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Renal cell carcinomas in trichloroethene (TRI) exposed persons are associated with somatic mutations in the von Hippel-Lindau (*VHL*) tumour suppressor gene

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Abstract Renal cell carcinomas (RCC) develop as a consequence of somatic mutations of the von Hippel-Lindau (*VHL*) tumour suppressor gene. Recent epidemiological studies show that high and prolonged occupational exposures to trichloroethene (TRI) are associated with an increased incidence of RCC. Tumour tissues from 23 RCC patients with occupational histories of very high TRI exposure were analysed for somatic mutations within the *VHL* gene. DNA was isolated from microdissected tumour cells, amplified by polymerase chain reaction (PCR), and analysed in single strand conformation polymorphism (SSCP) and sequencing. RCC tissues of all 23 TRI exposed persons analysed thus far showed aberrations of the *VHL* gene, with 30% having aberrations in exon 1, 44% in exon 2, and 26% in exon 3. By comparison to much lower reported *VHL* mutation frequencies of 33–55% in TRI-unexposed RCC patients, these results indicate a specifically high mutation frequency at the *VHL* gene in TRI-exposed RCC patients; four of these aberrations have thus far been confirmed as *VHL* mutations by sequence analysis. This finding indicates the *VHL* gene being a susceptible and specific target in TRI induced renal carcinogenesis. Furthermore, the frequent involvement of exon 2 identifies potential 'hot spots' for this carcinogen. In addition to the available epidemiological studies the results are now further proof for human renal carcinogenicity induced by high occupational exposures to TRI.

Key words Trichloroethene · Renal cell cancer · *VHL* · Tumour suppressor genes

Introduction

Trichloroethene (TRI) has been used for many decades as a degreasing solvent in the metal and electronic industries (Bruckner et al. 1989). Recently, high and prolonged occupational exposures to TRI have been reported to cause increased incidence of renal cell carcinoma (RCC; Henschler et al. 1995; Vamvakas et al. 1996). Moreover, TRI-exposed RCC patients show increased frequency of abnormal urinary protein patterns compared to unexposed RCC patients, indicating a TRI-associated tubular damage preceding or enhancing its nephrocarcinogenic effect (Brüning et al. 1996). The nephrocarcinogenicity of TRI has been linked to specific metabolites generated by the glutathione (GSH) dependant pathway. The initial GSH conjugate of TRI is processed to dichlorovinylcysteine (DCVC), which is cleaved by renal tubular β -lyase to highly reactive chlorinated thioketenes. Such intermediates react readily with proteins and DNA (Vamvakas et al. 1993; Müller et al. 1994).

A genetic hallmark of common RCC is the deletion of genetic material from the short arm of chromosome 3 (3p; Zbar et al. 1987). Based on these findings, a gene responsible for renal cancer was hypothesized to be located within this chromosomal region. Recently, the *VHL* gene has been isolated from that region by positional cloning, and it could be demonstrated that this gene fulfilled the criteria of a tumour suppressor gene (Latif et al. 1993): both homologous *VHL* alleles are mutated in the tumour tissue, and hereditary as well as sporadic forms of RCC follow the same two-hit mutational mechanism (Knudson 1971). The coding region of the *VHL* gene contains 639 nucleotides (nt) distributed in three exons of 340 nt, 123 nt and 176 nt, respectively. The identification of these sequences allows direct in-tragenic mutation analyses. Somatic *VHL* mutations

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have been identified by different groups to occur in 33–55% of sporadic RCC (Gnarra et al. 1994; Shuin et al. 1994; Foster et al. 1994; Whaley et al. 1994). About half of these known mutations affect exon 2, which contributes to less than 20% of the coding *VHL* sequence. Such disproportionate distribution of somatic *VHL* mutations has been viewed as an indication of a preferential target site for environmental carcinogens. In order to elucidate a possible genotoxic effect of TRI on the *VHL* gene it is important to establish its mutational pattern in high-TRI exposed RCC patients. This report indicates a specific role of TRI in human renal carcinogenesis.

Materials and methods

Twenty-three patients with histologically verified RCC and with a specific history of prolonged occupational exposure to high concentrations of TRI were studied. These patients were obtained from two clinical case-control studies (one finalized, one ongoing) and/or were pending legal compensation cases regarding an occupational disease due to TRI. An extensive review of the occupational history was performed in all patients. Paraffin-embedded tumour tissue of these patients was obtained from the respective clinical pathology units which were involved in setting the original histological diagnosis.

All TRI-exposed RCC patients had been regularly handling TRI under presently unacceptable conditions. Room temperatures ranged frequently between 30 and 50 °C in small and poorly ventilated working areas. According to the symptoms described by the patients, concentrations of TRI must have exceeded manyfold the current German occupational exposure limit (MAK) of 50 ppm. The patients reported that they often had noticed a massive smell of TRI at work and often described frequently recurring symptoms such as dizziness, headache, nausea and drowsiness. Many times a day the patients left their workplaces for short periods of time, to get fresh air and to recover from these acute symptoms (see also the descriptions by Vamvakas et al. 1996). The average time of exposure to TRI of the patients studied was 21.8 years. Semiquantitative assessments of levels of TRI exposure are included in Table 1.

DNA of normal and tumour tissue was isolated using QiaAmp tissue kit. Amplification of individual *VHL* exons was with primer sets *VHL* 28/22 (exon 1), 15/13 (exon 2) and YH1A/6b (exon 3). Single strand conformation polymorphism (SSCP) analysis of the polymerase chain reaction (PCR) products was in 8 and 10% polyacrylamide gels, containing 0.09 M TRIS-phosphate buffer, 0.02 M EDTA and 2% glycerol. Running buffer comprised 0.02 M TRIS-borate and 0.01 M EDTA. Temperature during electrophoresis was 4, 15, or 25 °C. Sequencing of both strands of DNA was carried out by the dye terminator method on a 373 DNA sequencer (Sequiseq). Detailed descriptions of the PCR, SSCP and sequencing methodologies are given by Glavac et al. (1996).

Results

Twenty-three RCC tumours were analysed for somatic mutations in the *VHL* gene. According to the semi-quantitative exposure assessment by Vamvakas et al. (1997), nine patients were graded a TRI exposure history of level (++) and 14 patients of level (+++; Table 2). In comparison to *VHL* wild-type sequences, all patients showed an abnormal SSCP pattern in either one of the *VHL* exons [Fig. 1]. The SSCP variations were located within exon 1 in 7/23 (30%) patients, within exon 2 in 10/23 (44%) patients and within exon 3 in 6/23 (26%) patients (Table 2). Currently we are performing further sequencing analyses of these variations by SSCP, so far we have confirmed four SSCP band shifts as *VHL* mutations. Tumour number 1696 had a TC deletion of nucleotides 632 and 633, and tumor no. 1680 had a G deletion at nucleotide 637, both located within exon 2. The SSCP variation of tumor no. 1677 revealed an 18-bp deletion at nucleotide 512 of exon 1. The SSCP band shift of tumour no. 1685 was due to a nucleotide change from C to T at position 811 in exon 3. The mutations in exon 1 and 2 were of frameshift type, the mutation in exon 3 was a missense mutation.

Discussion

The carcinogenicity of TRI is currently being controversially discussed. Chronic exposure to TRI led to an increased incidence of malignant liver, lung and kidney tumours in rats and mice (Maltoni et al. 1988; NTP 1990). In human epidemiology, four large cohort studies on workers exposed to TRI showed no significant increase of cancer morbidity or mortality (Weiss 1996). However, exposure concentrations might have been low in these studies, and analysis of mortality instead of incidence might have influenced the results in cases of non-fatal tumours. The observation of an increased incidence of RCC in workers with extremely high and prolonged occupational exposure to TRI again raised the question of a carcinogenic effect of TRI in humans (Henschler et al. 1995). The results of this particular study have been corroborated by a further case control study in the same geographical area (Vamvakas et al.

Table 1 Semiquantitative exposure assessment of persons occupationally exposed to trichloroethene (TRI), according to Vamvakas et al. (1996). At least one criterion of each column had to be fulfilled in order to categorize the exposure levels

Exposure level grading	Duration/frequency of exposure	Handling of TRI	Adverse effects: dizziness, vertigo, headaches
+++	>5 years and/or daily for at least 1 h	TRI temperature >60 °C and/or 200 l Open TRI tubs	>3 x/week, severe prenarcoctic symptoms
++	>3 years and/or >2 x/week for at least 1 h	Open TRI tubs	>1 x/week, moderate symptoms
+	>3 years but <2 x/week and/or <2 h/week	Others	Only occasionally light symptoms

Table 2 von Hippel-Lindau (VHL) mutations in renal cell carcinoma (RCC) tissue of patients, related to age and TRI exposure data

Tissue/patient no.	Age at diagnosis (years)	TRI exposure (see Table 1)	TRI exposure, months	VHL-variation in exon no.
1654	67	+++	240	2
1655	53	++	62	2
1657	56	++	56	2
1662	67	++	34	1
1663	66	++C	136	2
1669	61	+++	288	3
1670	62	+++	540	1
1676	84	+++	168	1
1677	57	++	%	1
1679	65	++	192	1
1680	73	++	480	2
1683	55	++	246	2
1684	57	+++	132	2
1685	57	+++	360	3
1687	83	+++	390	2
1689	60	++	48	1
1691	59	+++	251	3
1694	74	+++	420	3
1695	38	+++	132	3
1696	57	+++	58	2
1697	55	++	312	3
1698	61	+++	300	1
1700	56	+++	460	3

1996). In view of the strong and somewhat emotional debate of this issue (Swan 1995; Bloemen and Tomenson 1995; Henschler et al. 1995b) further, independent lines of argumentation concerning the human renal carcinogenicity of TRI are helpful. Therefore, the introduction of new arguments based on specific molecular biological features of TRI-induced renal cell tumours are of utmost scientific and practical importance.

A known causative molecular event in RCC are somatic *VHL* mutations (Gnarra et al. 1994). The *VHL* mutation patterns heretofore observed within exon 2, a frequent site of mutations in sporadic RCC, led to the conclusion that environmental carcinogens might play a major role in RCC pathogenesis. In the light of a sta-

tistically significant correlation between TRI exposure and the occurrence of RCC (Vamvakas et al. 1996), the present study investigates whether the *VHL* gene is a specific molecular target for TRI. This is the first report to link a known molecular genetic cause of RCC with exposure to a specific, and practically important chemical toxicant.

We have identified aberrations of the *VHL* gene in all 23 RCC patients studied with high and long-term occupational exposure to TRI. Initial sequencing results confirm these alterations as being *VHL* mutations with a potential loss or change of the biological function of the *VHL* protein. Thus, it is most likely that the high frequency of *VHL* aberrations observed here may be due to specific mutational events within the *VHL* gene. The mutational events affected exon 1 in 30%, exon 2 in 44% and exon 3 in 26% of the tumours in TRI-exposed persons.

These results are the first indication of differentiation between TRI-exposed and non-TRI-exposed RCC patients with regard to *VHL* mutation rates and frequencies. Whereas only 33–55% of non-TRI-exposed RCC showed *VHL* mutations (Gnarra et al. 1994; Shuin et al. 1994; Foster et al. 1994; Whaley et al. 1994), 100% of the highly TRI-exposed RCC patients are affected by such mutations. Also, despite the previous observations of frequent exon 2 mutations in sporadic non-TRI-exposed RCC, these mutations accounted for only 24% of all tumours analysed (Gnarra et al. 1994). The high frequency (44%) of exon 2 mutations in TRI-exposed RCC patients, compared to the lower mutation frequency in non-TRI-exposed patients, is apparently due to the potential of TRI to form chemical intermediates capable of interactions with DNA. The responsible mechanism involves specific bioactivation reactions. There are two competing pathways in the metabolism of TRI. The

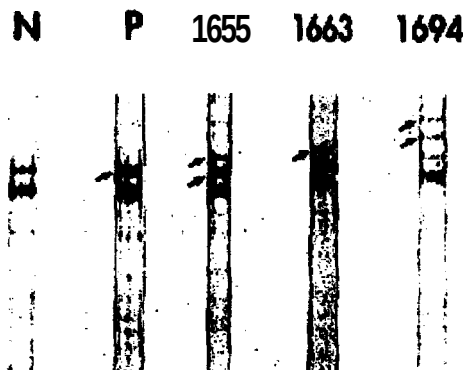


Fig. 1 Example of von Hippel-Lindau (VHL) mutation screening in exon 2 by single strand conformation polymorphism analysis. N is a normal control, P is a known control with an additional band (arrowed). Tumour nos. 1655, 1663, 1694 are from trichloroethylene exposed renal cell carcinoma patients showing additional bands (arrowed) indicative for various mutations

major pathway involves biotransformation by cytochrome P 450. In the second pathway, TRI is conjugated by glutathione S-transferases in a dose-dependant manner. Glutathione conjugates derived from TRI are formed, especially following high exposure, and are further processed into reactive intermediates with proteins and DNA (Müller et al. 1994). The present observations are in accordance with this mechanistic concept (Müller et al. 1994) and provide further proof for the human renal carcinogenicity of high exposures to TRI. In order to confirm further the mutational effects of TRI on the *VHL* gene, we are currently exploring the mutational patterns of the *VHL* gene in more detail, to establish a specific 'genetic fingerprint' of human RCC caused by TRI exposure.

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