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Lung Cancer Incidence and Survival in Chromium Exposed Individuals with Respect to Expression of Anti-apoptotic Protein Survivin and Tumor Suppressor P53 Protein

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Abstract

Objective: Workers chronically exposed to hexavalent chromium have elevated risk of lung cancer. Our study investigates the incidence of lung cancer types, age at onset of the disease, and survival time among chromium exposed workers with respect to the expression of anti-apoptotic p53 and pro-apoptotic survivin proteins.

Material and methods: 67 chromium exposed workers and 104 male controls diagnosed with lung cancer were analyzed. The mean exposure time among workers was 16.7 ±10.0(SD) years (range 1- 41 years). To investigate the possible regulation of survivin by p53 we examined the expression of both proteins using immohistochemical visualization.

Results: Chromium exposure significantly decreases the age of onset of the disease by 3.5 years (62.2 ±9.1 in the exposed group vs. 65.7 ±10.5 years in controls; P=0.018). Small cell lung carcinoma (SCLC) amounted for 25.4% of all cases in chromium exposed workers and for 16.3% in non-exposed individuals. The mean survival time in the exposed group was 9.0 ±12.7 vs. 12.1 ±21.9 months in controls, but this difference was not significant. Survivin was predominantly expressed in both cell nucleus and cytoplasm, whereas p53 was expressed in the nucleus. There was a negative correlation between survivin and p53 expression. A decreased intensity of expression and fewer cells positive for survivin was detected in SCLC compared with other types of lung cancer. p53 was expressed in 94.1% and survivin in 79.6% of the samples analyzed.

Conclusion: The study calls attention to decreased expression of survivin, as opposed to p53, in small cell lung carcinoma.

Key words: lung cancer, chromium exposure, survival, onset age, survivin, p53

Introduction

Lung cancer is the world's leading cause of cancer death. It is primarily due to the inhalation of carcinogens and highly accessible to prevention by diminishing exposure to lung carcinogens. Smelters are regularly exposed to higher levels of chromium (Cr) at the workplace in comparison with non-exposed individuals; respiratory tract being the major route of exposure. Based on in vitro and animal data as well as on epidemiological [1-5] and cytogenetic studies in humans [6], IARC has classified hexavalent chromium as a carcinogen of the group I.

Entering cells, chromium induces formation of reactive intermediates, resulting in enhanced oxidative stress [7]. Oxidative stress caused by intermediates formed during chromium reduction has cyto- and genotoxic effect [8, 9]. During Cr(VI) reduction, a diverse range of genetic lesions are generated including Cr-DNA binary (mono) adducts, Cr-DNA ternary adducts, DNA protein cross-links, bi-functional (DNA inter-strand cross-links) adducts, single-strand breaks, and oxidized bases. Cr(VI) exposure elicits a classical DNA damage response within cells including activation of the p53 signaling pathway and cell cycle arrest or apoptosis [10].

Apoptosis or programmed cell death is needed for maintenance of cell homeostasis and to destroy cells that represent a threat to the integrity of the organism. Apoptosis can be induced by either specific extracellular signals or internal stimuli. The molecular mechanisms involved in apoptotic enzymatic pathway have been sufficiently reviewed [11]. Protein p53 plays an important role in apoptosis induction. It acts as a transcription factor which is in humans encoded by the TP53 gene [12, 13]. p53 is activated by various stress signals as radiation (UV, gamma), carcinogens (polycyclic aromatic carbohydrates, heavy metals), oxidative stress, hypoxia, oncogene activation, telomere shortening, and others [14]. Apoptosis induction is one of the main functions of p53.

The expression and activity of p53 are precisely regulated at many levels [15]. p53 prevents tumor formation through cell cycle, blocking and eliminating damaged cells. Mutations or inactivation of p53 are the most frequent changes in human tumorous cells [16]. On the other hand, survivin is a member of *IAP* gene family, which has been implicated in both inhibition of apoptosis and mitosis regulation [17]. Survivin up-regulates genes in tumor tissues [18]. High survivin expression is related to poor prognosis in many cancer types [19, 20] Some investigations have shown that

p53 leads to the repression of survivin expression in non-small lung cancers [21]. There are many studies that show the expression of the mentioned proteins in non-small cell lung cancer, but only few regarding small cell carcinomas (SCLC) [22-24].

The present study focuses on the investigation of the incidence of lung cancer types, age at onset of disease, and survival time among chromium exposed workers (smelters, tapers, crane operators) with respect to the expression of anti-apoptotic p53 and pro-apoptotic survivin proteins.

Material and Methods

Subjects and Sampling

The study was performed in accordance with the Declaration of Helsinki for Human Research and study protocol was approved by a local Ethics Committee. Data were analyzed available at the Department of Pathology of Dolny Kubín Hospital and of the Slovak National Cancer Register covering the period 1985–2005 (278 men diagnosed with lung cancer). A hundred and seventy one cases were selected for the present study with a clear histopathological lung cancer type. According to chromium exposure two groups were formed. The exposed group consisted of 67 former workers who had contact with ferrochrome alloys, and who were diagnosed with lung cancer. The mean time of exposure was 16.7 ±10.0 years. The control group consisted of 104 men, who also were diagnosed with lung cancer, but were never exposed to Cr or any other known carcinogen.

Exposure Data

Chromium analysis in soil and air was made in the vicinity of the workplaces. Samples were examined by atomic absorption spectrometry (Varian Spectrophotometer AA30-P, Varian B.V. Scientific Instruments, Middelburg, The Netherlands). The mean all-shift concentrations of total chromium in the air of the smelting plant were 0.03–0.19 mg m^{-3}, the values of hexavalent chromium were between 0.019-0.03 mg m^{-3}. The mean concentrations of total chromium in the air in the environment surrounding the workplaces and in the control area (0.0113 μg m^{-3}) did not reach the recommended norm (0.01 – 0.0117 μg m^{-3}). In the soil, in a distance of 200 m from the workplaces, the chromium content was 137 mg kg^{-1}, which is slightly exceeding the recommended norm of 100 mg kg^{-1}. The chromium contents in the soil at a farther distance and from the control area were below the recommended norm (60.2 mg kg^{-1} and 46.0 mg kg^{-1}, respectively).

Sixty seven samples from the study patients were suitable for the evaluation for survivin and p53 expression. The remaining specimens had to be discarded due to damage. The hematoxylin and eosin stained slides from each case were independently reviewed by two pathologists to ascertain the diagnosis based on morphological and immunohistochemical parameters and were correlated with clinical data. Three sections 4 μm thick, obtained from each paraffin block, were stained for p53 and survivin proteins. To achieve greater adherence of the sections to glass surface, silanized slides (DAKO, Denmark) were used, which had been heated for 2 h in an oven at 56 °C. Then the sections were deparaffinized in xylene for 20 min, rehydrated in a series of descending ethanol concentrations and washed with phosphate-buffered saline (PBS). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigen unmasking was achieved by heating the sections which had been immersed in the target solution (DAKO) within hot water bath (96 °C) for 45 min. Immunohistochemical staining was performed using monoclonal mouse anti-p53 antibody (DAKO, Clone DO-7, dilution 1:50) and monoclonal mouse anti-survivin antibody (DAKO, Clone12C4, dilution 1:50). After overnight incubation, the p53 and survivin antigens were visualized by means of the LSAB Visualization System (DAKO) using 3, 3'- diaminobenzidine chromogen as a substrate; according to the manufacturer's instructions. All sections were counterstained with Mayer's hematoxylin (DAKO). Negative controls were obtained by omitting the primary antibodies.

In each case, the following features were assessed: 1) the intensity of staining; 2) the relative number of positively stained cells; and 3) the subcellular localization of p53 and survivin antigens.

Statistical elaboration was performed with a Chi^2 test or Fischer's exact test to compare differences in the observed parameters between survivin and p53 immunoreactivity. Spearman's coefficient was used to estimate the correlation between parameters. All statistical calculations were performed using Microsoft Excel and MedCalc v.5 software for Windows.

Results

The age at onset of disease and survival time are given in Table 1. Chromium exposure significantly decreased

Table 1. Number of cases, mean age at onset of lung cancer, and survival time in patients exposed and non-exposed to chromium.

Group	No. of cases	Age at onset range (yr)	P	Survival range (mo)	P
Exposed	67	62.2 ±9.1 39-82		9.0 ±12.7 0.3-60	
Non-exposed	104	65.7 ±10.5 43-87	0.018*	12.1 ±21.9 0.5-210	0.473

*Significant difference between exposed and non-exposed patients by t-test.

the age at which disease began by a mean of 3.5 years (62.2 ±9.1 years in the exposed group compared with 65.7 ±10.5 years in the unexposed group; P = 0.018). No significant correlation between the age at which disease began and the time of exposure was found (P>0.05). The mean survival time in the exposed group was 9.0 ±12.7 months compared with 12.1 ±21.9 months in the unexposed group; but this difference was not significant (P = 0.47). Survival of more than 5 years concerned only 3 (1.7%) men.

Table 2 shows the analysis of lung cancer types. Small cell lung carcinoma (SCLC) formed 25.0% of all cases in the chromium exposed workers and 16.3% in the non-exposed individuals. No correlation was found between the age at which disease began and the time of exposure.

Table 2. Number and percentage of cases according to lung cancer type in patients exposed and non-exposed to chromium.

	Exposed No. of cases (%)	Non-exposed No. of cases (%)
Non-small cell lung cancer	50 (74.6)	87 (83.7)
Small cell lung cancer	17 (25.4)	17 (16.3)

Table 3 shows the results of p53 and survivin expression profiles. Survivin was predominantly expressed in both nucleus and cytoplasm in 58 cases (96.7%), whereas p53 was expressed in 56 (88.9%) in the nucleus only. A majority of cases - 61 (92%) showed more than 25% of positively stained cells per field of view for p53 in comparison with only 18 cases (29%) with more than 25% of positively stained cells per field of view for surviving; the difference being significant (Chi^2 = 53.8, P<0.001). There was a negative correlation (r = -0.72) between survivin and p53 expression. It seems that p53 down-regulated the survivin expression. A comparison of non-small and small cell lung cancer types for the survivin expression and its intensity showed a significant decrease in the intensity and a fewer number of cells positive for survivin in small cell lung cancer (Chi^2 = 15.3, P<0.001; Chi^2 = 8.4, P<0.05, respectively). There was no significant difference in the intensity of expression and in the number of cells positive for p53 between small cell and non-small cell lung cancer types (Chi^2 = 1.8, P>0.06; Chi^2 = 0.1, P>0.75, respectively). Neither was there an appreciable difference in the survival time between the patients with or without p53 and survivin expression.

Discussion

Lung cancer is currently the most common cause of cancer mortality in males worldwide. This is largely due to the effect of cigarette smoking and to exposure to other carcinogens. Our previous studies [8, 25, 26] and many other epidemiological studies [27-31] show that workers in ferrochromium industry have excess risk for chromosomal injury and lung cancer and that the onset of disease starts at younger age. However, the information on the influence of chromium exposure on the age of disease onset is missing in the literature. Studies on the issue point to genetic predispositions and conclude that genetic constitution can play a role [32-37] in that the appearance of lung cancer in first-degree relatives can increase the risk of the early onset of lung cancer 5-fold [38, 39].

Concerning different lung cancer types we found that small cell lung carcinoma made up 25.0% of all cases in chromium exposed workers and 16.3% in non-exposed individuals. Similar findings were published by Kavcova et al [40], who found spinocellular lung cancer was the predominant type and 25.0% of patients had small cell lung cancer. Etzel et al [41] analyzed 230 early onset lung cancer (EOLC) and 426 later-onset cases (LOLC). In their study, median survival time was 16.7 months for EOLC and 19.2 for LOLC, and the 24-month survival time was 20.6 and 29.5%, respectively. Our findings did not show an appreciable difference in the median survival time between the exposed and non-exposed groups; 9.0 ±12.7 and 12.1 ±21.9 months, respectively. Only did the survival time exceed 5 years in 3 patients.

p53 is a multifunctional protein that regulates cell division and activates apoptosis. On the other hand, survivin can act as an apoptosis inhibitor which is overexpressed in many malignancies, including lung carcinoma. A lot of studies have been focused on the relationship between survivin and p53 expression, but the results obtained are quite controversial. Jin et al [42] and Nakano et al [43] have suggested that survivin expression is negatively regulated by p53. They conclude that survivin gene is negatively regulated by p53 in NSCLC, and that survivin expression could inhibit apoptosis and accelerate tumor proliferation to produce more aggressive carcinomas. Some of the above outlined findings are in accordance with our results. We found a neg-

Table 3. Expression of survivin and p53 in 67 biopsies from patients with lung cancer.

	I				%		S.L.		
	0	+	++	++	<25	>25	N	C	NC
Survivin	7	20	32	8	43	18	1	1	58
p53	4	13	29	21	5	61	56	1	6

I-intensity of immunoreactivity: + weak, ++ moderate, +++ strong; % - of labelled cells; S.L. - Subcellular localization of surviving and p53 positivity: N-nuclear, C- cytoplasmic, NC-nuclear and cytoplasmic.

ative correlation between p53 and survivin expression, which confirms a clear relationship between these two opposite-acting proteins. However, we did not find a significant difference in survival time between patients with or without p53 and survivin expression.

Contrary results have been published by Akjurek et al [24]. The aim of his immunohistochemical study was to investigate the role of survivin in the early steps of lung carcinogenesis and non-small cell carcinomas, and its relationship with the expression of p53. The authors have found no correlation between survivin and p53 expression; however, the patients in whom survivin was expressed had a significantly worse prognosis. Other studies demonstrate a prognostic importance of p53 mutations and overexpression in lung cancer tissues [44, 45].

Molecular mechanisms of tumor progression and apoptosis are still unclear. Several predictors, such as nodal involvement, tumor stage, survivin and p53 expressions have been reported. However, the relationship between p53 or survivin and the prognosis of lung cancer patients is still controversial [46-48]. Our study calls attention to the expression of survivin in relation to p53 in small cell lung carcinoma. The results of this study suggest that survivin expression in small cell lung carcinoma is decreased in comparison with other lung cancer types. Further studies are required to confirm this suggestion, which for the time being remains speculative.

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