Molecular epidemiological study of non-small-cell lung cancer from an environmentally polluted region of Poland

M Rusin¹, D Butkiewicz¹, E Malusecka¹, A Zborek¹, J Harasim², K Czyzewski², WP Bennett*, PG Shields¹, A Weston³, JA Welsh³, S Krzyzowska-Gruca¹, M Chorazy¹ and CC Harris³

¹Department of Tumour Biology, Institute of Oncology, 44-100 Gliswice, Poland; ²Department of Thoracic Surgery, Silesian Medical Academy, 41-800, Zabrze, Poland; ³Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4255, USA; *Toxicology and Molecular Biology Branch, National Institute for Occupational Safety and Health, Centers for Disease Control, Morgantown, WV 26505, USA

Summary The p53 mutation spectrum can generate hypotheses linking carcinogen exposure to human cancer. Although it is well-documented that tobacco smoking is a major cause of lung cancer, the contribution of air pollution is less well-established. We determined the molecular and immunohistochemical changes (p53 gene mutations, p53 protein accumulation and WAF1 protein expression) and genetic polymorphisms of GSTM1, CYP1A1 and CYP2D6 genes in a case series of non-small-cell lung cancers from Silesia. This region of southern Poland is highly industrialized with considerable environmental pollution. More than 50% of lung cancers (90/164) contained p53 mutations and 75% showed the combined alteration of the p53 gene and protein accumulation. Males occupationally exposed to coal-derived substances showed a relatively high frequency of squamous and large-cell carcinomas, relatively frequent mutations in codon 298 of p53 and a low frequency of p53 immunohistochemically positive tumours. Codon 298 GAG→TAG mutations have rarely been found in lung cancers in other populations. We found no correlation between WAF1 protein expression and mutations in the p53 gene or p53 protein accumulation. No statistically significant relationship was found between p53 mutations and GSTM1, CYP1A1, CYP2D6 genotypes. Never smokers with lung cancers from Silesia had a higher frequency of G:C→T:A transversions than previously reported of the p53 mutation spectrum in never smokers (6/15 vs 4/34; P = 0.06 by χ²). These data are a tentative indication that occupational and environmental exposure to polycyclic aromatic hydrocarbons, such as benzo(a)pyrene, in polluted air contributes to the molecular pathogenesis of lung cancer in never smokers.

Keywords: p53, WAF1; GSTM1; CYP1A1; CYP2D6; tobacco smoke

The p53 tumour suppressor gene codes for protein that regulates the expression of various genes and affects many cellular functions including: DNA repair, cell cycle checkpoints and apoptosis (reviewed in Harris, 1996; Ko and Prives, 1996; Levine, 1997). The p53 protein activates transcription of the WAF1/CIP1, encoding a 21 kDa protein (El-Deiry et al, 1993) associating with the cyclin D1–cyclin-dependent kinase (cdk)4 complex and inhibiting its kinase activity. This in turn prevents the cell from entering the S phase of the cell cycle (Harper et al, 1993; Xiong et al, 1993). Mutations in p53 lead to the loss of tumour suppressor function and also can result in a gain-of-function phenotype of mutant protein (Gualberto et al, 1998 and refs therein). Missense mutations lead to the abnormal accumulation of p53 protein which can be visualized by immunohistochemical methods (Iggo et al, 1990). Abnormal accumulation of p53 and p53 mutations are frequent in precancerous lesions (dysplasia) of lung epithelium (Sozzi et al, 1992; Vahakangas et al, 1992; Bennett et al, 1993).

p53 mutation spectra may give clues to the aetiology of cancer (Hollstein et al, 1991; Harris 1993; Greenblatt et al, 1994; Hussain and Harris, 1998). Thirty per cent of p53 point mutations in lung cancers from smokers are G:C→T:A transversions, and most of the G residues are on the DNA non-transcribed strand (Greenblatt et al, 1994). The non-transcribed strand of the p53 is repaired at a slower rate than the transcribed strand (Evans et al, 1993). G:C→T:A transversions are significantly less frequent in never smokers (Takeshima et al, 1993; Greenblatt et al, 1994; Hainaut et al, 1998: the database for p53 mutations). These transversions may be attributed to either bulky chemical carcinogens (e.g. benzo(a)pyrene) or, perhaps, oxiyradical exposure resulting, for example, from tobacco smoking (Ruggeri et al, 1993; Denissenko et al, 1998).

Tobacco smoking is a predominant cause of human lung cancer (reviewed in IARC Monograph, 1986). The contribution of air pollution to the aetiology of lung cancer is less clear, which may reflect the limited sensitivity of epidemiological studies. The role of occupational and environmental exposure to polluted air is supported by some studies (Miller, 1992; Petersen, 1994; Speizer and Samet, 1994).

Silesia is a highly industrialized and densely populated region in southern Poland with considerable air pollution (Chorazy et al, 1994). Previous studies have shown that non-occupationally exposed Silesian residents have more chromosomal aberrations, sister chromatid exchanges and PAH-DNA adducts than rural controls (Motykiewicz et al, 1992; Perera et al, 1992; Grzybowska et al, 1993; Motykiewicz et al, 1995).

Gene–environment interactions may influence the risk of cancer. Aryl hydrocarbon hydroxylase (AHH) and debrisoquine
hydroxylase, encoded by CYP1A1 and CYP2D6 genes respectively, have been studied extensively in lung cancer patients with varying results. Initial CYP2D6 phenotyping studies suggested overrepresentation of the dominant EM (extensive metabolizer) phenotype in lung cancer patients when compared to patients with chronic obstructive pulmonary disease (Ayesh et al., 1984; Caporaso et al., 1989). Subsequent genotyping reports have not confirmed these findings (Sugimura et al., 1990; Tefre et al., 1994; Stucker et al., 1995). Caporaso et al. (1995) discussed the possibility of an explanation for these apparent paradoxical findings. For CYP1A1, the polymorphic minor allele codes for valine in exon 7 (codon 462) instead of isoleucine. A significant preponderance of the susceptible Val/Val genotype among individuals with lung cancer has been reported in a Japanese population (Hayashi et al., 1992). The glutathione-S-transferase (GSTM1) gene encodes glutathione S-transferase class μ that catalyses conjugation reactions of glutathione with electrophiles (e.g. activated PAHs). Some reports have observed the lack of GSTM1 activity due to the homozygous deletion confers increased lung cancer risk (Hirvonen et al., 1993; Alexandrie et al., 1994; Kihara et al., 1994; reviewed by Rebbeck, 1997). This increased lung cancer risk is compounded for carriers of p53 susceptibility genotypes (Hayashi et al., 1992; Nakachi et al., 1993).

The specific aim of our investigation was to determine molecular and immunohistochemical changes (p53 gene mutations, p53 protein accumulation and WAF1 protein expression) in a case series of non-small-cell lung cancer (NSCLC) samples from a heavily polluted area of Poland. We investigated the hypothesis that the p53 mutational spectrum in the Silesian residents reflects the mutagenic activity of air pollution. Our analyses also examined possible relationships between the mutational spectrum of p53 and selected functional genetic polymorphisms involved in carcinogen metabolism.

MATERIALS AND METHODS

Sample collection

The cases of NSCLC were resected at the Department of Thoracic Surgery, Silesian Medical Academy between 1991 and 1995. The patients were interviewed at the hospital by either a physician or a trained nurse. Data on demographics, medical history, family history of cancer, occupational exposure and smoking habits were collected by questionnaire. Tumour and non-tumour samples were either frozen on dry ice (all non-tumour lung and 45 tumour samples) or fixed (see below) and paraffin-embedded (119 tumour samples). Forty-five tumour samples were not available for paraffin embedding. DNA from fresh tissues was extracted, after crushing in liquid nitrogen, using standard procedures (Sambrook et al., 1989). Cancer cells were not microdissected from tumour samples. DNA from dewatered paraffin sections of tumour tissues was extracted by sodium dodecyl sulphate (SDS)–proteinase K treatment (0.5 mg ml−1 in 1% SDS) at 55°C for 18–24 h followed by phenol–chloroform extraction and ethanol precipitation with glycogen as a carrier.

Immunohistochemistry

Immediately after the surgical removal, the tissues were fixed for 24 h in either ice-cold 10% formalin in phosphate-buffered saline (PBS), or in methacarn (methanol:chloroform:acetic acid 6:3:1) and embedded in paraffin. A portion of each specimen was sectioned. The sections were dewaxed and counterstained with Mayer’s haematoxylin. Immunohistochemical staining was performed on dewaxed 7-μm sections. Formalin-fixed tissues sections were heated 2 × 5 min in a microwave oven in 0.01 M citric buffer, pH 6.0. The p53 protein was detected using either the polyclonal antibody CM1 (Signet Laboratories) or one of the following monoclonal antibodies: Ab 1801 (Ab 2, Oncogene Sciences, Gaithersburg, MD, USA), DO-1 (Ab 6, Oncogene Sciences) recognizing epitopes on the amino-terminus of the p53 protein (amino acids 40–65 and 37–45 respectively), HR231 (a gift of Dr T Soussi) that reacts with amino acids 351–393 on the carboxy-terminus of the p53 protein (Legros et al., 1993). All used antibodies recognize both the wild-type and mutant p53. The four anti-p53 antibodies yielded a very similar staining pattern, but the intensity of staining varied slightly. There was no instance of a positive result with one antibody contradicted by lack of staining with another.

For detection of the p21WAF1/CIP1 protein, sections of tissue fixed in formalin and exposed to microwave antigen retrieval were used. The sections were incubated overnight at 4°C with the primary antibody (mouse monoclonal antibody Ab-1 from Oncogene Sciences). Indirect immunoperoxidase staining with avidin–biotin complex and 3,3′-diaminobenzidine as a substrate was applied for visualization of the antigens. The sections were counterstained with Mayer’s haematoxylin.

p53 and p21WAF1/CIP1 immunohistochemical analysis of the intensity of positive staining was scored as follows: + 2–10% of cells distinctly positive or more cells weakly stained, ++ 10–50% distinctly positive or more cells weakly stained, +++ more than 50% of cells strongly stained.

Mutational analysis of the p53 gene

Exons 5 to 8 of p53 were amplified by polymerase chain reaction (PCR) with intronic primers (Lehman et al., 1991) and sequenced with Sequenase Version 2.0 DNA sequencing kit (USB-Amersham, Cleveland, OH, USA). When PCR amplification was strong, the PCR product was sequenced with Sequenase PCR product sequencing kit (USB-Amersham). A DNA fragment with mutation was reamplified and resequenced to confirm that the mutation was not introduced by an error of the thermostable polymerase used for PCR. Germline mutations were excluded by PCR amplification and sequencing of relevant exons from non-tumour lung DNA.

Genotyping of GSTM1, CYP2D6 and CYP1A1

The CYP1A1 exon 7 and GSTM1 polymorphisms were determined as described previously (Shields et al., 1993). In a multiplex PCR reaction, a CYP1A1 fragment served as an internal control for the detection of the GSTM1 gene deletion. The CYP1A1 fragment was analysed by NcoI restriction enzyme digestion. The CYP2D6-A allelic variant was detected using allele-specific double-step PCR according to Heim and Meyer (1990). The CYP2D6-B allele was identified using PCR and BstNI digestion as previously described by Kato et al. (1995).

Statistical analyses

Descriptive statistics and standard measures of association (e.g. cross-tabulations using χ2 test for association) were used for data

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exploration and to test the significance. These analyses were performed using the Statistica Version 5.0 program for PCs (StatSoft, Inc., Tulsa, OK, USA) or Statistical Analysis System (Cary, NC, USA). Mutational spectra of the p53 were compared using a computer program developed by Cariello et al (1994). It can be obtained at http://sunsite.unc.edu/dnam/mainpage.html. We refer to it as Monte Carlo analysis. The differences were considered statistically significant if the $P$-value was 0.05 or less in two-tailed tests.

### RESULTS

Exons 5–8 of p53 were sequenced directly after PCR amplification from genomic DNA of 164 patients without pre-selection of samples. Ninety-two somatic mutations were found in 90 patients (55%). In two patients, we found two p53 mutations. In sample no. 288, which stained positively for the p53 protein, one base pair deletion in exon 5 (codon 159) and a mis-sense mutation in exon 8 (codon 275) were found. In sample no. 217, which stained positively

#### Table 1

| All mutations | SQ | AD | LG | Smokers | Never smokers | Coal-exposed | Coal non-exposed |
|---------------|----|----|----|---------|---------------|--------------|-----------------|
| G:C→T:A      | 31 | 20 | 55 | 50      | 29            | 39           | 37              | 24              |
| G:C→A:T      | 29 | 33 | 30 | 0       | 31            | 20           | 20              | 34              |
| G:C→C:G      | 9  | 12 | 0  | 10      | 9.5           | 7            | 8               | 12              |
| A:T→T:A      | 4.5| 5  | 0  | 10      | 4             | 7            | 2.5             | 5               |
| A:T→C:G      | 5.5| 5  | 5  | 10      | 5             | 7            | 5               | 10              |
| A:T→G:C      | 9  | 10 | 10 | 0       | 9.5           | 7            | 12.5            | 5               |
| del/ins      | 12 | 15 | 0  | 20      | 12            | 13           | 15              | 10              |
| Number of mutations | n = 90 | n = 59 | n = 20 | n = 10 | n = 75 | n = 15 | n = 40 | n = 41 |

Except for the bottom row the numbers are per cents. Two mutations are not included because they represent either tandem or composite mutation. One mutation is not included in the histological column because the tumour was of mixed type. Coal-exposed and non-exposed patients were males. Using mutational spectra comparison software, the only significant differences were found between spectra in SQ vs AD ($P = 0.042$; Monte Carlo analysis) and between AD vs LG ($P = 0.029$; Monte Carlo analysis). Moreover, the difference of G:C → T:A frequencies in SQ vs AD was also significant ($P = 0.008$; $\chi^2$).

#### Table 2

| Tumour types, p53 immunohistochemical staining and p53 mutations in coal-exposed and non-exposed NSCLC cases (males only) |
|---------------------------------------------------------------|
| **Coal-exposed** | **Non-exposed** | **Significance** |
| SQ + LG (84%) | 59 | 46 (68%) | $P = 0.04 \chi^2$ |
| AD (16%) | 22 | 11 (16%) | $P = 0.01 \chi^2$ |
| p53 IHC-positive (50%) | 24 | 40 (77%) | $P = 0.01 \chi^2$ |
| p53 IHC-negative (50%) | 24 | 12 (23%) | $P = 0.01 \chi^2$ |
| p53 mutations (56%) | 41 | 41 (60%) | $P = 0.01 \chi^2$ |
| No p53 mutation detected (44%) | 31 | 27 (40%) | $P = 0.01 \chi^2$ |
| p53 missense mutation (67%) | 26 | 30 (75%) | $P = 0.01 \chi^2$ |
| Other p53 mutations (33%) | 13 | 10 (25%) | $P = 0.57 \chi^2$ |
| Non-codon 298 mutation (90%) | 35 | 41 (100%) | $P = 0.05$ Fisher’s |
| Codon 298: GAG → TAG (10%) | 4 | 0 | $P = 0.05$ Fisher’s |

*One exposed patient had two mutations; *Non-sense mutations, deletions and frameshifts; *One silent mutation was not included. SQ, squamous cell carcinoma; AD, adenocarcinoma; IHC, immunohistochemistry.

#### Table 3

| Gender, smoking status and *GSTM1*, *CYP1A1* and *CYP2D6* genotypes |
|---------------------------------------------------------------|
| **GSTM1** | **CYP1A1** | **CYP2D6** |
| Null | Positive | Ile/Ile | Ile/Val | BB or AB | WT + A or B | WT/WT |
| Females | 13 | (62%) | 8 | (38%) | 17 | (81%) | 4 | (19%) | 2 | (9.5%) | 8 | (38%) | 11 | (52.5%) |
| Males | 70 | (52%) | 65 | (48%) | 115 | (88%) | 20 | (15%) | 19 | (5%) | 5 | (38%) | 77 | (57%) 3* |
| Smokers | 69 | (52%) | 64 | (48%) | 114 | (86%) | 19 | (14%) | 8 | (6%) | 50 | (38%) | 75 | (56%) |
| Never | 14 | (61%) | 19 | (39%) | 18 | (78%) | 5 | (22%) | 4 | (4%) | 1 | (39%) | 13 | (57%) 6* |

1* $P = 0.53 \chi^2$; 2* $P = 0.86$ Yates corrected $\chi^2$; 3* $P = 0.77$ Yates corrected $\chi^2$ (BB or AB vs others); 4* $P = 0.57 \chi^2$; 5* $P = 0.54 \chi^2$; 6* $P = 0.87$ Yates corrected $\chi^2$ (BB or AB vs others).
for p53 protein, a non-sense mutation in exon 8 (codon 298) and a missense mutation in exon 7 (codon 246) were detected.

The frequency of p53 mutations in large-cell carcinomas (LG), squamous cell carcinomas (SQ) and adenocarcinomas (AD) was: 83% (10/12), 56% (60/107) and 43% (19/44) respectively (one tumour was diagnosed as of mixed histology).

A statistically significant difference of p53 mutation frequency was found between AD and LG ($P = 0.02$, Fisher’s exact test), the other differences were not statistically significant. No statistically significant association was detected between the presence of p53 gene mutations and gender, T stage (characterizing primary tumour size), N stage (characterizing presence of cancer invasion to local lymph nodes) or general stage (I, II or III) of lung cancer (combining the data on: primary tumour size, spread of cancer tissue to local lymph nodes, presence on distant metastases).

Five mutational hotspots were detected (with at least four mutations in each) at codons: 155 ($n = 4$), 158 ($n = 4$), 248 ($n = 7$), 273 ($n = 5$) and 298 ($n = 5$). Mutational spectra of the p53 gene in the selected groups of patients and the spectrum of all mutations are shown in Table 1. It was found that the spectra in smokers and never smokers do not differ significantly, although G:C $\rightarrow$ T:A transversions were more frequent in never smokers (39%) compared with smokers (29%; Table 1). We did not find a dose–response relation between the number of smoked cigarettes (pack-years) and either the frequency of mutations or the frequency of G:C $\rightarrow$ T:A transversions (data not shown).

In our series of patients, 70 males were occupationally exposed to either coal dust or coal processing substances (54 coal miners, but also foundry workers and others). The percentage of histological types in coal-exposed versus non-exposed individuals (males only) is shown in Table 2. The observed differences are statistically significant ($P = 0.04$, $\chi^2$ test). Four non-sense mutations in codon 298 (GAG $\rightarrow$ TAG; Glu $\rightarrow$ STOP) were found among 39 coal-exposed males with mutated p53, whereas no non-exposed male patient out of 41 with mutated p53 showed this mutation ($P = 0.05$ by Fisher’s exact test). The overall spectra in coal-exposed and in non-exposed male patients did not differ (Table 1). We observed that tumours of non-coal-exposed patients tended to stain positively for p53 (40/52, 77%) when compared to coal-exposed patients (24/48, 50%); ($P = 0.01$, $\chi^2$; Table 2). No significant association between the exposure to coal and the presence of p53 gene mutations (Table 2) was detected, although, consistent with immunohistochemical data, males exposed to coal had more mutations (frameshifts, splice site mutations, non-sense mutations) coding for truncated, rapidly degraded p53 molecules, than non-exposed males (33% and 25% respectively; $P = 0.57$ by $\chi^2$, Table 2).

G:C $\rightarrow$ T:A transversions were more frequent in males (26/82, 32%) than in females (2/8, 25%), but the difference was not statistically significant. The frequency of all p53 mutations was lower in females (8/21, 38%) compared to males (82/143, 57%; $P = 0.16$ by $\chi^2$). The gender difference in the frequency of p53 mutations (although not significant) is likely related to lower frequencies of p53 mutations in AD which have, in our sample set, a higher prevalence in females (10/21; 48%) compared to males (34/143; 24%; $P = 0.04$ by $\chi^2$).

In our case series AD were more frequent in never smokers (9/23; 39%) than in smokers (35/140; 25%; $P = 0.25$ by $\chi^2$). But in never smokers with a mutated p53 gene (15 individuals) AD constitute only 26% of cases (four in 15) and only one of the AD had G:C $\rightarrow$ T:A transversion. Thus, most of G:C $\rightarrow$ T:A transversions frequently observed in never smokers (39%) are found in SQ and LG.

G:C $\rightarrow$ T:A transversions showed a strong strand bias: 96% of G nucleotides were located on the DNA coding (non-transcribed) strand and only 4% on the non-coding (transcribed) strand. The bias was stronger in smokers (100%) than in never smokers (86%). In contrast, the DNA strand bias was not observed for G:C $\rightarrow$ A:T transitions (54% of G nucleotides on coding strand, 46% on non-coding strand).

The GSTM1, CYP1A1 (exon 7 polymorphism) and CYP2D6 (alleles A and B) genotypes were determined in 156 NSCLC patients (Table 3). Normal tissue from eight cases was not available. Eighty-three patients were found to be GSTM1-null (53%) and 24 patients were heterozygous for exon 7 polymorphism of CYP1A1 gene (15%). We did not find any patient who was homozygous for the minor Val allele of CYP1A1 (allele frequency 0.08). For CYP2D6, we found 88 (56%) patients who had neither A nor B alleles (predicted phenotype EM), 59 (38%) patients who were heterozygous for one of the mutant alleles A or B (predicted phenotype IM – intermediate metabolizers), and nine (6%) patients who carried either two B alleles or A and B alleles (predicted phenotype PM – poor metabolizers).

No statistically significant gender differences were found of genotype frequencies (Table 3) although the GSTM1-null genotype was more frequent in females. The genotype frequencies in smokers and in never smokers are shown in Table 3. Analysis of genotype frequencies by smoking history showed that frequencies of ‘at risk’ genotypes of GSTM1 (null) and CYP1A1 (Ile/Ile) were higher in never smokers than in smokers, but the differences were not statistically significant (Table 3). The relation between genotypes and p53 mutations and p53 protein overexpression are shown in Table 4. No statistically significant associations between the variables were observed.

We did not observe any statistically significant association between combined GSTM1 and CYP1A1 polymorphisms (GSTM1-positive/CYP1A1 Ile/Ile, GSTM1-positive/CYP1A1 Ile/Val, GSTM1-null/CYP1A1 Ile/Ile, GSTM1-null/CYP1A1 Ile/Val, GSTM1-null/CYP1A1 Ile/Ile) and the muta-


tions in p53 in a group of 133 smokers for whom the genotyping data were available. We also compared the mutation spectra of the p53 gene among GSTM1-positive and GSTM1-null smokers. The p53 mutational spectra in neither group showed statistically significant differences although the deletions/insertions were more frequent in GSTM1-positive individuals (7/40; 18%) than in GSTM1-null ones (2/34; 6%; P = 0.17 Fisher’s exact test). In never smokers we found that almost all (12/13; 92%) GSTM1-null individuals stained positive for the p53 protein whereas only 38% (3/8) GSTM1-positive individuals stained positive for the p53 protein (P = 0.014 by Fisher’s exact test). A similar relation was observed in never smokers for the mutations of the p53 gene; 79% (11/14) GSTM1-null individuals had a mutated p53 gene, whereas only 44% (4/9) GSTM1-positive individuals had mutations in p53; however, this difference was not statistically significant (P = 0.18; Fisher’s exact test).

The accumulation of p53 protein determined by immunohistochemical methods was examined in 119 cases. Immunostaining for p53 was most prominent in the formalin-fixed tissue after microwave treatment, distinct in methacarn-fixed tissue and often either a low or false-negative in formalin-fixed material without antigen retrieval. The p53 immunostaining was found almost exclusively in nuclei of cancer cells. In some cases, single positive cell nuclei were visible in bronchial epithelium or among cells of lymph nodules. Distinct cytoplasmic localization was found in positive cases in some mitotic cancer cells. Accumulation of p53 protein (21/89) or both (54/89). Statistical analyses (Student’s t-test) showed that the mutation of the p53 gene is significantly associated with strong immunohistochemical staining of the p53 protein.

The immunohistochemical analyses of the p21\textsuperscript{WAF1/CIP1} antigen were studied in 119 NSCLC cases immunostained also for p53 protein. Of three fixatives used, both PAF and formalin yielded positive immunostaining of p21\textsuperscript{WAF1/CIP1} after antigen retrieval, while methacarn proved to be unsuitable for the purpose. The p21\textsuperscript{WAF1/CIP1} staining was found to be localized in tumour cell nuclei. In some cases, single nuclei of the bronchial epithelium or cells within lymph nodules were also positive. A positive reaction for p21\textsuperscript{WAF1/CIP1} was present in 82 cases (69%). The majority of p21\textsuperscript{WAF1/CIP1}-positive cases also were reactive with anti-p53 antibodies (56/82; 68%). Samples showing a strong immunohistochemical reaction for p21 protein (+++ more frequently showed strong (++ or ++++) (19/25; 76%) rather than a weak or negative (6/25; 24%) staining for p53 protein. Among 37 cases negative for p21\textsuperscript{WAF1/CIP1} staining, only 19 (51%) showed a positive immunostaining for p53.

In some tumour foci of well-differentiated squamous cell carcinomas, the immunoreaction for p53 was observed in basal cell layers, whereas p21\textsuperscript{WAF1/CIP1}-positive cells were localized in layers of differentiating cells, sometimes displaying the features of keratinization. In other cases, the positive cells were randomly distributed in the cancer tissue. Co-localization of both p53 and p21\textsuperscript{WAF1/CIP1} antigens in the same cell nuclei was also observed.

No association was found between p21\textsuperscript{WAF1/CIP1} IHC staining and any of the following: gender, smoking status, histology, occupational exposure, the stage of the disease or the mutation of the p53 gene (data not shown). When immunohistochemical staining for p21 and p53 was combined, no association between combined staining (both negative, either positive, both positive) and gender, histology or disease stage was found (data not shown).

**DISCUSSION**

The DNA sequence was determined for exons 5–8 of p53 for 164 primary NSCLC from Silesia, a heavily polluted region of Poland. Mutations of p53 were found in tumours of 90 patients (55%). The most frequent mutation type was G:C\rightarrow T:A transversions (31%), which is in agreement with the data of other authors (Figure 1) (Greenblatt et al, 1994; Hainaut et al, 1998: the database of p53 mutations and air pollution 1449

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Table 4  GSTM1, CYP1A1 and CYP2D6 genotypes and p53 gene and protein

| Genotype | p53 seq | p53 seq + IHC |
|----------|---------|--------------|
|          | WT/WT   | WT/A         | WT/B or AB | WT/A or AB | WT/WWT |
| GSTM1    |         |              |            |            |
| Null     | 39 (47%)| 44 (53%)     | 19 (32%)   | 41 (68%)   | 13 (16%)|
|          |         |              | 22 (42%)   | 30 (58%)   | 14 (19%)|
| Positive | 30 (41%)| 43 (59%)     | 51 (31%)   | 60 (69%)   | 25 (19%)|
| CYP1A1   |         |              | 36 (38%)   | 60 (62%)   | 25 (107) |
| Ile/Ile  | 57 (43%)| 75 (57%)     | 12 (51%)   | 11 (49%)   | 2 (2%) |
| Ile/Val  | 12 (50%)| 12 (50%)     | 5 (31%)    | 11 (69%)   | 8 (8%) |
| CYP2D6   |         |              | 3 (50%)    | 3 (50%)    | 2 (22%) |
| BB or AB | 3 (33%) | 6 (67%)      | 38 (58%)   | 68 (68%)   | 25 (122) |
|          | 66 (45%)| 81 (55%)     | 1 (36%)    | 64 (84%)   | 1 (17%) |
|          |         |              | 7 (8%)     | 9 (9%)     | 9 (83%) |

1* P = 0.56 \chi^2; 2* P = 0.33 \chi^2; 3* P = 0.71 \chi^2; 4* P = 0.69 \chi^2; 5* P = 0.84 \chi^2; 6* P = 0.33 Yates corrected \chi^2; 7* P = 0.74 Yates corrected \chi^2; 8* P = 0.79 Yates corrected \chi^2; 9* P = 0.96 Yates corrected \chi^2.
mutations. Surprisingly, G:C→T:A transversions were more frequent, although not significantly, in Silesian never smokers than in smokers (39% and 29% respectively, Table 1 and Figure 1). This was in contrast with p53 mutational spectrum in never smokers from other populations (Figure 1) which showed a predominance of G:C→A:T transitions (47%). Unfortunately, the lung cancer incidence in Silesian never smokers is not known. Our data are a tentative indication that occupational and environmental exposure to polluted air contributes to the molecular pathogenesis of lung cancer in never smokers. To test this hypothesis further, the p53 mutation data from never smokers living in less polluted areas are needed.

In contrast to other reports (Takeshima et al, 1993; Wang et al, 1995; Kondo et al, 1996), we did not find a dose–response relation between the number of smoked cigarettes (pack-years) and either the frequency of all p53 mutations or the frequency of p53 G:C→T:A transversions, which may be explained by the confounding effect of environmental air pollutants. Also it is of interest that 100% of the G:C→T:A transversions in smokers (22 mutations) showed DNA strand bias for the DNA coding strand, which was higher than in other populations studied (reviewed by Greenblatt et al, 1994). The similarity of the mutational spectrum in smokers from our case series and spectra reported by others from Caucasian and Oriental populations may result from a considerable influence that the carcinogens in tobacco smoke have on the mutational processes in target lung cells apart from either the differences in local environment or the differences in genetic background of populations. The spectrum of mutations in p53 in never smokers may reflect also differences in either local environment or in the genetic makeup of populations. Future molecular epidemiological studies on p53 mutations from never smoking lung cancer patients of various ethnic, environmental and occupational backgrounds will clarify these issues.

We detected five p53 mutational hotspots. The most intriguing one is codon 298. Four of its G to T transversions are found in coal-exposed males (all were coal miners) and only one in a non-miner. Two of the miners were never smokers. This mutation is very rare in lung cancers from other geographical regions of the world. The frequency difference is significant for all p53 mutations in NSCLC in the database (5/92 vs 6/495; \( P = 0.03 \); Yates corrected \( \chi^2 \); Hainaut et al, 1998). These data generate the hypothesis that the codon 298 GAG to TAG mutation in coal miners is induced by a carcinogen to which the miners are exposed. According to our knowledge this is the first report of the spectrum of p53 mutations in lung cancers from coal miners. We also noted that males exposed to coal dust and coal-processing substances differed significantly from non-exposed males in frequency of histological types. Adenocarcinomas were much less frequent in exposed than in non-exposed males and non-adenocarcinomas were associated with coal exposure. It is of further interest that tumours in males occupationally non-exposed to coal stained more frequently for the p53 protein than tumours of occupationally-exposed males (\( P = 0.01 \) by \( \chi^2 \)). This, in part, could be explained by a higher frequency of loss-of-function mutations in coal-exposed males (33%) compared with non-exposed ones (25%; Table 2). The apparent discrepancy between the p53 immunohistochemistry status and p53 sequencing data in Table 2, can result from the fact that small deletions, insertions and non-sense mutations generally lead to no detectable p53 protein and, therefore, negative p53 immunohistochemistry staining. Conversely, positive p53 immunohistochemistry staining can result from other mechanisms than p53 mutations that are not understood at present.

The epidemiological data indicate that lung cancer mortality is not elevated in Polish coal miners (Starzynski et al, 1996). This is consistent with the results of studies performed on coal miners from other countries, e.g. UK, USA, The Netherlands (Miller and Jacobsen, 1985; Meijers et al, 1991; Kuempel et al, 1995). We do not suggest that there is a substantial lung cancer risk in coal miners. Most of the coal miners from our patient series were smokers. It is conceivable that some carcinogens in coal mines with concurrent tobacco smoking do not substantially increase lung cancer risk but induce a different ‘pathway’ of cancer formation, hence, for example, an elevated frequency of non-adenocarcinomas and p53 immunohistochemistry-negative tumours in coal exposed patients (Table 2). The similar analyses performed on coal miners from other countries would be of great value.

Significant differences in the p53 mutational spectra between adenocarcinomas and squamous cell carcinomas and between adenocarcinomas and large cell carcinomas were detected in our case series. The most striking difference in mutational spectra between adenocarcinomas and squamous cell carcinomas was the frequency of G:C→T:A transversions, which are much more frequent in adenocarcinomas (55%) than in squamous cell carcinomas (20%; \( P = 0.008; \chi^2 \); Table 1). When only G:C→T:A transversions, showing DNA strand bias (G base located on DNA coding strand) were considered, the difference was even more obvious (55% vs 19%).

The higher susceptibility to lung cancer was associated with the exon 7 polymorphism of CYP1A1 gene in Japanese population. The frequency of Val allele in Japanese lung cancer patients is 0.26 (Nakachi et al, 1993). It was also shown that the p53 gene is more frequently mutated in patients with the CYP1A1 Val/Val genotype (Kawajiri et al, 1996; Przygodzki et al, 1998). We have not detected the similar relationship in our case series (not shown). However, only 15% of the patients were heterozygous for the Val allele so that our study does not have sufficient statistical power to adequately test the hypothesis proposed by Kawajiri et al (1996).

**GSTM1-null** heavy smokers have a higher risk of transversion mutations in the p53 gene (Ryberg et al, 1994), although it was not observed by other investigators (Kawajiri et al, 1996). In the Silesian cases, the spectrum of p53 mutations is similar among smokers who are GSTM1 wild-type and null. Moreover, in contrast with the report of Ryberg et al (1994) GSTM1-positive patients from our case series tend to have a higher p53 mutation frequency than GSTM1-deficient individuals (Table 4). Taken together, our data indicate that the influence of GSTM1 on p53 mutagenesis is small in the Silesian cases.

The role of CYP2D6 in modulating lung cancer susceptibility is controversial. So far, two tobacco smoke components: 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane (NNK) and nicotine, have been proposed as substrates for CYP2D6 (Crespi et al, 1991; Cholerton et al, 1994). The frequency of predicted phenotypes for our group of patients is almost identical to the frequency of phenotypes determined by pharmacogenetic methods in a healthy Polish population (EM: 58% by genotyping vs 57% by metabolite measurements, IM 37% vs 37%, PM 5% vs 6%) (Kunicki et al, 1995). Considering the CYP2D6 genotype (presence of alleles A and B), we classified the patients by the two predicted phenotypes (EM or IM and PM). Since we detected only nine patients with predicted PM phenotype, it was not possible to reasonably compare mutational spectra between CYP2D6 proficient and deficient individuals.

Our study demonstrated that the use of the anti-p21(raf)CIP1 anti-
body stained nuclei of tumour cells selectively, leaving the normal lung tissue unstained. In our material, the \( p21_{WAF1/CIP1} \) product occurred independently of the status of p53 protein or gene. Study performed on a different NSCLC case series from the USA yielded similar results (Bennett et al., 1998). In conclusion, we observed that more than 50% of NSCLC patients had mutations within p53 in DNA isolated from tumour tissues. In contrast with other studies (reviewed in Greenblatt et al, 1994; Hainaut et al, 1998; Hussain and Harris, 1998), the never smokers had a high frequency of G:C - T:A transversions. We observed that males exposed occupationally to coal dust and coal processing substances (mostly coal miners) constitute a group of patients that can be characterized by a relatively high frequency of squamous and large-cell carcinomas, relatively frequent nonsense mutations in codon 298 of p53, and a relatively low frequency of p53 immunohistochemistry-positive tumours. These data are a tentative indication that occupational and environmental exposure to polluted air contributes to the molecular pathogenesis of lung cancer in Silesian residents.

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