-exposed subjects ($p = 0.02$), as shown in Table I and Figure 3. No association between COX-2 expression and tumor site (Table I) or tumor grade (data not shown) was observed.

COX-2 expression could also be detected at mRNA level from the tumor tissue by real time quantitative PCR analysis. This analysis was performed only on a subset of adenocarcinomas due to limitations with tissue material available. The levels of COX-2 mRNA expression determined by the real time quantitative PCR assay showed variation between the tumors (range 6–384 RU; Fig. 4) and were in parallel with the expression seen in IHC in most cases (Pearson correlation coefficient between IHC index and RU was 0.63).

p53

Overall, about half of the 41 cases that underwent p53 IHC analysis expressed p53 (Table I). No statically significant associations were found; however, some tendencies could be seen. Collectively, the SNC cases with wood-dust exposure in the past more often exhibited strong staining for p53 as compared with the nonexposed ($p = 0.06$; Table I), resembling the staining pattern observed for COX-2 expression. In the adenocarcinomas, all the more strongly positive p53 accumulations (levels ++/+++) were found in nonsmokers ($p = 0.061$) and wood dust-exposed cases ($p = 0.15$). In SCC, on the contrary, smokers tended to show more p53 accumulation (from cases with p53 accumulation levels of ++/+++ and data available for smoking history, 7/7 were smokers). Unlike COX-2, p53 accumulation was not associated with tumor histology (Table I). The association between p53 and COX-2 expressions was close to statistical significance in adenocarcinomas ($p = 0.054$) but not in SCCs.

Exposure and tumor histology

From the 17 SNC cases with work-related wood dust exposure in the past, 82% (14/17) presented with adenocarcinoma (all ITAC) and 18% (3/17) with SCC. The association between the histological tumor type and wood dust exposure was statistically significant ($p < 0.001$). There was a nonsignificant association between smoking and histology; 63% (12/19) of smokers presented with squamous cell carcinoma and 64% (7/11) of the nonsmokers displayed adenocarcinoma tumors. Smoking was not associated with wood-dust exposure. The cancer site and the histological type were also significantly associated ($p < 0.001$; data not

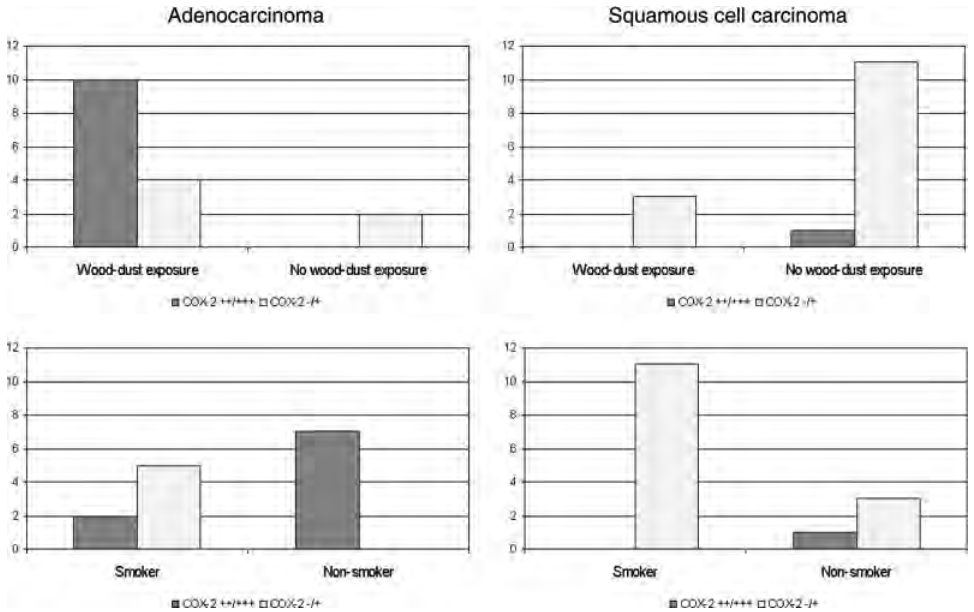

FIGURE 3 – Distribution of COX-2 expression in sinonasal carcinomas by exposure and tumor histology. (−/+): weak or no expression of COX-2; (++/+++): moderate or strong expression of COX-2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

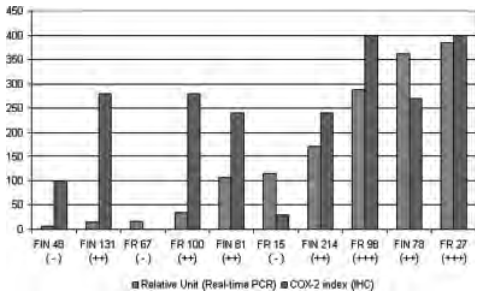

FIGURE 4 – Comparison between COX-2 mRNA expression (real-time quantitative PCR assay) and COX-2 immunohistochemisty (IHC) in sinonasal adenocarcinoma. Real-time quantitative PCR results are expressed as relative units, and IHC results as COX-2 index (percentage of COX-2 positive cells × intensity of COX-2 staining). The +/− sign in parentheses under the tumor codes mark the IHC classification as in Figures 3 and 4. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shown); almost all ethmoidal cancers were adenocarcinomas, whereas almost all cancers of maxillary sinus were SCCs.

Discussion

In the present study, we observed a significantly elevated COX-2 expression in human sinonasal adenocarcinoma (mostly ITAC), as compared to squamous cell carcinoma. Our IHC findings on COX-2 expression were supported by further analysis of COX-2 mRNA levels in a subset of adenocarcinoma tumors. These results are in accordance with increased COX-2 expression reported for other human cancers, such as lung cancer,[11] colon cancer,[17] pancreatic cancer[18] and stomach cancer.[19] Earlier, COX-2 expression has been reported to be related to smoking in oral mucosa,[20] but in our study smoking did not have an effect on COX-2 expression. However, we observed that COX-2 expression in SNC was statistically significantly associated with wood dust exposure.

In the scientific literature, involvement of multifactorial biological pathways in development of wood dust-associated sinonasal cancers has been proposed.[2,7] In line with this, our recent experimental work lends support for a role of wood dust exposure in eliciting inflammatory response. We showed that dusts from various wood species were able to induce the release of proinflammatory mediators in lung macrophages and pulmonary cells *in vitro* and *in vivo*. In addition, our data suggest that generation of oxygen species (ROS) is likely to be involved in the inflammatory process.[4–6] We have further preliminary data indicating that wood dusts are able to induce COX-2 expression in a dose-dependent manner in mouse pulmonary macrophages *in vitro* (Holmila et al., unpublished data).

Interactions between COX-2 and p53 have been shown *in vitro* and *in vivo*. It has been demonstrated that p53 can upregulate COX-2,[12,21,22] or suppress the transcription of COX-2.[9,23] Additionally, COX-2 has been observed to exhibit strong inhibitory effects on p53 transcriptional activity.[21,22] Benoit *et al.*[12] found a correlation between COX-2 expression and *TP53* wild-type status in esophageal adenocarcinoma with Barrett's esophagus as a precursor lesion, but not in SCC, providing evidence that the participation of p53 in the regulation of COX-2 expression in cancer may be dependent on tumor histology. They proposed also, interestingly, that chronic inflammation could represent a physio-path-

ological context in which p53 and the transcription factor NF-kappaB could cooperate to activate COX-2.[12]

In a recent review, de Moraes *et al*.[24] propose that the seemingly contradictory data on the cross talks between COX-2 and p53[5,21–23] can be explained by two different mechanistic scenarios, not mutually exclusive though, where the variable patterns between COX-2 and p53 expressions are dependent largely on the inflammatory context. The authors further hypothesize that both the suggested mechanisms—the one involving cooperation between NF-kappaB and p53, as well as the other one in which COX-2 activation occurs independently from those two regulators, with *TP53* gene mutation coexisting as an additional independent effector—may have synergistic effects in inhibiting apoptosis. They see that such a situation may prevail in many squamous cancers as well as in cancers where *TP53* mutation occurs prior to the development of a lesion as a result of intense exposure to environmental carcinogens, exemplified by lung cancer of smokers, irrespective of histology.[24] Finally, it is pointed out that most likely the activation of COX-2 as in the latter mechanistic alternative may be relevant for most tumors, including those where inflammation stress occurs.[24]

Our present results suggest an association between p53 accumulation and COX-2 expression in sinonasal adenocarcinomas, with no such relation seen in SCCs. It is known that a major cause of p53 accumulation in tumors is inactivating mutation in the *TP53* gene; our observations thus propose that COX-2 expression may arise through mechanisms independent of p53, in at least sinonasal tumors with adenocarcinoma histology. On the basis of the mechanisms envisaged for the interplay between COX-2 and p53,[24] it seems likely that partially different and partially shared conditions and regulatory events of COX-2 and p53 expressions prevail in adenocarcinoma and SCC histologies of sinonasal cancer.

Immunostaining against the tumor suppressor p53 protein indicated that accumulation of p53 is common in sinonasal tumors; about half of the cases had a high level of p53 accumulation. The two histologies did not differ in this respect. In the literature, the data on sinonasal cancer are sparse but there are a few studies that have reported accumulation of p53 in sinonasal cancer.[25,26] In the study by Valente *et al*.,[25] the number of normal epithelial cells showing p53 nuclear accumulation was significantly higher in ethmoidal mucosa of subjects with a long history of exposure to wood dust in comparison with patients not known to have been exposed to wood dust (i.e. those who had undergone an esthetic nasal surgery). Differences between wood dust-exposed and non-exposed adenocarcinoma cases were also seen in p53 expression in normal epithelium and neoplastic cells.[25] We did not find statistically significant associations between p53 accumulation and the two exposures, wood dust and smoking, possibly due to the small number of cases. There was, however, a tendency ($p = 0.06$) for p53 accumulation to occur more frequently in tumors from the wood dust-exposed cases as compared to those from the nonexposed. In SCC, p53 accumulation was particularly seen in the group of smokers. Overall, the frequent p53 accumulation observed in this subgroup of sinonasal cancers is in accordance with preliminary results from our on-going study on TP53 mutations conducted in a larger collection of sinonasal cancers.[27]

Altogether, our present molecular biology findings in sinonasal tumors are well in line with the overall data from epidemiological studies establishing a consistent link between wood-dust exposure and sinonasal cancer.[2,7] The epidemiological data indicate highly elevated risks especially for adenocarcinoma histology, and considerably smaller risks have been found for SCC.[2,7] Our study demonstrated that 88% of the adenocarcinoma cases for which the exposure history was known, had been exposed to wood dust at work. All of the ethmoidal adenocarcinomas included in the study had occurred in wood dust-exposed cases.

Another risk factor reported for SNC is cigarette smoking, with a less pronounced, two- to threefold increased risk of nasal cancer observed among smokers and a reduction in risk among long-term quitters. The association has been suggested to be limited to squamous cell carcinoma rather than adenocarcinoma.[28,29] In our data, almost two-thirds of smokers presented with SCC, whereas a similar proportion of nonsmokers displayed adenocarcinoma tumors; the associations with tumor histology, however, remained statistically nonsignificant. Interestingly, COX-2 expression associated with nonsmoking.

In summary, our study demonstrates that COX-2 is expressed in high level in sinonasal adenocarcinomas, whereas in SCCs, the expression is much lower. Furthermore, COX-2 expression was associated with wood dust exposure, and with nonsmoking status. The accumulation of p53 was found to be common in sinonasal cancer, but no statistically significant associations with p53 and histological type or exposures were found. Our current findings suggest a role for inflammatory components in carcinogenesis of sinonasal cancer. Increased knowledge on molecular cancer mechanisms associated with carcinogenic exposures is highly needed for risk assessment; such data may eventually open prospects for prevention and treatment.

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III

Relationship between *TP53* tumour suppressor gene mutations and smoking-related bulky DNA adducts in a lung cancer study population from Hungary

by

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Relationship between *TP53* tumour suppressor gene mutations and smoking-related bulky DNA adducts in a lung cancer study population from Hungary

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Lung cancer rate in Hungary is one of the highest in the world among men and also very high among women, for reasons not clearly understood yet. The aim of the study was to explore characteristics of DNA damage and *TP53* gene mutations in lung cancer from Hungary. Tissue samples from 104 lung resections for lung cancer patients, both men and women, operated on for non-small cell lung cancer, specifically, primary squamous cell carcinoma or adenocarcinoma were studied. Of the cases, 37% smoked up to the surgery, 24% stopped smoking within 1 year before the surgery, 26% stopped smoking more than a year before the surgery and 13% never smoked. *TP53* mutations were detected by denaturant gradient gel electrophoresis, automated capillary electrophoresis single-strand conformation polymorphism and sequencing. Bulky DNA adduct levels were determined by ^{32}P-post-labelling in non-tumorous lung tissue. In total, 45% (47/104) of the cases carried *TP53* mutation. The prevalence of *TP53* mutations was statistically significantly associated with duration of smoking, tumour histology and gender. Smokers had approximately twice as high bulky adduct level as the combined group of former- and never-smokers (10.9 ± 6.5 versus 5.5 ± 3.4 adducts/10^8 nucleotides). The common base change G → T transversion (8/43; 19%) was detected exclusively in smokers. For the first time, we demonstrate that most carriers of G → T transversions had also a high level of bulky DNA adducts in their non-tumourous lung tissue. Our study provides evidence for a high burden of molecular alterations occurring concurrently in the lung of lung cancer patients.

Introduction

Lung cancer is among the leading causes of cancer death in the developed countries (1). In Hungary, incidence of lung cancer is very high, it is 138/100 000 among men and 73/100 000 among women according to statistics from 2007 (2–4). The primary risk factor for lung cancer is smoking (5). Besides smoking, aetiological factors for lung cancer include exposure to other lung carcinogens, such as asbestos and radon. In Hungary, cigarette consumption is high, with a prevalence of ~30% of regular daily smokers in the population at age of 15+ years (4), but the risks associated with other aetiological factors are not known to be different in Hungary from those that exist in many other European countries (6–8).

The tumour suppressor gene *TP53* is one of the most studied genes in cancer research. The protein product of *TP53* participates in cell cycle control, apoptosis and DNA repair and is central in cellular response to different kinds of stress (9). In non-stress situations, the p53 protein is expressed in almost all tissues at low level and degrades rapidly. DNA damage, activation of oncogenes and also non-genotoxic stresses, e.g. heat and hypoxia, can lead to activation and stabilization of the p53 by post-translational modifications (9).

TP53 gene mutations are common in human tumours and several types of environmental cancer have been shown to contain mutations associated with exposures (10,11). According to the International Agency for Research on Cancer (IARC) database, in average, 39% of lung cancers carry *TP53* mutations (12). In many cancers, the prevalence of *TP53* mutations tend to be higher in smokers than in non-smokers (13–15) and a special mutation spectrum attributed to tobacco smoking has been reported (16).

In the framework of a multi-end point molecular epidemiological study among Hungarian lung cancer patients, we explored molecular DNA alterations showing association with cigarette smoking. We investigated the frequency and the type of *TP53* gene mutations and analysed the results in relation to smoking characteristics and gender in the two major histological types of lung cancer. In parallel, we determined the levels of bulky DNA adducts in non-tumorous tissue specimens from the same patients, to search for association between *TP53* mutations and tobacco smoke-induced primary DNA damage.

Materials and methods

Study population

The sample set consisted of 104 lung cancer cases, 62 males (60%) and 42 females (40%), who underwent lung resection for primary lung cancer. This series of cases with adenocarcinoma (n = 67) and squamous cell carcinoma (SCC; n = 37), the two major histological types of lung cancer, were selected from a larger Hungarian study population comprising various histological types of lung cancer. In the sample set, among men, 55% (34/62) presented with adenocarcinoma and 45% (28/62) with SCC. Among women, 79% (33/42) of the cases had adenocarcinoma. After the selection for histology, there was no additional selection for smoking status. The sample set partially overlaps with the study population of a previously published work (17). The samples of lung tumour and histologically non-tumorous peripheral lung tissue were obtained with informed consent of the patients and the research was approved by the local and national ethics committee in Hungary. Information on smoking history was obtained from the patients by self-reporting. Three major smoking categories were defined as

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follows: (i) smokers, that included current smokers who smoked up to surgery, and those patients who stopped smoking within 1 year before surgery, (ii) former-smokers who gave up smoking >1 year before surgery and (iii) never-smokers. The smoking categories were set up on the basis of previous studies (17,18). Out of the 104 lung cancer patients, 37% (38/104) were current smokers, 24% (25/104) quit smoking within 1 year before the surgery, 26% (27/104) had given up smoking >1 year before the surgery and 13% (14/104) had never smoked. Heavy smoking was common among both men and women. Almost all the ever-smoking men consumed 20 or more cigarettes per day (93%, 51/55), and most of them had smoked longer than for 20 years (85%, 46/54). More than half of the smoking women (66%, 21/32) had similarly smoked at least one pack per day and had a long smoking history (≥20 years of smoking). Women were in majority among the never-smokers (9 out of 14). Demographic characteristics and data on smoking history of the present study population are given in Table I.

DNA isolation

Samples of macroscopically non-tumorous peripheral lung tissue (100–500 mg) and lung tumour tissue (20–500 mg) were obtained from the resected lobes of the patients and frozen within 2 h of dissection. The tissue samples were stored at −80°C prior to DNA isolation. DNA was isolated by a phenol–chloroform–isoamyl alcohol extraction procedure as described previously (17).

TP53 mutation detection

TP53 mutations were analysed in exons 5–9 and 11. The *TP53* gene sequences were amplified from the DNA samples by polymerase chain reaction (PCR). Denaturant gradient gel electrophoresis (DGGE) and automated capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) were applied to screen for *TP53* mutations according to Holmila and Husgafvel-Pursiainen (19). In short, in DGGE analysis, the PCR products were run in perpendicular acrylamide gels containing denaturing gradient in 1× Tris-acetate-EDTA buffer at 60°C (in DCode Universal Mutation Detection System apparatus; Bio-Rad, Hercules, CA) followed by ethidium bromide staining and detection by ultraviolet. For CE-SSCP, the diluted PCR products were denatured at high temperature and then plunged into ice directly. The CE-SSCP analysis was performed using ABI PRISM 310 capillary sequencer [Applied Biosystems or with ABI PRISM 3100 Avant capillary sequencer using a separation media of 5% GeneScan Polymer (Applied Biosystems, Foster City, CA)] in 1× running buffer containing ethylenediaminetetraacetic acid (Applied Biosystems) and 10% glycerol.

The locations and types of the mutations detected by DGGE or CE-SSCP were determined by direct sequencing (ABI Prism 310 capillary sequencer). As reported before (19), DNA was amplified by PCR reaction and the PCR products were purified with QIAquick PCR purification Kit (Qiagen, Hilden, Germany). The sequencing reaction was prepared with BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems). Polymorphisms were ascertained by sequencing the DNA derived from both tumour and non-tumorous tissues. All mutation analyses were performed at the laboratories of the Finnish Institute of Occupational Health (Helsinki, Finland).

Determination of bulky DNA adducts by ^{32}P-post-labelling

Bulky DNA adducts were determined in non-tumorous peripheral lung DNA by the ^{32}P-post-labelling method essentially as described before (17). Briefly, DNA (4 μg) was digested overnight with micrococcal nuclease (Sigma, St Louis, MO) and spleen phosphodiesterase (ICN and MP Biomedicals). Adduct enrichment was made with nuclease P1 (Sigma) digestion of the normal mononucleotides. Radiolabelling occurred with 50 μCi carrier-free [γ-^{32}P]ATP (end-labelling grade; ICN Biomedicals, Inc., Aurora, OH and MP Biomedicals, LLC, Irvine, CA) and 6 U of T4 polynucleotide kinase (USB Corporation, Cleveland, OH and Fermentas, Vilnius, Lithuania). Multidirectional thin-layer chromatography of the radiolabelled DNA digests was performed on 10 × 10 cm poly(ethyleneimine)cellulose sheets (Macherey-Nagel, Düren, Germany) as detailed earlier (17). Radioactivity patterns were detected by Cerenkov counting and electronic autoradiography (InstantImager; Packard Instrument Co., Inc., Meriden, CT). Background radioactivity of the blank area, corrected for the size of the adduct areas was subtracted from the radioactivity of the adduct areas. DNA adduct levels were calculated by normalization for an *in vitro*-modified benzo[*a*]pyrene diol-epoxide-DNA standard (110 adducts/10^8 nucleotides) as described earlier (17). Two to four replicate analyses were performed with each human DNA sample in separate assays.

Statistical analyses

The statistical analyses were performed with GraphPad Prism 4.0 software, using Fisher's exact test and Mann–Whitney *U*-test. Two-sided *P*-values are given.

Results

Tumour histology and smoking habits

The histological type of the tumour showed close association with smoking habits. All SCCs were among ever-smokers. The proportion of SCC against adenocarcinoma rose with the duration of smoking. Among those who had smoked for 35 years or less, adenocarcinoma predominated, but the difference disappeared over a longer duration of smoking. All never-smokers, mostly women, presented with adenocarcinoma (Table I).

Table I. Demographic characteristics and details of smoking for the lung cancer cases studied

Patient feature	Smokers[a] (n = 63), n (%)	Former-smokers[b] (n = 27), n (%)	Never-smokers (n = 14), n (%)	All cases (n = 104), n (%)
Gender (no. of cases)				
Male	37 (59)	20 (74)	5 (36)	62 (60)
Female	26 (41)	7 (26)	9 (64)	42 (40)
Tumour histology (no. of cases)				
Adenocarcinoma	38 (60)	15 (56)	14 (100)	67 (64)
SCC	25 (40)	12 (44)	–	37 (36)
Age at diagnosis (years)				
Age range	35–78	45–79	48–76	35–79
Mean age	56 ± 9	61 ± 3	59 ± 9	58 ± 9
Median age	57	60	58	58
Daily smoking				
4–25 cigarettes/day (no. of cases)	49 (78)	15 (56)	–	
26–60 cigarettes/day (no. of cases)	12 (19)	11 (41)	–	
ND[c]	2 (3)	1 (4)	–	
Mean consumption (cigarettes/day)	23 ± 10	28 ± 15	–	24 ± 12
Median consumption (cigarettes/day)	20	23	–	20
Duration of smoking				
5–20 yrs (no. of cases)	12 (19)	9 (33)	–	
21–35 yrs (no. of cases)	25 (40)	10 (37)	–	
36–60 yrs (no. of cases)	25 (40)	6 (22)	–	
ND[c]	1 (2)	2 (7)	–	
Mean yrs of smoking	33 ± 12	28 ± 9	–	32 ± 11
Median yrs of smoking	33	30	–	30

[a]Smokers included current smokers and those who quit smoking within 1 year before surgery.
[b]Former-smokers were defined as those, who gave up smoking >1 year before the surgery.
[c]ND, not defined.

TP53 mutations versus histology, gender and smoking characteristics

Forty-five percent of the subjects (47/104) carried a *TP53* mutation, and altogether, 51 *TP53* mutations were detected; 44 cases had a single mutation, two cases carried a double mutation and one case three different mutations (complete *TP53* mutation data are presented in the Supplementary Table I, available at *Mutagenesis* Online). The mutation frequency was significantly higher in SCC (70%, 26/37) than in adenocarcinoma (31%, 21/67) ($P = 0.0002$; Table II). About half of the smokers (i.e. those who smoked up to surgery and short-term quitters) carried a mutation, and they tended to carry more mutations (49%, 31/63) than former-smokers (33%, 9/27) ($P = 0.25$; Table II).

Overall, men exhibited statistically significantly higher mutation frequency than women (55 versus 31%), ($P = 0.045$; Table II). When stratified according to histological type and ever-/never-smoking status, there was no statistically significant difference between the genders. However, the difference between ever-smoker males (38%, 11/29) and ever-smoker females (13%, 3/24) was close to borderline significance ($P = 0.059$) in the adenocarcinoma group, whereas in SCC group, the mutation frequency was fairly similar among male and female ever-smokers. Self-reported never-smokers, all with adenocarcinoma, were also found to carry mutations, with an overall frequency of 50% (total 7/14; males 3/5 and females 4/9) (Table II).

The frequency of mutations increased with the duration of smoking. Among those who had smoked up to 20 years, mutations were found in 14.3% (3/21) of the cases, whereas among those who had smoked longer than for 20 years, more than half carried a *TP53* mutation (36/66, 54.5%) ($P = 0.002$) (Figure 1).

Codon distribution and types of TP53 mutations

Sequence identification was successful for 43 of the 51 mutations. In adenocarcinoma, mutations were more distributed along the gene, whereas in SCC, 86% (26/37) of the mutation were found in codons situated in exons 5 and 8. The most commonly mutated *TP53* codon was codon 175 (three mutations), followed by codons 146, 157, 248 and 266, each with two mutations. The codons where the mutations had occurred in never-smokers were different from those mutated in smokers, with the exception of one.

The most common sequence change detected was G → A transition (8/43 plus one intronic G → A transition found in a former smoker, 21%), followed by G → T transversion (8/43, 19%), frameshift mutation (7/43, 16%) and G → C transversion (7/43, 16%) (Figure 2). G → A transitions were more commonly seen among former-smokers (5 out of 10) and never-smokers (2 out of 6) than among smokers (2 out of 27 mutations) (Figure 2A). G → T transversions were found exclusively in smokers, as were the majority of G → C transversions (Figure 2A).

Almost all frameshift mutations were seen in ever-smokers. They were more frequent at a long smoking history with at least 35 years of smoking (4/12, 33%) as compared to fewer years of smoking (2/23, 9%). Among never-smokers, missense mutations dominated.

Mutations versus smoking-related DNA adducts

We analysed smoking-related bulky DNA adducts in non-tumorous lung tissue in relation to *TP53* mutation in the tumour tissue from the same patients.

Smokers, including those who smoked up to surgery and short-term quitters, had approximately twice as high bulky DNA adduct level as the merged group of former-smokers and never-smokers, 10.9 ± 6.5 versus 5.5 ± 3.4 adducts/10^8 nucleotides (mean ± standard deviation). The difference between the combined groups was statistically significant ($P < 0.0001$). Among smokers, bulky DNA adduct levels were the same in mutation carriers and non-carriers. However, among former-smokers and never-smokers, the mutation carriers tended to have higher level of DNA adducts in comparison to those with the wild-type *TP53* gene ($P \geq 0.1$) (Table III).

Furthermore, we investigated the different types of *TP53* mutations that had occurred in the tumour by the levels of bulky DNA adducts measured in the non-tumorous lung tissue. The carriers of a G → T transversion were those found with a high burden of adducts in their lungs (Figure 2B). Five out of eight (62.5%) cases with a G → T transversion exhibited >12 adducts/10^8 nucleotides, in one case, adducts were at the medium level and in only two cases (2/8, 25%) adducts were in the lowest range (<6 adducts/10^8 nucleotides) (Figure 2B).

Discussion

The present study provides evidence for a high burden of smoking-related molecular alterations occurring concurrently in the lungs of lung cancer patients. We observed frequent *TP53* mutations and a simultaneous high level of lung DNA adducts, predominantly detected in G → T mutation carriers, among lung cancer patients who smoked. We investigated a series of 104 lung SCC and adenocarcinoma cases from Hungary, in which both male and female cases were adequately represented (with 60 and 40%, respectively). The majority in

Table II. Lung cancer cases with and without *TP53* gene mutation by gender, tumour histology and smoking status

	Gender		Tumour histology		Smoking		
	Male, *n* (%)	Female, *n* (%)	SCC, *n* (%)	Adenocarcinoma, *n* (%)	Smokers,[a] *n* (%)	Former-smokers,[b] *n* (%)	Never-smokers, *n* (%)
TP53 mutation positive	34 (55)	13 (31)	26 (70)	21 (31)	31 (49)	9 (33)	7 (50)
TP53 mutation negative	28 (45)	29 (69)	11 (30)	46 (69)	32 (51)	18 (67)	7 (50)
Total	62	42	37	67	63	27	14
	$P = 0.045$[c]		$P = 0.0002$[d]		$P =$ NS[e,f]		

[a]Smokers include current smokers and those who quit smoking within 1 year before surgery.
[b]Former-smokers were defined as those, who gave up smoking >1 year before the surgery.
[c]Difference between females and males.
[d]Difference between adenocarcinoma and SCC.
[e]NS, not significant, difference between smokers and former-smokers.
[f]NS, not significant, difference between smokers and never-smokers.

both genders, >90% of men and 66% of women had smoked >20 cigarettes per day on average and had a long history of smoking with a median of 30 years of smoking, reflecting heavy smoking habits of Hungarian smokers (4).

In the current series of lung cancers, 45% of all cases carried a *TP53* mutation; 55% of men and 31% of women were positive for mutation. The overall mutation frequency was relatively high as compared to that detected in many lung cancer populations (20–24); however, some studies have also reported higher frequencies (25–28). We found that smokers (the combined group of those who smoked up to surgery and short-term quitters) representing 59% of the male and 41% of the female cases carried more mutations than the former-smokers. The frequency of mutations increased with the increasing duration of smoking, in line with earlier findings (27,29). A high mutation load in heavy smokers with long duration of smoking is in accordance with the epidemiological evidence of associating heavy and persistent smoking with high risk of lung cancer (30). Among never-smokers, half of cases carried a mutation, a frequency higher than that found in many studies (31) but not exceptional (27).

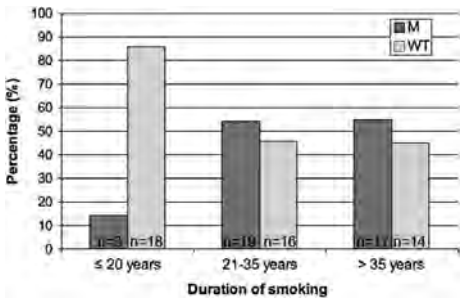

Fig. 1. Occurrence of *TP53* mutation by the duration of smoking. Percentages of *TP53* mutations or wild type are indicated: M, mutated; WT, wild type.

One of the most common base changes detected in the present set of lung tumours was G → T transversion that was found exclusively in smokers. According to the IARC *TP53* mutation database, the mutation spectrum charasteristic to lung cancer contains ∼30% G → T transversions, detectable on many of the frequently mutated codons (12). A similar mutation profile has been found in non-tumorous lung tissue from smoking lung cancer patients (32). This specificity is compatible with the reported presence of polycyclic aromatic hydrocarbon (PAH)-related bulky DNA adducts in lung tissue from smokers (33), as well as with the evidence of association between DNA adducts and types of mutations resulting from exposure to benzo[*a*]pyrene (B*a*P) and other PAH compounds (16,34,35). For the first time, we were able to demonstrate that a majority of the lung cancer cases exhibiting a G → T transversion were, indeed, those with high levels of smoking-related bulky adducts in non-tumorous lung tissue. Among former- and never-smokers, higher levels of bulky DNA adducts were detected in non-tumorous lung tissue from *TP53* mutation carriers as compared to non-carriers ($P = 0.076$). The present findings strongly support the postulation that the specific mutation spectrum of smokers originates from continued exposure to a complex mixture of carcinogenic and mutagenic PAHs (34–37). Other factors, such as selective DNA repair, are likely to participate to some extent in the

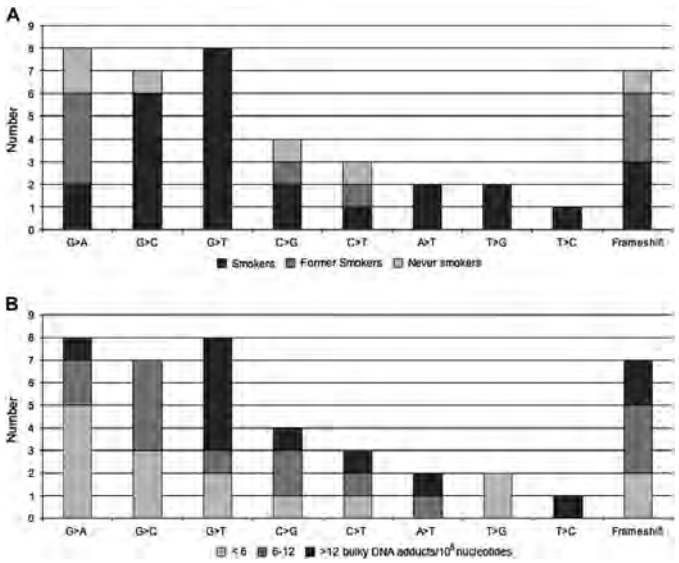

Fig. 2. Distribution of base changes in the exons according to smoking status (**A**) and bulky DNA adducts level as detected from paired non-tumorous lung tissues (**B**). Smokers include current smokers and those who quit smoking within 1 year before surgery.

Table III. Levels of bulky DNA adducts in non-tumorous lung tissue of lung cancer patients with and without *TP53* mutation in the tumour

Smoking status	Bulky DNA adducts/10^8 nucleotides, mean ± SD (*n*)		*P*-value[a]
	TP53 mutation carriers	*TP53* mutation negative cases	
Smoker[b]	10.9 ± 6.4 (31)	10.9 ± 6.7 (32)	>0.1
Former-smoker[c]	7.6 ± 4.2 (9)	5.0 ± 2.7 (18)	0.10
Never-smoker	5.7 ± 3.5 (7)*	4.0 ± 3.1 (7)**	>0.1

[a]Difference between *TP53* mutation positive and mutation negative cases.
[b]Smokers included current smokers and those who quit smoking within 1 year before surgery.
[c]Former-smokers were defined as those, who gave up smoking >1 year before the surgery.
*Difference between *TP53* mutation carrier smokers and never-smokers, $P = 0.065$.
**Difference between *TP53* mutation negative smokers and never-smokers, $P = 0.0008$.

process (34,36–39). It is also likely that other tobacco smoke carcinogens also contribute to the mutation profile (34,40,41).

Besides G → T transversions, also G → A transitions, frameshifts and G → C transversions were commonly found in this study population. G → C transversions were detected in smokers, almost exclusively, whereas G → A transitions were more prevalent among former- and never-smokers. Previous studies have also associated G → A transitions with never smoking (16,22,27). Frameshift mutations were frequently seen in smokers, who had smoked >35 years; none was detected in smokers who had smoked for 20 or fewer years.

The codon distribution of the mutations followed overall the general pattern found in lung cancer (31). The most commonly mutated codon in our study was codon 175, one of the mutation hot spots in human cancer, followed by codons 146, 157, 248 and 266. Codon 157 is frequently mutated in lung cancer associated with smoking and it has been experimentally identified as a site of adduct formation by B*a*P (27,32). Accordingly, we found both of the codon 157 mutations in smokers.

The prevalence of *TP53* mutations in SCC was particularly high (70%) in our study, similar to that observed by Le Calvez *et al*. (27) recently. The mutation frequency reported for SCC in the IARC database is clearly lower [48.7%; (12)] than the present one; this may again reflect the effect of heavy smoking in the present lung cancer series. On the other hand, the mutation frequency detected in adenocarcinoma was very close to that reported in the database (12), with 30.6% in the database Version R13 versus 31% in our series. In our study, adenocarcinomas from male patients carried more mutations than adenocarcinomas from female patients. The overall codon distribution of the mutations followed the pattern reported in database for lung cancer (12).

In summary, we investigated a series of non-small cell lung cancer, SCC or adenocarcinoma histological type in a Hungarian study population. We found statistically significant association between *TP53* mutations and duration of smoking, gender and histology. We explored that smoking-related bulky DNA adducts were at a high level in the non-tumorous lung tissue in those cases who carried a G → T transversion, a signature mutation of tobacco smoking. Collectively, our findings underline the impact of the mutagenic and carcinogenic tobacco smoke components in the lungs of smokers in inducing molecular alterations central to human lung carcinogenesis. Further studies are, however, required to unravel the whole range of factors that are unique for the high lung cancer risk in Hungarian population.

Supplementary data

Supplementary Table 1 is available at *Mutagenesis* Online.

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IV

Mutations in *TP53* tumor suppressor gene in wood dust related sinonasal cancer

by

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Mutations in TP53 tumor suppressor gene in wood dust-related sinonasal cancer

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The causal role of work-related exposure to wood dust in the development of sinonasal cancer has long been established by numerous epidemiologic studies. To study molecular changes in these tumors, we analyzed *TP53* gene mutations in 358 sinonasal cancer cases with or without occupational exposure to wood dust, using capillary electrophoresis single-strand conformation polymorphism analysis and direct sequencing. A significant association between wood-dust exposure and adenocarcinoma histology was observed [adjusted odds ratio (OR) 12.6, 95% confidence interval (CI), 5.0–31.6]. *TP53* mutations occurred in all histologies, with an overall frequency of 77%. *TP53* mutation positive status was most common in adenocarcinoma (OR 2.0, 95% CI, 1.1–3.7; compared with squamous cell carcinoma), and mutation positivity showed an overall, nonsignificant association with wood-dust exposure (OR 1.6, 95% CI, 0.8–3.1). Risk of *TP53* mutation was significantly increased in association with duration (≥24 years, OR 5.1, 95% CI, 1.5–17.1), average level (>2 mg/m^3; OR 3.6, 95% CI, 1.2–10.8) and cumulative level (≥30 mg/m^3 × years; OR 3.5, 95% CI, 1.2–10.7) of wood-dust exposure; adjustment for formaldehyde affected the ORs only slightly. Smoking did not influence the occurrence of *TP53* mutation; however, it was associated with multiple mutations ($p = 0.03$). As far as we are aware, this is the first study to demonstrate a high prevalence of *TP53* mutation-positive cases in a large collection of sinonasal cancers with data on occupational exposure. Our results indicate that mutational mechanisms, in particular *TP53* mutations, are associated with work-related exposure to wood dust in sinonasal cancer.

Cancer of the nose and paranasal sinuses is rare, with an incidence of 0.1–1.7 per year per 100,000 in men and 0.1–0.8 per 100,000 in women; the incidence varies markedly from country to country and even between different geographical regions. This variation has not been attributed to individual susceptibility, such as genetic differences, but rather to differences in exposure at work and in tobacco smoking.[1,2]

Numerous epidemiologic studies have established a causal role of exposure to wood dust in the development of sinonasal cancer.[2] High relative risks, up to 10–45-fold, have consistently been found for the adenocarcinoma cell type, especially the ethmoidal sinus adenocarcinoma, in association with occupational exposure to dusts from hardwood species (*i.e.*, deciduous trees or angiosperms).[2–4] In view of the firm epidemiologic evidence, wood dusts, especially from certain hardwood species, are classified as carcinogenic to humans.[2,5,6] However, it is unknown whether similar increased cancer risks are associated with all hardwood species or with softwood (conifers).[2,3,5,7] In addition, some but

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not all studies have found increased risks for other types of cancer, including lung cancer, in association with wood-dust exposure.[2,8–11]

Exposure to wood dust at work is common. In 2001–2003, it was estimated that about 3.6 million workers were occupationally exposed to inhalable wood dust in the European Union (EU) alone,[12] with over half a million workers probably exposed to levels exceeding 5 mg/m^3.[12] Mixed exposure to dusts from more than one species of wood and to dusts from wooden boards typically occurs in most branches of the wood-processing industries.[2,12]

Wood dust is a complex mixture of compounds. Chemically, it is a mixture of organic and inorganic components, and the composition varies according to the tree species.[2,5] Wood contains a wide variety of biologically active substances, including genotoxic and carcinogenic agents.[2,5] In addition, the capacity of wood dusts to induce DNA damage has been attributed, in part, to the particulate nature of the exposure inducing generation of reactive oxygen species in the cells[2,5,13–16] However, there are few experimental or human data on cellular mechanisms of wood dust-related sinonasal carcinogenesis.[2,3,5]

Little is known about genetic alterations in human sinonasal cancer, published findings being based on a relatively limited number of cases and mostly involving adenocarcinoma tumors; the adenocarcinomas have often been those from an ethmoidal location or classified as of intestinal type. In these studies, high frequencies of DNA copy number changes as detected by comparative genomic hybridization have been detected,[17,18] whereas mutation rates reported for the KRAS gene[19–24] and the *TP53* tumor suppressor gene have been lower.[23,25–27]

This study sought to clarify whether mutational mechanisms are involved in sinonasal carcinogenesis and their relation to wood-dust exposure. We investigated *TP53* mutations in a large series of sinonasal cancer. All tumors were reviewed by a panel of pathologists, resulting in 358 cases of histologically confirmed sinonasal cancers subjected to molecular analysis. Emphasis was also placed on careful collection of data on occupational exposure of the cases.

Material and Methods

Patients and samples

Paraffin-embedded tissue (PET) samples of sinonasal cancer tumors were collected from 3 European countries (Denmark, Finland and France). All incident cases of the cancer of the nose and paranasal sinuses (ICD-7 code 160, except 160.1, corresponding to ICD-10 code C30.0, C31) were identified from the nationwide cancer registries in Denmark for the years 1991–2001, as described earlier,[19] and in Finland for 1989–2002. In Finland, every other case of squamous cell carcinoma (SCC) was randomly included. Informed consent was obtained from the patients who were alive. For the deceased patients, permission was received from the appropriate authorities. The archival formalin-fixed PET samples were collected from pathology laboratories. In France, cases were identified in the regional cancer registries of Isère, Somme and Doubs for the years 1990–2002. The tissue fixation medium used in pathology laboratories was often something other than formalin (Bouin, which makes DNA-based molecular analyses difficult to perform), resulting in a more restricted collection of PET samples

A panel of 3 pathologists (M.D., T.S. and H.W.) reviewed tumor sections for the entire tumor collection (432 cases) without reference to clinical details, exposure background or mutations status. Common criteria for inclusion and exclusion were established as presented earlier.[19] In brief, tumors that corresponded to one of the main histopathologic categories of cancer of the nose and paranasal cavities as indicated in the World Health Organization (WHO) classification of tumors were included.[19,28] Benign tumors, metastatic lesions and malignant tumors outside of these categories were excluded, as were cases that were miscoded in the registries. The latter included, for example, intranasal melanomas, skin cancers, olfactory neuroblastomas and adenoid cystic carcinomas. When appropriate, immunohistochemistry confirmed the tumor type in excluded cases. A common reason for exclusion was location of the tumor in the vestibulum nasi or in the nasopharynx. In addition, some cases were not included in the study because of missing pathology reports, inadequate hospital records, tissue sample, or poor yield of DNA from PET. The final collection for the molecular analysis consisted of 358 SNC cases (170 cases from Denmark, 109 cases from Finland and 79 cases from France), each case with a consensus histology diagnosis of adenocarcinoma, SCC, or carcinoma not otherwise specified (NOS). Demographic and other data for the cases included are shown in Table 1. SCC was the commonest tumor histology (59%, 213 of 358), followed by adenocarcinoma (34%, 122 of 358) and carcinoma NOS (6%, 23 of 358) (Table 1). Of the adenocarcinomas, 88 of 122 (72%) fulfilled the WHO criteria[28] for intestinal type of adenocarcinoma (Fig. 1), and 92% (81 of 88) of this type of tumor were in men.

The study was approved by the appropriate national and institutional ethical review boards in Finland, Denmark and France.

Exposure assessment

Work history. In all study centers, patients or next-of-kin (preferably the last spouse or a child) of the deceased patients were interviewed using a structured questionnaire designed for this study; in Denmark and Finland by telephone; in France by personal interviews. The interviews provided data on demographics, tobacco use and employment histories, including occupational exposure to wood dust, formaldehyde, chromium (VI) compounds, nickel and its organic compounds and textile and leather dust. Interview data were received in 84 (49%) of the Danish cases, 69 (63%) of the Finnish cases and 51 (65%) of the French cases. In Denmark and Finland, the information obtained through the interviews

Table 1. Demographic, clinical and exposure information for the sinonasal cancer cases investigated

Characteristic	All cases, n (%)
Center	
Denmark	170 (47)
Finland	109 (30)
France	79 (22)
Total	358 (100)
Sex	
Male	247 (69)
Female	111 (31)
Total	358 (100)
Histology	
Adenocarcinoma	122 (34)
Male/female	104/18 (29/5)
Squamous cell carcinoma	213 (59)
Male/female	131/82 (37/23)
Carcinoma NOS	23 (6)
Male/female	12/11 (3/3)
Total	358 (100)
Wood-dust exposure	
Exposed	100 (28)
Male/female	98/2 (27/1)
Nonexposed	183 (51)
Male/female	107/76 (30/21)
ND[1]	75 (21)
Male/female	42/33 (12/9)
Total	358 (100)
Smoking	
Smoker	149 (42)
Male/female	119/30 (33/8)
Nonsmoker	52 (15)
Male/female	28/24 (8/7)
ND[1]	157 (44)
Male/Female	100/57 (28/16)
Total	358 (100)
Age at diagnosis (yr), mean ± SD	
All	65.2±12.6
Male	64.4±11.8
Female	66.9±14.0

NOS: not otherwise specified.
[1]ND: no data were available.

on lifelong employment history was supplemented with data from additional sources. First, employer records were retrieved from the Danish National Pension Fund (Danish cases) and the Finnish Centre for Pensions (Finnish cases). These registries record employment histories for all companies; if a person has been employed, the working history and period of employment can be retrieved back to 1964 in Denmark and 1963 in Finland. In Denmark, the commercial classification of each company was obtained from Statistics Denmark, and in Finland, the companies were classified by an industrial hygienist according to an extended version of International Standard Industrial Classification of all economic activities in 1990. Finally, job titles of the subjects were extracted from the Central Person Registry in Denmark (data available since 1968) and from Statistics Finland (data available at 5-year intervals; retrieved for 1970, 1975, 1980, 1985 and 1990). In all, data on wood-dust exposure were available for 79% (Table 1) of the cases, on other occupational exposures, including formaldehyde, chromium (VI) compounds, nickel and its organic compounds and textile and leather dust (with overlap in exposures), for 59% (210 of 238) of the cases, and on smoking for 56% (Table 1) of the cases.

Exposure assessment. For each case, exposure to wood dust and to other risk factors was assessed based on the entire work history by an experienced industrial hygienist in each study center. Criteria and procedure for exposure assessment were harmonized between the study centers in a common meeting where each rater assessed exposures of 5 cases to minimize variation of exposure estimates between the assessors and discuss differences in exposure characteristics between the countries. Wood-dust exposure estimates were subdivided by wood species and products used (softwood, hardwood and wooden boards). The industrial hygienists involved in the exposure assessment were blinded to the results of the mutation analysis.

In Finland and France, for each job held, the concentration of exposure to wood dust was assessed by the industrial hygienists into 6 categories: unexposed, very low (<0.3 mg/m^3), low (0.3–<1 mg/m^3), medium 1 (1–<2 mg/m^3), medium 2 (2–<5) or high (≥ 5 mg/m^3). Quantitative values (midpoints of the intervals) were assigned to each category of concentration. The frequency of exposure (proportion of working time during which exposure occurred) was assessed separately. A level of exposure was then calculated for each job by multiplying concentration by frequency. The classification of exposure levels was based on measurement data available in Finland and France[29,30] and on exposure estimates generated for the EU member states in another arm (WOOD-RISK) of the larger study program (WOOD-RISK) to which this study also belongs.[12] In Denmark, exposure levels were estimated from the Danish exposure estimates in WOODEX,[31] mainly based on measurements of inhalable wood dust concentrations measured in different Danish and Finnish woodprocessing industries in the 1990s. A 6% yearly decline as compared with the level in 1990 was assumed for the years 1975–1990, and exposures were assumed constant before the year 1975. This assumption was based on a large number of random measurements in the Danish wood

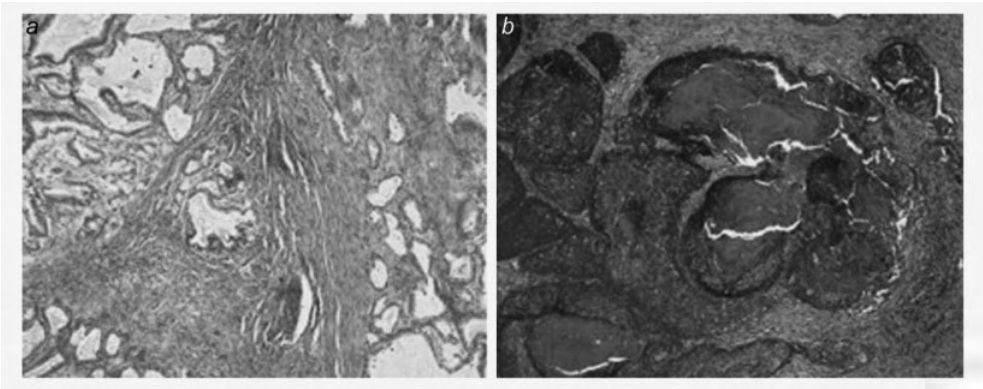

Figure 1. (*a*) Intestinal-type adenocarcinoma of nasal cavity, origin ethmoidal (case #SNCFIN176), found not to carry a TP53 mutation. (*b*) Squamous cell carcinoma of ethmoidal sinus (case #SNCFIN37), positive for mutation. Haematoxylin–eosin staining (original magnification, 5×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

industry from 1985 to 2003.[32,33] They showed quite consistently a 6–7% yearly decline. A 7% yearly decline has been reported from 1978 to 1997 for the US wood industry as well.[34] Exposure level estimates were also adjusted based on the average working distance from the exposure source.

Molecular analysis of TP53 mutations

Tissue samples, DNA extraction and PCR. For each eligible case, 10–25 tissue sections of 5–20 μm were cut from the PET blocks approved for DNA extraction. DNA was extracted using a standard phenol–chloroform extraction protocol. Extracted DNA was stored in 100 μm/ml concentration in aliquots at −20°C. For PCR amplification of the gene sequences under study, 100 ng of genomic DNA was used in a 25-μl reaction, with the reaction performed according to the instructions of the manufacturer of the enzyme (TaqGold, Applied Biosystems, CA)

Capillary electrophoresis single-strand conformation polymorphism. For the *TP53* tumor suppressor gene mutation detection, DNA samples were amplified by PCR, exon by exon, using primers labeled with fluorescent dyes (6-carboxyfluorescein, 6-FAM, for the forward primer and 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein, HEX, for the reverse primer). The capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) analysis was performed as described earlier.[35] In brief, after the PCR amplification, the samples were diluted 1:20, and 1 μl of the diluted sample was mixed with 10.5 μl deionized formamide, 0.5 μl NaOH (0.3 M) and 0.5 μl GeneScan 350 TAMRA size standard (Applied Biosystems, CA). The samples were denatured at 95°C for 5 min and put directly in ice. The CE-SSCP analysis of *TP53* exons 5–9 was performed in ABI PRISM 310 capillary sequencer or in ABI PRISM 3100-avant capillary sequencer (Applied Biosystems) at 30°C. In some cases with unclear results, additional running temperatures were used. Mutations were detected as mobility shifts and/or changes in the electrophoregrams. The CE-SSCP analysis of *TP53* gene could not be successfully performed for all of the exons in 9 cases.

Direct sequencing. Mutations found in CE-SSCP analysis were identified, and the location and type of the sequence change were determined by direct sequencing. In brief, DNA was first amplified by PCR, and the PCR products were purified with QIAquick PCR purification Kit (Qiagen Nordic, Sweden) or by using ExoSAP-IT (USB, OH). The sequencing reaction was prepared with BigDye Terminator v3.0 Cycle Sequencing ready Reaction kit (Applied Biosystems), and sequencing was performed with an ABI Prism 310 capillary sequencer (Applied Biosystems) or an ABI Prism 3100-avant capillary sequencer (Applied Biosystems). Mutations that could not be identified by direct sequencing because of the quality of DNA from PET samples were confirmed by at least one, but in most cases several independent CE-SSCP runs from a new PCR reaction. In all, there were 136 cases positive for at least one CE-SSCP-detected *TP53* mutation identified by sequencing, and for 141 cases, the presence of 1 or more mutations was detected and confirmed using CE-SSCP.

Statistical analysis

Several exposure variables were used to summarize the lifetime exposure to wood dust: ever exposed *versus* never exposed, total duration of exposure, cumulative level of exposure (calculated by summing over the total work history the job-specific products of level and duration) and average level of exposure (calculated as the cumulative level divided by total duration).

Table 2. Main histologic types of sinonasal carcinoma subdivided according to wood-dust exposure and smoking, adjusted for age, sex and study center

	Wood-dust exposure[1]					Smoking[2]				
Tumor histology	Yes, n (%)	No, n (%)	Total, n (%)	OR	95% CI	Yes, n (%)	No, n (%)	Total, n (%)	OR	95% CI
Squamous cell carcinoma	33 (20)	134 (80)	167 (100)	1	Reference	87 (78)	24 (22)	111 (100)	1	Reference
Adenocarcinoma	65 (66)	34 (34)	99 (100)	8.8	4.2–18.3	57 (74)	20 (26)	77 (100)	0.5	0.2–1.1
Carcinoma NOS	2 (12)	15 (88)	17 (100)	0.5	0.1–2.7	5 (38)	8 (62)	13 (100)	0.2	0.1–0.9
Total	100	183	283			149	52	201		

OR: odds ratio; CI: confidence interval; NOS: not otherwise specified.
[1]From the total study population, no data were available on occupational wood-dust exposure for 46 squamous cell carcinomas, 23 adenocarcinomas and 6 carcinoma NOS. [2]No data available on tobacco smoking for 102 squamous cell carcinomas, 45 adenocarcinomas and 10 carcinoma NOS.

The average daily consumption of cigarettes was calculated by dividing the total lifetime number of cigarettes by the total duration of smoking. Cumulative tobacco consumption was expressed as pack-years (pack-years = total duration × average number of packs smoked per day; 1 pack = 20 cigarettes).

Odds ratios (ORs) for *TP53* mutations and the corresponding 95% confidence intervals (CI) were calculated by unconditional logistic regression. All ORs were adjusted for age, sex and country. Quantitative variables related to wood-dust exposure and smoking were categorized in 3 classes according to the distribution among all exposed subjects (unexposed, <50th percentile and ≥50th percentile). Other occupational exposures were coded in 2 categories (unexposed and exposed). Comparisons of proportions were performed using χ^2 or the Fisher's exact tests. All analyses were performed using the STATA statistical package (Stata statistical software: Release 9. College Station, TX: Statacorp LP 2005)

Results

Tumor histology and exposure

From the cases included in the study, a history of occupational exposure to wood dust was documented for 100 cases (28%); only 2 of those were women (Table 1). We observed a strong, statistically significant association between wood-dust exposure and tumor histology. Adenocarcinoma was the prevailing histology among the wood dust-exposed cases (65 of 100, 65%; Table 2; Fig. 2*a*). In contrast, those not exposed to wood dust exhibited mainly SCC (134 of 183, 73%; Table 2; Fig. 2*a*). After adjustment for age, sex and study center, exposure to wood dust was associated with an 8-fold increased risk of adenocarcinoma compared with SCC (Table 2). Most cases with a histopathologic diagnosis of intestinal type adenocarcinoma were encountered in individuals who had been exposed to wood dust (64%, 25 of 39).

With regard to the types of wood dust to which the cases were exposed, those who had mostly (>50% of wood-dust exposure) been exposed to hardwood dust exhibited almost exclusively adenocarcinoma (19 of 20; 95%), whereas those mainly exposed to softwood dust also presented with SCC (23 of 43; 53%; Fig. 2*b*; $p < 0.001$).

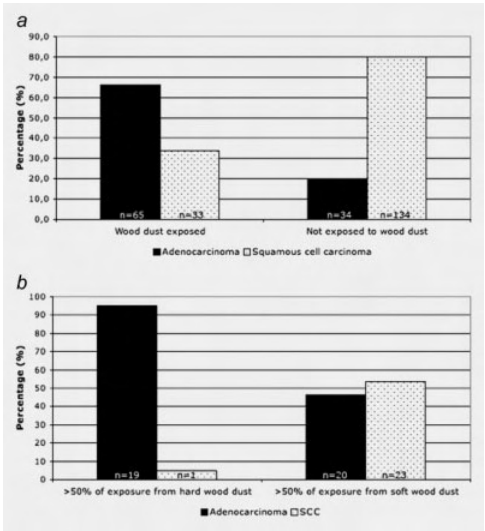

Figure 2. Distribution of tumor histology by wood-dust exposure. (*a*) Adenocarcinoma *versus* squamous cell carcinoma histology in sinonasal cancer cases with history of wood-dust exposure and those without wood-dust exposure. (*b*) Tumor histology subdivided according to the main type of wood-dust exposure (hardwood *versus* softwood).

The study included 201 patients with interview data available on smoking habits (149 smokers and 52 nonsmokers; Table 1). The majority of patients with SCC and adenocarcinoma were smokers (Table 2). The proportion of smokers among SCCs (78%) was close to that observed in adenocarcinomas (74%). When adjusting for age, sex and study center, smoking was associated with a nonsignificantly decreased risk of having an adenocarcinoma (Table 2). None of the 15 wood-dust-exposed patients who were nonsmokers presented with SCC (13 adenocarcinomas and 2 carcinoma NOS).

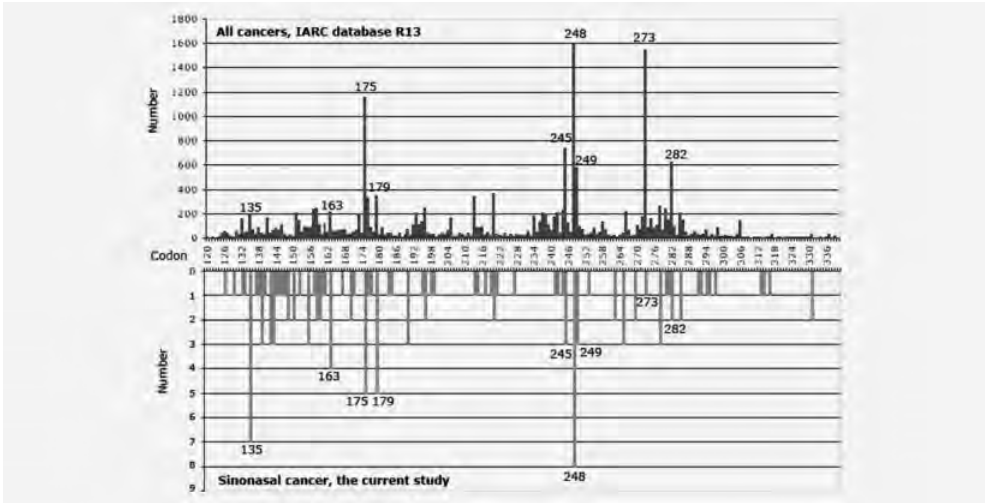

Figure 3. The codon distribution (base changes) of *TP53* mutations in human cancer in the IARC database[54] compared with the distribution detected in sinonasal cancer in this study. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Additional analyses were performed on the subgroup of subjects with data available on both smoking and wood-dust exposure ($n = 200$). The association between wood-dust exposure and histology remained significant when further adjusting for smoking (OR for adenocarcinoma: 12.6, 95% CI, 5.0–31.6). After additional adjustment for wood-dust exposure, smoking was associated with a significantly decreased risk of having an adenocarcinoma (OR= 0.4, 95% CI, 0.2–0.96).

Finally, there were 59 cases of sinonasal cancer with a history of occupational exposure to formaldehyde (22 SCCs, 36 adenocarcinomas and 1 carcinoma NOS). Of these, 19 had been exposed to formaldehyde but not to wood dust (15 SCCs, 3 adenocarcinomas and 1 carcinoma NOS).

TP53 gene mutations: association to exposure and tumor histology

Mutations of the *TP53* gene were common, with an overall frequency of 77% (277 mutation-positive cases of 358 cases). The mutations were scattered over the whole coding region of the gene, and overall, the codon distribution followed the general distribution of *TP53* mutations in human cancer (Fig. 3). Adenocarcinoma patients carried a *TP53* mutation significantly more frequently than those presenting with either of the 2 other histologic types (Table 3). After adjusting for smoking and wood-dust exposure, a 3-fold increased OR for *TP53* mutation remained for adenocarcinoma histology, as compared with SCC (OR 3.4, 95% CI, 1.4–8.5). No difference in the *TP53* mutation frequency was found between intestinal type of adenocarcinoma (86%, 76 of 88) and adenocarcinoma of nonintestinal type (85%, 29 of 34).

The OR for *TP53* mutation among the subjects ever exposed to wood dust was increased, although statistically nonsignificantly, compared with those never exposed (OR 1.6, 95% CI, 0.8–3.1; Table 3). A statistically significant 5-fold increased OR was associated with duration of wood-dust exposure of $\geq$24 years (OR 5.1, 95% CI, 1.5–17.1; Table 3). Similarly, the estimated average level of wood-dust exposure above 2 mg/m^3, and the cumulative level of exposure $\geq$30 mg/m^3 $\times$ years both exhibited an association to a significantly increased risk of *TP53* mutation (OR 3.6, 95% CI 1.2–10.8, and OR 3.5, 95% CI, 1.2–10.7, respectively; Table 3).

Smoking habits were not found to be associated with the overall occurrence of a *TP53* mutation, *i.e.*, the OR was not changed according to any smoking characteristic (duration of smoking, average number of cigarettes or pack-years; Table 3). Consequently, further adjustment for smoking only marginally affected the OR associated with wood-dust exposure, and adjustment for wood-dust exposure did not substantially modify the OR associated with smoking habits (data not shown).

Information on exposure for both wood dust and formaldehyde was available for 210 cases. Forty-nine percent (40 of 81) of the wood-dust-exposed cases were also exposed to formaldehyde, but the adjustment for formaldehyde exposure affected only slightly the OR for *TP53* mutation. *TP53* mutation was found among 86% (51 of 59) of the cases exposed to formaldehyde (*versus* 103 of 151, 68%, of those nonexposed to formaldehyde), resulting in an OR of 2.6 (95% CI,

Table 3. *TP53* mutations in sinonasal cancers by wood-dust exposure, smoking and histology, adjusted for age, sex and study center

	Mutated TP53, n (%)	Wild-type TP53, n (%)	OR	95% CI
Wood dust				
Nonexposed	133 (73)	50 (27)	1	Ref
Exposed	81 (81)	19 (19)	1.6	0.8–3.1
Duration of exposure (yr)				
<24	33 (70)	14 (30)	1.0	0.5–2.1
≥24	42 (93)	3 (7)	5.1	1.5–17.1
Average level of exposure (mg/m^3)				
≤2.0	33 (72)	13 (28)	1.1	0.5–2.5
>2.0	42 (91)	4 (9)	3.6	1.2–10.8
Cumulative level of exposure (mg/m^3 × yr)				
<30	34 (72)	13 (28)	1.1	0.5–2.5
≥30	41 (91)	4 (9)	3.5	1.2–10.7
Smoking[1]				
Never smoker	39 (75)	13 (25)	1	Ref
Ever smoker	112 (75)	37 (25)	0.7	0.3–1.6
Duration of smoking (yr)				
≤35	39 (74)	18 (26)	0.7	0.3–1.8
>35	48 (72)	19 (28)	0.5	0.2–1.3
Average consumption (nb cig/d)				
<20	46 (71)	19 (29)	0.6	0.2–1.4
≥20	49 (77)	15 (23)	0.8	0.3–2.1
Pack-years				
≤29.6	46 (71)	19 (29)	0.6	0.2–1.4
>29.6	49 (77)	15 (23)	0.8	0.3–2.0
Histology				
Squamous cell carcinoma	156 (73)	57 (27)	1	Ref
Adenocarcinoma	105 (86)	17 (14)	2.0	1.1–3.7
Carcinoma NOS	16 (70)	7 (30)	0.9	0.3–2.5

OR: odds ratio; CI: confidence interval; NOS: not otherwise specified.
[1]Never smokers were lifelong nonsmokers; ever smokers consisted of current smokers and former smokers.

1.0–7.0), when adjusting for age, sex and study center. However, this OR decreased notably after additional adjustment for the duration (1.8; 95% CI, 0.6–4.9), average level (1.7; 95% CI, 0.6–4.4) and cumulative level (1.7 95%; 95% CI, 0.6–4.8) of wood-dust exposure. Finally, 81% (29 of 36) of the cases with exposure in the past to 1 or several other risk factors (chromium, nickel, leather or textile dust) carried a mutation. However, the occurrence of *TP53* mutation was not significantly associated with any of these exposures (data not shown).

We further analyzed the 2 main histologic types separately. In adenocarcinomas, 89% (58 of 65) of the wood-dust-exposed cases were positive for *TP53* mutation, and 95% (35 of 37) of the cases with a long duration of wood-dust exposure carried a *TP53* mutation as compared with 82% (28 of 34) of the nonexposed cases. For SCCs, no significant difference between exposed (67%, 22 of 33) and nonexposed (72%, 96 of 134) was seen. A higher frequency of mutations was found in cases with a long history of wood-dust exposure (88%, 7 of 8). In both histologic types, after adjustment for age, sex and country, duration of wood-dust exposure of 24 years or more was associated with an increased, but statistically nonsignificant, OR (OR 2.1, 95% CI, 0.3–14.2 for adenocarcinoma; OR 3.5, 95% CI, 0.4–30.8 for SCC). Additional adjustment for formaldehyde exposure did not change these results. In comparison, after adjustment for the duration of wood-dust exposure, the OR for *TP53* mutation associated with formaldehyde exposure was 0.6 (95% CI, 0.1–5.5) for adenocarcinoma and 1.7 (95% CI, 0.5–5.4) for SCC.

Finally, 51% (131 of 268) of the sinonasal cancer cases positive for *TP53* mutation possessed 1 or more concurrent mutations that were situated in different exons. Among

Table 4. Distribution of single and multiple mutations by wood-dust exposure and smoking, adjusted for age, sex and study center

Exposure status	One exon mutated, n (%)	Multiple exons mutated, n (%)	OR	95 % CI	p
Wood dust					
Nonexposed	45 (53)	40 (47)	1	Ref	
Exposed	50 (63)	29 (37)	0.5	0.3–1.1	0.071
Smoking					
Nonsmokers	26 (70)	11 (30)	1	Ref	
Smokers	54 (50)	55 (50)	2.7	1.1–6.5	0.029

OR: odds ratio; CI: confidence interval.

mutation-positive cases, after adjustment for age, sex and country, the proportion of cases with >1 mutated exon was significantly higher in smokers than nonsmokers ($p = 0.03$, Table 4); this was pronounced in both wood-dust-exposed and nonexposed groups (Fig. 4). There were also more cases with multiple mutations among the subjects never exposed to wood dust compared with wood-dust-exposed subjects ($p = 0.07$, Table 4). In particular, the cases with multiple *TP53* mutations predominated among non–wood dust-exposed smokers (Fig. 4) in both adenocarcinomas and SCCs.

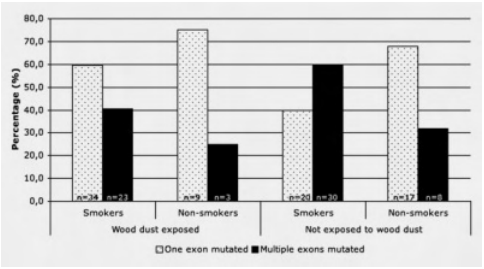

Figure 4. Distribution of cases with single or multiple exon mutations in the different exposure groups.

Discussion

In this study, a high overall frequency (77%) of *TP53* gene mutation-positive cases was discovered in a series of 358 systematically collected and histologically confirmed cases of sinonasal cancer. *TP53* mutations were observed in all tumor cell types, and the distribution of mutations with sequence information in the coding region of *TP53* gene followed the general distribution reported in human cancer. Importantly, our findings indicate that *TP53* mutations occurred most frequently in sinonasal adenocarcinoma, and the occurrence of mutations was associated with exposure to wood dust.

Epidemiologic evidence indisputably has demonstrated large excess risks of sinonasal adenocarcinoma among wood-dust-exposed workers, particularly for those with occupational exposure to hardwood dust and whose tumor originates in the ethmoid sinus and shows intestinal-type histology.[2–4,36] We found in this study a >8-fold increased occurrence of adenocarcinoma compared with SCC among the wood-dust-exposed cases; this association remained after adjustment for smoking. The present sinonasal cancer cases with wood-dust exposure had mostly been exposed to both hardwood and softwood dusts originating from different tree species; in fact, exposure to hardwood dust only or to dusts from individual tree species was rare. Nonetheless, the histologic type found almost exclusively among cases exposed mainly to hardwood dust was adenocarcinoma. The cases mostly exposed to softwood dust exhibited SCC more often than adenocarcinoma.

Our current data suggest an increased overall occurrence of *TP53* mutations in association to wood dust. Furthermore, the occurrence of *TP53* mutation was increased over 5- to 3-fold in association to a long duration of wood-dust exposure of ≥24 years, to an estimated average level of exposure above 2 mg/m^3, and to cumulative exposure at or above 30 mg/m^3 × years, respectively. The association between mutation and a long duration of wood-dust exposure was seen in both histologic types, when analyzed separately and adjusted for age, sex, country and formaldehyde, even though the increased OR for *TP53* mutation remained without statistical significance. These results are in concordance with epidemiologic data demonstrating an association between duration and level of wood-dust exposure and increased risk of sinonasal adenocarcinoma.[2,3]

The previous studies on *TP53* mutations in sinonasal adenocarcinoma investigated small numbers of cases, reported variable occurrence of *TP53* mutations (18–60%), and had less detailed information on exposure.[23,26,27] In addition to mutations, p53 overexpression has been studied; the results obtained indicate that p53 accumulation is a common feature in adenocarcinoma, with immunopositivity ranging between 20 and 80%.[24,27,37] Overexpression of p53 in epithelial cells of ethmoidal mucosa was studied in biopsy samples from 60 woodworkers with a minimum of 10 years of occupational exposure to wood dust and no malignancy, in comparison to 50 nonexposed controls who underwent functional or esthetic nasal surgery, as well as in 15 cases of intestinal type adenocarcinoma.[38] The percentage of cells positive for p53 was significantly higher in wood-dust-exposed subjects in metaplastic epithelium (28.6 *versus* 7.9%; $p < 0.001$), in ciliated epithelium (11.7 *versus* 2.1%; $p < 0.001$) and in seromucous

glands of the nasal stroma (12.5 *versus* 1.0%; $p < 0.001$).[38] Overexpression of p53 was observed in neoplastic cells as well as in the adjacent normal ciliated epithelium in patients with intestinal type adenocarcinoma and long occupational history of wood-dust exposure compared with cases without such exposure.[38] In line with these data, we found the highest frequency of mutations in the wood-dust-exposed adenocarcinoma cases (89%), with the proportion of cases carrying a *TP53* mutation being the greatest among those with a long duration of exposure.

Of the present adenocarcinomas, the pathology panel classified 72% as representing the intestinal type.[39] Our current results indicate that *TP53* gene was commonly mutated in these tumors, with no difference to adenocarcinomas of nonintestinal type. In an earlier work, we investigated mutations of the *KRAS* gene in the cases diagnosed in Denmark and found that *KRAS* was rarely mutated but the mutations mostly occurred in adenocarcinoma (7 cases of 58 adenocarcinomas, 12%), with 2 intestinal type adenocarcinomas exhibiting a *KRAS* mutation.[19]

SCC is another prevalent histology of sinonasal cancer suggested to have at least partially different etiology.[2] There are not many studies concerning *TP53* mutations in sinonasal SCC. A study investigating a series of 70 SCC tumors of the maxillary sinus reported that 29% of the cases were positive for *TP53* mutation and 56% displayed p53 accumulation.[25] In the present study, the frequency of *TP53* mutations in SCCs was found to be higher (74%). In general, SCCs of the head and neck have been reported to exhibit relatively high rates of *TP53* mutation, in agreement with our current finding.[40,41] Nevertheless, the current adenocarcinoma cases had a statistically significant, 2-fold increased occurrence for *TP53* mutation in comparison to SCC.

In addition to wood dust, cigarette smoking is a risk factor for sinonasal cancer, with a 2- to 3-fold increased risk of nasal cancer observed among smokers and a reduction in risk among long-term quitters.[42,43] This effect may, however, be limited to SCC, as opposed to adenocarcinoma.[42,43] In this study, SCC seemed to be more common in smokers, even though this association was not statistically significant. It is noteworthy that smoking was not associated with the overall risk of having a *TP53* mutation, but smokers were found to exhibit more frequently multiple mutations than nonsmokers. Concurrent mutations of the *TP53* gene were found in about half of the mutated cases and in both histologic types. These did not show association with wood-dust exposure. Interestingly, multiple mutations have been reported earlier in SCC of the head and neck,[44,45] for which smoking and alcohol consumption are known to be the most important risk factors.[40] We discuss the mutation profile detected in sinonasal cancer in detail elsewhere.[46]

Approximately half of the wood-dust exposed cases had at least some history of formaldehyde exposure. Formaldehyde is a known human carcinogen and a cause of nasopharyngeal cancer[47,48]; in addition, at high exposure levels, it has been shown to induce SCC in nasal cavity in rats, although not in mice or hamsters.[2] However, in humans, no substantial risk of sinonasal cancer has been observed in association with formaldehyde exposure.[47,48] In our current data, adjustment for formaldehyde affected the risk estimate for *TP53* mutation associated with wood-dust exposure only slightly. Furthermore, even if the cases with exposure to formaldehyde presented mainly with adenocarcinomas, the cases exposed only to formaldehyde and not to wood dust predominantly developed SCC. Overall, SCC was the prevailing histology in cases not exposed to wood dust. These observations lend support to the hypothesis that there may be differences between wood-dust-related carcinogenesis and carcinogenesis related to the other risk factors of sinonasal cancer.

Deeper insight into the molecular mechanisms involved in sinonasal cancer would be highly valuable for the assessment of cancer risk associated with exposure to wood dusts.[2,5,6] The *TP53* gene encodes a transcription factor that is known to induce DNA repair or apoptosis in response to cellular DNA damage.[49] Thus, it is a highly plausible molecular target in sinonasal epithelial cells in association with regular, long-term exposure to a complex substance with carcinogenic potential, such as wood dust. Our current findings on wood dust-related sinonasal cancers resemble the highly frequent occurrence of *TP53* mutations in other types of human cancers linked with carcinogen exposures.[50,51] Another cellular mechanism implicated in the carcinogenesis of sinonasal cancer is inflammation.[2,52] In our recent study on a subset of the current sinonasal cancers, cyclooxygenase-2 protein, an enzyme associated with inflammation and DNA damage, and known to interact with p53, was shown to be highly expressed in adenocarcinomas in association to wood-dust exposure.[53]

In summary, our current molecular epidemiology study demonstrates a high load of *TP53* mutations in human sinonasal cancer, with the occurrence of *TP53* mutation significantly increased in association to long duration and high level of exposure to wood dust. Smoking was not observed to influence the overall risk of having a *TP53* mutation, but was associated with multiple *TP53* mutations. Our present results, together with earlier data, suggest that mutational mechanisms, in particular mutations of the tumor suppressor gene *TP53*, are important in sinonasal carcinogenesis. In addition, the carcinogenesis of wood dust-related sinonasal adenocarcinoma probably involves inflammatory mechanisms that may, in turn, contribute to the overall mutational load.

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by

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Profile of *TP53* gene mutations in sinonasal cancer☆

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ABSTRACT

Genetic alterations underlying the development of the cancer of the nose and paranasal sinuses (sinonasal cancer, SNC), a rare cancer that can be included in the group of head and neck cancers, are still largely unknown. We recently reported that *TP53* mutations are a common feature of SNC, with an overall frequency of 77%, and they show association to adenocarcinoma and wood-dust exposure [15]. In this study, we report in detail the sequence change for 159 *TP53* mutations identified by direct sequencing. More than half of the mutations (60%, 95/159) were missense mutations; there were also 28 (18%) frameshift or nonsense mutations, and 36 (23%) intronic or silent mutations. In coding region, the most common base change detected was C → T transition (43/125; 34% of base changes in the coding region). G → T transversions occurred at a frequency of 10% (12/125), which is less than reported in mutation databases for head and neck squamous cell carcinoma (24%). Characteristically, in our SNC series, the mutations were scattered over a large number of codons, codon 248 being the most frequent target of base substitution. Codon 135 was the second most frequently mutated codon; this nucleotide position has not been reported before as frequently mutated in head and neck cancer or human cancer in general. About half of all tumours with *TP53* mutations carried more than one mutation. Interestingly, 86% (19/22) of the silent mutations detected had occurred in tumours with multiple mutations.

1. Introduction

Head and neck cancers are among the most common malignancies worldwide [1,2]. They also have one of the lowest 5-year survival rates of all cancers [3]. The aetiology of head and neck cancers is relatively well defined in terms of risk factors, with the role of tobacco and alcohol consumption being established [1,2]. Other risk factors include environmental and occupational exposure to dusts or fibres, low intake of vegetables and fruits, and the presence of human papilloma virus infection (HPV16) [1].

Head and neck cancer refers to tumours at different anatomical sites; most frequently they occur in the oral cavity, pharynx and larynx, and rarely in the nose and paranasal sinuses (sinonasal cancer, SNC). A special feature of SNC is that exposure to wood dust, as demonstrated in numerous epidemiological studies, constitutes an overwhelming risk factor for this cancer type [4,5]. In addition, although head and neck cancers are mostly squamous cell carcinomas, a significant proportion of SNCs, depending to some extent on the geographical region and exposure factors, are adenocarcinomas [4,5]. Exceptionally high relative risks for the development of SNC of the adenocarcinoma cell type-histology have been reported for individuals who have a history of exposure to hardwood dust, a known human carcinogen [4,5].

Mutation of the tumour suppressor gene *TP53* is the most common genetic alteration in human cancers; mutations are found in about half of human malignancies. It has been well documented that several types of environmental cancers exhibit an association between the nature and type of *TP53* mutations and the causative exposures [6,7]. In head and neck squamous cell carcinomas (HNSCC), the prevalence of *TP53* mutation reported in the literature varies from 30% to 70%, with at least part of the variation

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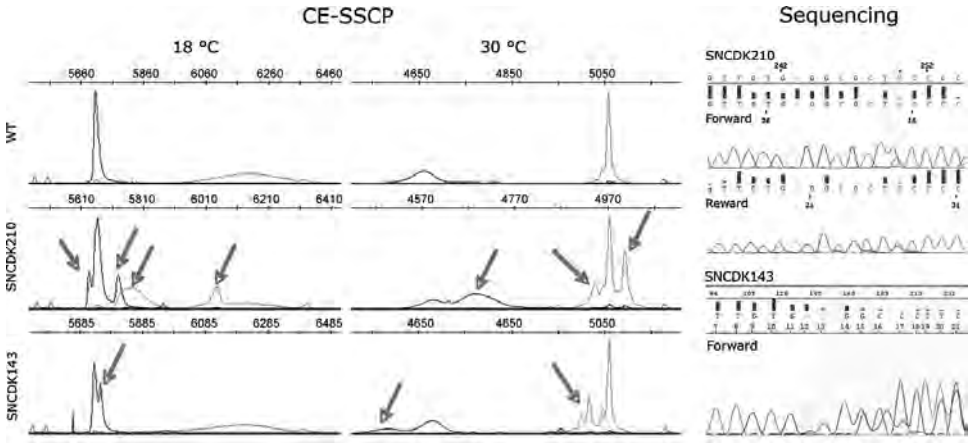

Fig. 1. Detection of exon 5 *TP53* gene mutations in sinonasal tumors by Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) using two different temperatures (left panel), and sequence identification of the same mutations (right panel). In the panel illustrating the CE-SSCP analysis, the red arrows point to changes in the electrophoregrams resulting from a mutation (the blue peaks correspond to forward strand and green ones to the reverse strand). The wild type (WT) sample was human leukocyte DNA. Sequencing of the tumour samples identified the mutations as a G to T mutation in codon 176 (case # SNCDK210), and a frameshift mutation, insertion of A, in codon 173/174 (case # SNCDK143). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

explained by different tumour localisations, mutation detection techniques used, or different exposure backgrounds [1].

It has been proposed that in HNSCC, *TP53* mutations occur mostly prior to the conversion to the invasive state and that they are associated with two carcinogenic exposures, i.e. tobacco and alcohol consumption [3]. In contrast, little is known about occurrence of genetic alterations in SNC, including *TP53* mutations, since there are very few studies reported so far and typically these have examined very limited numbers of cases [8–12]. Therefore, in the *TP53* databases, information of *TP53* mutations for SNC is in practice lacking [13,14]. Furthermore, for the larger anatomical entity of head and neck cancer, mutation information based on any substantial numbers of cases is available only for squamous cell carcinoma histology [13,14].

In this study, we analysed the mutation profile of SNC based on a large series of SNCs collected systematically in a European multicentre study, with 358 primary, histopathologically confirmed SNCs included in the analysis. We recently reported a high frequency of *TP53* mutations, 77% (277 mutation positive cases out of 358) in this SNC series, with the highest frequency of mutations found in adenocarcinoma and in cases exposed to wood dust at work [15]. Here we report the sequence data for 159 mutations identified by direct sequencing, representing by far the largest set of data for sinonasal cancer so far published.

With the current detailed analysis, we wanted to create a *TP53* mutation profile for SNC, and, for further understanding, we compared that with database profiles existing for HNSCC. In addition, we analysed the types and locations of the mutations in relation to patients' exposure to wood dust and smoking habits, in order to identify possible exposure-specific features.

2. Materials and methods

2.1. Patients and samples

Paraffin-embedded tissue (PET) samples of SNC tumours were collected for the study in three European countries (Denmark, Finland and France), as described in detail previously [15]. Briefly, incident cases of primary cancer of the nose and paranasal sinuses (ICD-7: 160, except 160.1) were systematically collected in collaboration with the national cancer registries. In Denmark, incident cases for the years 1991–2001 (211 cases), and in Finland for the years 1989–2002 (133 cases), were identified in the records of national cancer registries, and archival PET samples were collected from pathology laboratories. In France, due to the regional organisational bases of cancer registries, cases were identified in three registries in the areas of Isère, Somme and Doubs for the years 1990–2002. Another type of tissue fixation (Bouin) often used in the pathology laboratories in France, also restricted the collection of PET samples. A panel of three pathologists reviewed histologic sections in the whole tumour collection [15]. Only cases for which the tumour histology and tumour location were confirmed by consensus to fulfil the study criteria were included. In brief, the included tumours corresponded to the main histopathologic categories of cancer of the nose and paranasal cavities as indicated in the WHO classification of tumours [16–18]. The final study population comprised 358 cases of SNC [15]. Histories of occupational exposures and smoking habits were collected by telephone interviews and employers' or pension registries for the cases, as described in detail elsewhere [15]. The appropriate national Ethical Review Boards in Denmark, Finland and France approved the study.

2.2. Mutation analysis

DNA was extracted from paraffin-embedded tissue (PET) samples and amplified by PCR. CE-SSCP was used to detect sequence alterations [15]. In all, a total of 476 mutations were detected by CE-SSCP in 277 mutation positive cases among the 358 cases of sinonasal cancers that were eligible for the study according to the study inclusion criteria described in detail elsewhere [15]. From the collection of mutations first detected by a CE-SSCP analysis, the location and type of mutation were identified by direct sequencing (Fig. 1). In the sequencing protocol, extracted DNA was first amplified by PCR and the PCR-products were purified with QIAquick PCR purification kit (Qiagen) or by using ExoSAP-IT (USB). The sequencing reaction was prepared with BigDye Terminator v3.0 Cycle Sequencing ready Reaction kit (Applied Biosystems) and sequencing was performed with ABI Prism 310 capillary sequencer (Applied Biosystems) or ABI Prism 3100-avant capillary sequencer (Applied Biosystems). Due to the PET quality of DNA, sequence identification of all of the mutations detected with CE-SSPC analysis could not be successfully performed; however all those that remained CE-SSCP positive were confirmed in one or several independent CE-SSCP analysis, each carried out on a new PCR from genomic tumour DNA [15]. *TP53* polymorphisms were carefully excluded.

2.3. Statistical analysis

Statistical analyses were performed using the Stata software (Stata statistical software: Release 9. College Station, TX: Statacorp LP 2005), Fisher's exact test (two-sided) was used to compare proportions.

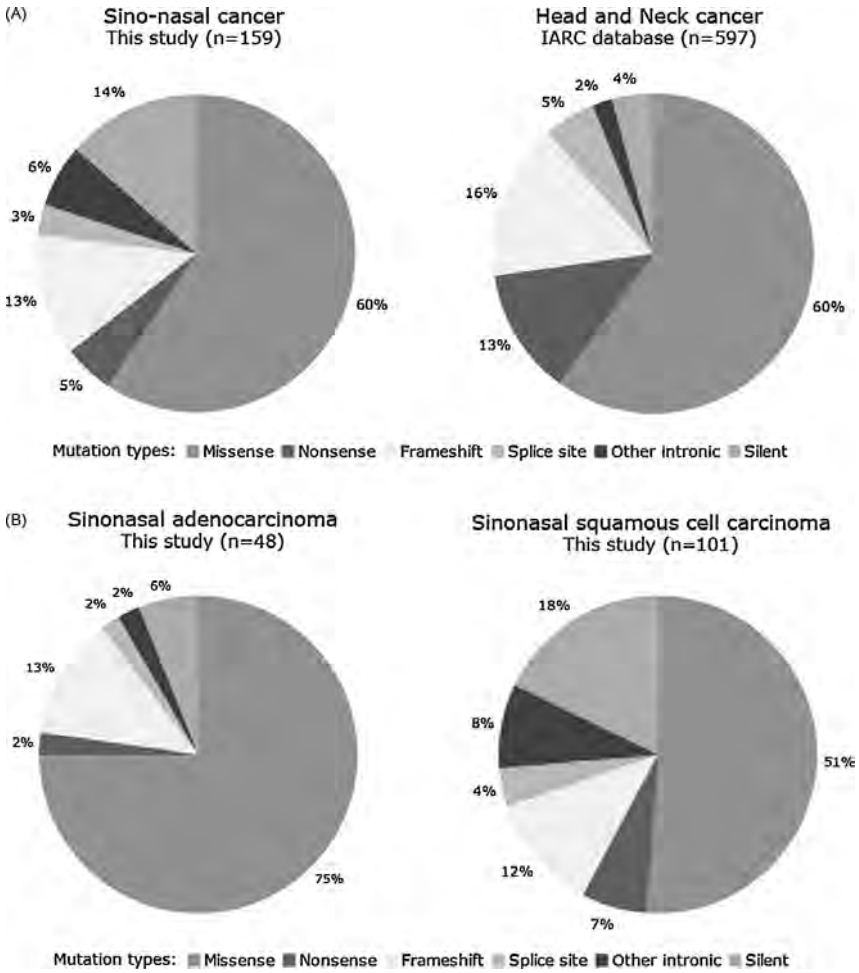

Fig. 2. *TP53* mutation profile in sinonasal cancer. (A) Distribution of mutation types of the *TP53* gene in sinonasal cancer as identified in the present study, and in head and neck cancer according to the IARC TP53 mutation database version R13 [14]. (B) Distribution of mutation types of the *TP53* gene in sinonasal adeocarcinoma and squamous cell carcinoma as detected in the present study (mutation types in carcinoma NOS, $n = 10$, not indicated).

3. Results

In this study, we report sequence data in detail for 159 *TP53* mutations, which we successfully identified with direct sequencing (Fig. 1). A clear majority of the sequenced mutations, 95 mutations (60%) out of the total 159, were missense base substitutions. Additionally there were 20 (13%) frameshift mutations of various types (9 deletions, 7 insertions and 4 deletions with insertions) and 8 (5%) nonsense mutations (Fig. 2A). Also, 14 (9%) intronic mutations and 22 (14%) silent mutations were found. Five of the intronic mutations occurred at a splice site, representing 3% of all mutations detected (Fig. 2A). Detailed data concerning the sequence change and location identified, as well as the demographic and exposure data are available for each case in the supplementary table; data on the mutations detected by CE-SSCP but left without accurate sequence information are also given (Supplementary Table 1).

The most common type of sequence alteration detected in the *TP53* coding region was a C to T transition (Table 1). We detected 43 of this kind of base substitution, representing 34% of the 125 base changes that occurred in the coding region. In particular, C to T transitions predominated among the silent mutations (16/22, 73%, Table 1), and they were most often encountered in non-wood-dust exposed SNC cases (Fig. 3A). The G to A transition was the second most common base change with 27 mutations (22% of base changes in the coding region; Table 1) found mainly in smokers (Fig. 3B). Frameshift mutations represented the third most frequent type of mutation (20/159, 13%, of all mutations). A G to T transversion was discovered to be only the fourth most common type of sequence alteration with 12 occurrences (10% of base changes in the coding

Table 1
Types of *TP53* mutations in the coding region of the gene found in sinonasal cancer. Base changes and their distribution in different mutation type categories are indicated.

Base substitution	Total		Missense		Silent		Nonsense	
	n	%	*n*	%	*n*	%	*n*	%
C>T	43	34	22	23	16	73	5	63
G>A	27	22	23	24	3	14	1	13
G>T	12	10	11	12	0	0	1	13
A>G	10	8	9	9	1	5	0	0
Other	33	26	30	32	2	9	1	13
Total	125		95		22		8	

region; Table 1). However, they were detected predominantly in smokers (Fig. 3B). G to T transversions typically resulted in missense mutations (11/12, 92%; Table 1).

The mutations in SNC were scattered over a large number of codons; altogether 72 codons were affected by a base substitution. The codons most frequently carrying a mutation in SNC were those located in the central DNA-binding region of the *TP53* gene; they were also, with one exception, at nucleotide sites commonly mutated in other human cancers as well. In this series, codon 248 was the most frequently mutated codon with 8 mutations (5 C**G**G → C**A**G and 3 **C**GG → **T**GG), followed in frequency by codon 135 (7 mutations; 5 T**G**C → T**T**C and 2 T**G**C → T**A**C), codon 175 (5 mutations; all C**G**C → C**A**C), and codon 179 (5 mutations; 2 C**A**T → C**T**T, 1 C**A**T → C**G**T and 1 **C**AT → **T**AT).

A relatively high number of the SNC tumours with a *TP53* mutation (49%, 131/268 [15]) were found to carry another mutation in a different exon. In some cases, two or more additional mutations were discovered (polymorphisms excluded). The distribution of the types of mutations between the cases with only one mutation and those with multiple mutations differed significantly ($p = 0.001$) (Fig. 4). Most of the mutations found in tumours exhibiting a single mutation were missense base substitutions (75%, 56/75), whereas in those with multiple mutations, missense substitutions represented only 45% (38/84) of all mutations found in these tumours. In particular, the proportion of silent mutations was larger in tumours carrying multiple mutations; almost all silent mutations (19/22, 86%) detected were found in such tumours. In addition, frameshifts and intronic mutations were mostly found in tumours with multiple mutations (13/20, 65% of all frameshift mutations, and 9/14, 64%, of all intronic mutations, respectively). Overall, silent and non-splice site intronic mutations represented 33% of all mutations in tumours carrying multiple mutations.

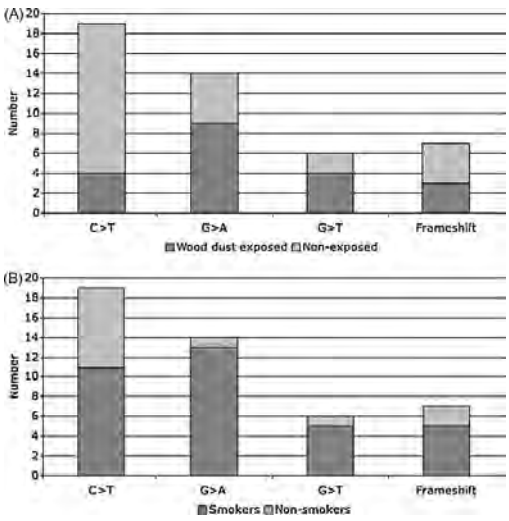

Fig. 3. The main mutation types in the coding region of the *TP53* gene according to exposures among sinonasal cancer patients. (A) Wood-dust exposure; (B) smoking.

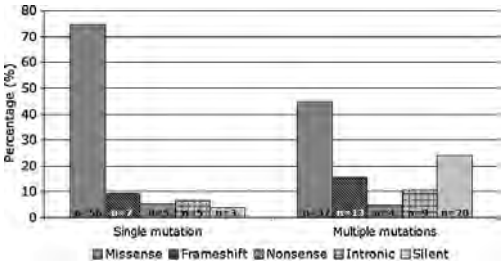

Fig. 4. Distribution of mutation types in sinonasal tumours with single or multiple exons of the *TP53* gene mutated. The difference in distribution between the two groups was statistically significant ($p = 0.001$).

The distribution of mutation types differed significantly depending on exposure to wood dust. The wood-dust exposed cases exhibited more missense base substitutions and less silent substitutions than the cases without such exposure ($p = 0.006$, Table 2). With respect to frameshift mutations, half (4/8) of the cases with a frameshift mutation and data available for exposure were smokers with wood-dust exposure. Multiple mutations were associated with smoking [15], and, accordingly, silent mutations were mostly found in smokers. There were 10 SNC cases with data on smoking habits available and presenting with a silent mutation; eight out of those (80%) were smokers.

The overall distribution of mutation types in the two main tumour histologies, adenocarcinoma and squamous cell carcinoma, differed significantly ($p = 0.041$, Fig. 2B). Adenocarcinomas harboured more missense mutations (36 of 48 mutations detected, 75%) than squamous cell carcinomas (52 of 101 mutations detected, 51%). Squamous cell carcinomas tended to contain more silent mutations (18/101, 18%) than adenocarcinomas (3/48, 6%), and almost all of the intronic splice site mutations were found in squamous cell carcinoma (4/5).

4. Discussion

We report here the sequence of 159 tumour suppressor gene *TP53* mutations identified in sinonasal cancer. The present study found that a majority of the identified *TP53* mutations were missense base substitutions. The frameshift mutations in SNC occurred at a frequency that is similar to what has been reported for HNC before, whereas the percentage of silent mutations was higher

Table 2
Distribution of mutation types in wood-dust exposed and non-exposed cases of sinonasal cancer.

Mutation type	Wood-dust exposure		No wood-dust exposure		*p*
	n	%	*n*	%	
Missense	34	79	43	52	
Silent	1	2	13	16	0.006
Other	8	19	27	33	
Total	43		83		

than normally has been found for HNC. We have reported earlier that tumours with multiple mutations were common in sinonasal cancer [15]. In the current study, we discovered a statistically significant difference in the distribution of mutation types between the tumours with a single mutation and tumours with multiple exon mutations. These multiple mutation tumours harboured almost all of the silent mutations discovered.

Almost 80% of *TP53* gene mutations in human cancer are missense mutations, followed by 10% of frameshift mutations, and 9% of nonsense mutations [14,19]. This is different than for most other tumour suppressor genes, which are primarily inactivated by frameshift or nonsense mutations that lead to absence or aberrant synthesis of the gene product. The pattern of *TP53* mutations can be explained by the critical role of the core domain of the p53 protein for recognising the p53 DNA binding sites; it is well characterised that most of the base changes found in this region can cause a loss of p53 function [19]. In addition, it has been proposed that some of the common *TP53* mutations convey oncogenic gain-of-function activity [20]. Each residue in this region has been found to be the target of at least one mutation in human cancer [19].

The frequency of missense base substitution (60%) we detected in sinonasal cancer is somewhat lower in comparison to the overall *TP53* mutation profile in human cancer, and, accordingly, the prevalences of frameshifts (13%) and silent mutations (14%) were higher than those generally found in mutations of the *TP53* gene. The frequencies we found were, however, not different from the data on 600 mutations reported in the database for the larger anatomical entity, head and neck cancer (Fig. 2A). In SNC, we observed a significant difference in the *TP53* mutation profiles between adenocarcinomas and squamous cell carcinomas; the former histology also exhibited a significantly higher mutation frequency [15].

About half of the sinonasal tumours with TP53 mutations carried a mutation not only in one but in two or more of the exons. It is likely that they represent different cellular clones developed over time; our cloning experiments supported this alternative but such data were unfortunately available for a limited number of cases only (unpublished data). We discovered that the overall mutation profiles between the single mutation and multiple mutation carrying tumours differed statistically significantly. The prevalence of silent mutations in tumours with a single mutation in our data (4%) corresponded to that generally reported for *TP53*, while almost all (86%) of the silent mutations had occurred in multiple mutation tumours. Overall, silent and non-splice site intronic *TP53* mutations represented 33% of all mutations we detected in SNC tumours carrying multiple mutations.

The frequency of multiple mutations was statistically significantly higher among smokers as compared to nonsmokers in this study population of SNC patients [15]. Our present analysis indicated that the majority of silent mutations, accordingly, had occurred in smokers. The tumours with multiple mutations may reflect a process in which the presumably first *TP53* mutation may lack the ability to function as the driving force for malignant transformation or growth in the tissue in question. Subsequent mutations may then accumulate in the tumour. Interestingly, multiple mutations have earlier been reported in squamous cell carcinoma of the head and neck [21,22]. Further, in HNCC, the timing of occurrence of *TP53* mutations is still a somewhat controversial issue, but the current consensus view is that they occur before the invasive state [3].

The frequency of frameshift mutations (13%) in our series of SNCs is close to the data from head and neck cancers, in which frameshift mutations have been reported to occur somewhat more frequently, in about 16% of tumours; this is higher compared to other cancer types [1,14]. In HNSCC, frameshift mutations are often found in the subgroup of patients using both tobacco and alcohol [1]. It can be speculated that similarly in SNC, a regular, continued presence of two mutagenic and carcinogenic exposures, wood dust and tobacco smoke [4,23,24], may create a constant high genotoxic stress in the target cells, resulting in a relatively high occurrence of frameshift mutations. Moreover, we detected significantly more missense and less silent mutations in tumours from the wood-dust exposed cases than in tumours from the non-exposed cases; this further points to a role for mutagenic carcinogen exposure.

The most common of the sequence alterations detected in SNC was a C to T transition that was particularly common among the silent mutations. The second most common base change was a G to A transition followed by a G to T transversion. G to T transversion are known to be associated with tobacco smoking in lung cancer; accordingly, the G to T transversions were primarily found in SNC cases who were smokers. The prevalence of G to T transversions in this study (10%) was lower than in general reported for other head and neck cancers (24%) [1,3]. This is in accordance with our previous finding showing that smoking was not significantly associated with the overall risk of having a *TP53* mutation among the SNC cases [15]. Epidemiological studies have reported smoking as a risk factor for SNC, with two- to threefold increased risk of nasal cancer observed among smokers and a reduction in risk among long-term quitters [24,25]. The association between smoking and risk of SNC has been suggested to be restricted to squamous cell carcinoma and no clear association has been observed for the adenocarcinoma histology [24,25].

The localisation of the *TP53* mutations in SNC followed the overall codon distribution found in human cancer [1,14] and mutations were scattered over a large number of codons [15]. The most commonly mutated codon in this study was 248, followed by 135, 175 and 179. Codons 248 and 175 are among the most frequently mutated *TP53* codons in human cancer in general [26], and 179 is often mutated in lung cancer and head and neck cancers [13,14]. However, codon 135 mutations are less commonly found in cancer; yet the types of base changes we observed in codon 135 in sinonasal cancers code for proteins that have highly impaired transactivation [27]. In the databases [13,14], codon 135 has been reported to be mutated more than 200 times altogether. Cancers, where codon 135 mutation had been found more than 10 times, include breast cancer, colorectal cancer, HNSCC, lung cancer, ovarian cancer, oesophageal cancer and bladder cancer [13]. Interestingly, in the UMD p53 database, almost half of the codon 135 mutations from HNSCC (11/23) are of the type T**G**C → T**T**C, which was also the most common change in this codon observed in our data; it represented 42% of the G to T transversions we detected. In comparison, the most frequent change in codon 135 in breast cancer (11/26) or colorectal cancer (13/25) found in the database is T**G**C → T**A**C.

The differences in predominant types of mutation seen between the cancer types may in part reflect differences in the biology of organs and tissues; a certain type of mutation may offer a selective growth advantage in a specific cellular context. There are examples of this phenomenon, such as R249S in liver cancer and R337H in association with adrenal cortical carcinoma, as indicated in a recent review [28]. Spontaneous codon 135 mutations have also been found in untreated human p53 knock-in (Hupki) murine embryonic fibroblast cells [29]. This was proposed to be due to radicals arising from the high level of oxygen in cell culture incubators in comparison to normal tissue [29]. We have earlier suggested that generation of reactive oxygen species (ROS) is likely to be one of the consequences of wood-dust exposure and the subsequent inflammatory process [20,27]. A ROS-related mechanism for the codon 135 mutations might fit this well. We have also reported data on COX-2 expression in SNC tumours that may further suggested involvement of an inflammatory mechanism [30].

There is a paucity of data on *TP53* mutations in sinonasal cancer. In this study, we analysed the profile of *TP53* mutations in a large series of SNCs. We found that the overall profile resembles

much what has been reported for human head and neck cancer in general, but features specific to SNC emerged in our analysis of the mutation data. We also conclude that sinonasal tumours from individuals with a history of wood-dust exposure exhibited predominantly missense mutations, whereas tumours from smokers contained *TP53* multiple mutations and an excess of frameshift and silent mutations. The mutation profile we observed in SNC, together with the high overall frequency of *TP53* mutations [15], is likely to reflect the high genotoxic stress generated by a regular exposure to carcinogenic substances, such as wood dust and, to a minor extent, tobacco smoking. The high frequency of TP53 mutations is a feature SNC shares with HNSCC; in the latter, the high pressure for DNA damage is believed to be associated with use of tobacco and alcohol.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2009.12.003.

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In 2008, cancer killed almost eight million people and that number is predicted to increase. A large proportion of human cancers are preventable; many cancers have environmental risk factors, such as tobacco smoking and work-related exposures. Carcinogenesis is driven by alterations in the sequence and function of genes involved in many crucial cellular processes. Understanding these molecular mechanisms will clarify the role and biological effects of these risk factors.

In this work, mutations in the tumour suppressor gene, *TP53*, and their associations with exposure were studied in sinonasal cancer and lung cancer. Another important mechanism in cancer is inflammation and its contribution was explored by analyzing the expression of COX-2 in sinonasal cancer.

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