

Trichloroethylene Exposure and Specific Somatic Mutations in Patients With Renal Cell Carcinoma

Hiltrud Brauch, Gregor Weirich, Maria Anna Hornauer, Stefan Störkel, Thorsten Wöhl, Thomas Brüning

Background: The development of renal cell carcinoma (RCC) has been associated with both genetic and environmental factors—with mutations in the von Hippel–Lindau (VHL) tumor suppressor gene for clear-cell RCC specifically and with long-term exposure to high doses of trichloroethylene (TRI), an industrially important solvent, for RCC generally. We investigated whether TRI exposure produces RCC through a specific mutational effect on the VHL gene by analyzing VHL sequences in the RCCs of patients exposed to high, cumulative doses of TRI. **Methods:** The level of exposure for each of 44 patients with RCC who had known industrial exposure to TRI was classified according to the duration, frequency, and mode of exposure. Samples of normal and cancerous tissues were microdissected from paraffin-embedded tissue. DNA was isolated from these samples, and somatic VHL mutations were identified by polymerase chain reaction analysis, single-strand conformation polymorphism analysis, DNA sequencing, and restriction enzyme digestion. Control samples included RCC DNA from 107 patients without known TRI exposure and lymphocyte DNA from 97 healthy subjects. **Results:** RCCs of TRI-exposed patients showed somatic VHL mutations in 33 (75%) of 44 cases. The mutations were frequently multiple and accompanied by loss of heterozygosity, and there was an association between the number of mutations and the severity of TRI exposure. We observed a specific mutational hot spot at VHL nucleotide 454 in the RCCs of 13 (39%) of the patients, and this mutation was present in adjacent non-neoplastic kidney parenchyma in four of these patients. The nucleotide 454 mutation was neither detected in any of the RCCs from patients without TRI exposure nor in any of the healthy subjects. **Conclusion:** Our results suggest that RCC in patients with high, cumulative TRI exposure is associated with a unique mutation pattern in the VHL gene. [J Natl Cancer Inst 1999; 91:854–61]

Clear-cell renal cell carcinoma (CCRCC) is one of the few human tumors known to evolve from mutations of a specific gene (1). Mutations of the von Hippel–Lindau tumor suppressor gene (VHL) were detected both in patients with hereditary von Hippel–Lindau syndrome (2) and in about 60% of patients with sporadic CCRCC (3–6). Germline and somatic VHL mutations are distributed within a 470-base-pair region that comprises all three exons of the gene. The germline mutation spectrum is characterized by a high percentage of missense mutations (2). Somatic mutations, however, mostly involve promoter methylation (6) and deletions or insertions that may lead to frameshifts (3–5). Moreover, for unknown reasons, mutations in VHL exon 2 are much more frequent in sporadic CCRCCs than in hereditary tumors (3).

Because the kidneys function primarily as excretory organs, they are exposed to a variety of different metabolites. Thus, kidneys are likely targets for exogenous carcinogens. On the basis of epidemiologic studies, for example, tobacco use is considered to be involved in nephrocarcinogenesis (7). Evidence is accumulating that a defined substance, the industrial solvent trichloroethylene (TRI), plays a role in renal carcinogenesis (8). Whereas acute effects of exposure to high doses of TRI are well known (i.e., dizziness, vertigo, nausea, and headache), long-term effects are still a matter of debate. An increased incidence of renal cell carcinoma (RCC) was reported among workers exposed to high doses of TRI (9). Independently, a case–control study (10) disclosed an association between high-dose TRI exposure and the incidence of RCC. However, a molecular basis for this association between exposure to a specific carcinogen and occurrence of RCC remains to be established.

Because RCC may be initiated by mutations of the VHL gene (1) together with the nephrocarcinogenic effect of TRI in the kidneys (8–10), one might expect to find an association between TRI exposure and VHL mutations. We reasoned that any carcinogenic impact of TRI on the VHL gene ought to be found in RCCs of exposed patients; hence, we sought to document this relationship.

SUBJECTS AND METHODS

Case patients and control subjects. Because TRI is considered a potential carcinogen, public health regulations in some countries mandate the registration of TRI-exposed employees. In Germany, occupational TRI exposure is monitored by the employer's liability insurance association (Berufsgenossenschaft). All 44 RCC case patients enrolled in this study were registered former employees of metal-processing factories with histories of high, cumulative TRI exposure. An extensive medical review covered each subject's occupational history and smoking habits. Subjects were interviewed individually and asked to complete a questionnaire. Paraffin blocks of RCCs from all patients were reviewed histologically by two independent observers (G. Weirich and S. Störkel). Lymphocyte DNA, tumor DNA, and DNA from morphologically normal adjacent parenchyma were obtained from all 44 RCC patients. Control samples consisted of tumor DNA from 107 RCC patients not registered as TRI exposed. Thirty-four patients with RCC were recruited from the same geographic area as TRI-exposed patients with RCC (the study panel), and 73 RCC specimens were obtained from university hospitals in other regions in Germany. Controls for analysis of germline VHL gene status included lymphocyte DNA of 97 subjects without RCC, of whom 47 were employees registered as TRI exposed. TRI

Affiliations of authors: H. Brauch, T. Wöhl, Research Laboratory of the Women's Hospital Eppendorf, University of Hamburg, Germany; G. Weirich, M. A. Hornauer, Institute of Pathology, Technical University Munich, Germany; S. Störkel, Institute of Pathology, University of Witten-Herdecke, Wuppertal-Barmen, Germany; T. Brüning, Institute of Occupational Physiology, University of Dortmund, Germany.

Correspondence to present address: Hiltrud Brauch, Ph.D., Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Auerbachstrasse 112, 70376 Stuttgart, Germany (e-mail: hiltrud.brauch@ikp-stuttgart.de).

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exposure and sample origin for patients and control individuals are listed in Table 1. The study was approved by an institutional review board, and all patients gave written informed consent.

Questionnaire. The questionnaire covered occupational activities and smoking habits. Individuals were questioned about exposure to potential carcinogens and nephrotoxic agents, such as TRI, perchloroethylene, lead, cadmium, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, asbestos, tar, tar products, cutting fluids, and any other chemicals. In addition, smoking habits and medical and family histories—especially history of kidney diseases—were recorded. For deceased patients, information was obtained from relatives and former co-workers. Data on TRI exposures were also obtained from occupational hygienists and from records of the employer’s liability insurance association. TRI exposure was ranked as one of three levels by a defined scoring system that integrates total exposure time as well as frequency and duration of acute adverse effects (10).

VHL mutations and loss of heterozygosity (LOH) analysis. DNA for VHL mutation screening was obtained from whole blood, fresh and frozen tumor tissues, and formalin-fixed, paraffin-embedded tissues. Tissue sections from paraffin blocks were microdissected before DNA extraction (11). An improved polymerase chain reaction (PCR) protocol using high-pressure liquid chromatography-purified primers for specific and reproducible amplification of DNA from archival tissue (11,12) was applied to all three VHL exons. PCR products were analyzed for mutations in comparison to wild type by single-strand conformation polymorphism (SSCP) analysis. Mutations were identified, respectively, by direct sequencing of purified PCR products (12). LOH was determined by microsatellite analysis at polymorphic loci D3S1350 and D3S1038 within a 300-kilobase interval of the VHL gene (13). To control for PCR-generated errors in mutational screening, all procedures including microdissection and DNA preparation were repeated independently at least twice. Results of multiple somatic mutations or of the nucleotide (nt) 454 C>T mutation were reproduced at least four times, independently and in different laboratories.

Restriction enzyme-based nt 454 C>T mutation detection assay. For fast analysis of the presence of a frequent mutation at nt 454 (C>T), a restriction endonuclease-based assay was used. We amplified a short 154-base-pair (bp) sequence with primers VHL 28 (12) and primer 454r (5'- AGCGTTGGG-TAGGGCTGC-3') that comprised a central *AcI*I restriction site. This site was cleaved in wild-type sequences, resulting in two fragments of 60 and 54 bp but remained uncut in a 104-bp fragment when the cytosine at position 454 was mutated. DNA fragments were analyzed electrophoretically on 3% agarose gels, stained with ethidium bromide, and visualized with the use of an on-chip integrating system (INTAS, Göttingen, Germany).

Statistical analysis. An association between TRI exposure level and number of VHL mutations was calculated with an exact version of the Mantel–Haenszel chi-squared test comparable to the Cochran–Armitage trend test (14). An association between the latency from onset of TRI exposure to diagnosis of RCC and the TRI exposure level (+++, ++, and +) and an association between the latency and the number of VHL mutations (≥2, 1, and 0) were tested by use of the Spearman rank correlation (14). The nonparametric Wilcoxon rank sum test was used for pairwise comparisons between subgroups of patients (14). Two-sided *P* values were used and were considered statistically significant when less than .05.

RESULTS

Clinical Data: Assessment of TRI Exposure and Development of RCC

Forty-four patients enrolled in this study were continuously employed over periods of several years in metal-processing fac-

tories, where TRI was used as a degreasing solvent for metal parts. Subjects were between 14 and 47 years of age (mean, 24 years of age) when they began to work with TRI. At the time, minimal precautions were taken to prevent exposure to the solvent. Manual handling of liquid TRI included unprotected cleaning of metal parts in large, open tubs filled with TRI, wiping off TRI residues, and using TRI for cleaning floors and clothes and even for cleaning the skin of hands and arms. In addition to skin contact, workers were exposed to high concentrations of volatile TRI, especially during heating of TRI (boiling point, 87 °C) in degreasing tubs and during use of compressed-air devices. The effects of these exposures were exacerbated by a lack of sufficient ventilation in working areas and by the absence of respiratory protection. Concentrations of volatile TRI were estimated retrospectively, by the reported clinical symptoms of dizziness, headache, and nausea. Estimated concentrations of volatile TRI exceeded many-fold today’s exposure threshold of 50 ppm (German Occupational Exposure Limit). We scored three intensity levels of TRI exposure based on the duration and frequency of working with TRI, the prevalent way of handling liquid TRI, and the clinical symptoms reported. Among these criteria, clinical symptoms and adverse effects were given priority in the assessment of exposure levels.

The mean age at diagnosis of RCC was 60 years (range, 38–84 years). Tumors occurred as single lesions, and no metastases were reported. Forty-two RCCs were of the clear-cell type, and two RCCs were of the papillary (chromophilic) type. The time from onset of TRI exposure to diagnosis of RCC ranged from 9 to 59 years (see Table 3).

Molecular Data: Somatic VHL Mutations

DNA from RCC specimens and from corresponding normal tissue of 44 patients with known TRI exposure was subjected to PCR amplification of all three exons of the VHL gene. We identified VHL mutations in 33 (75%) of 44 RCCs. Fourteen (42%) of the RCCs with VHL mutations had multiple (i.e., two, three, or four) mutations (Table 2, Fig. 1). Twenty-seven (54%) of a total of 50 mutations were missense, 15 (30%) of the mutations were frameshifts, four (8%) of the mutations affected splice consensus regions, three (6%) were in-frame deletions or insertions, one mutation was silent, and one sequence change affected an intronic site. Twenty-six (52%) of the 50 mutations were located within exon 1, 10 (20%) of the mutations were located within exon 2, and 14 (28%) of the mutations affected exon 3.

Missense mutations involved a cytosine change in 24 (89%) of 27 missense mutations, a C>T change in 19 cases and a C>G change in five cases, respectively. In two of 27 missense mutations, there was a G>A change and in one case a T>A change.

Table 1. Case patient and control subject selection for questionnaire evaluation and molecular analyses of the relationship between trichloroethylene (TRI) exposure and renal cell carcinoma (RCC)

TRI exposure	Patients or individuals	Total No.	Questionnaire	Lymphocyte DNA	Tumor DNA	DNA of adjacent normal tissue
Exposed	Patients with RCC (TRI industrial region)	44	44	44	44	44
	Individuals without RCC (TRI industrial region)	48	48	47	—	—
Unexposed	Patients with RCC (TRI industrial region)	34	—	—	34	—
	Patients with RCC (other regions)	73	—	—	73	—
	Healthy, normal individuals (other regions)	50	—	50	—	—

Table 2. Somatic von Hippel–Lindau gene mutations in renal cell carcinoma of patients with high, cumulative exposure to trichloroethylene*

Patient†	nt‡	Change‡	Codon	Amino acid	Mutation type
1695	454	C>T	81	Pro>Ser	Missense
	570	C>G	119	Phe>Leu	Missense
	699	C>G	162	Cys>Trp	Missense
	775	C>G	188	Leu>Val	Missense
1665	454	C>T	81	Pro>Ser	Missense
	657	delT	148		Frameshift
	677 – 2	a>g	155		Splice
1693	454	C>T	81	Pro>Ser	Missense
	811	C>T	200	Arg>Trp	Missense
	677 – 1	g>c			Splice
1654	454	C>T	81	Pro>Ser	Missense
	473	delTAT	87		Deletion
1657	454	C>T	81	Pro>Ser	Missense
	639	delTGAC	142		Frameshift
1658	454	C>T	81	Pro>Ser	Missense
	713	G>A	167	Arg>Gln	Missense
1659	454	C>T	81	Pro>Ser	Missense
	712	C>T	167	Arg>Trp	Missense
1667	470	C>T	86	Pro>Leu	Missense
	553 + 8	c>t			Intronic
1670	397	delG	62		Frameshift
	454	C>T	81	Pro>Ser	Missense
1672	713	G>A	167	Arg>Gln	Missense
	795	G>T	194	Val>Val	Silent
1676	418	C>T	69	Arg>Cys	Missense
	685	delCT	158		Frameshift
1677	418	delCGCG	69		Frameshift
	781	delG	190		Frameshift
1685	570	C>G	119	Phe>Leu	Missense
	811	C>T	200	Arg>Trp	Missense
1694	454	C>T	81	Pro>Ser	Missense
	570	C>G	119	Phe>Leu	Missense
1653	454	C>T	81	Pro>Ser	Missense
1656	454	C>T	81	Pro>Ser	Missense
1668	454	C>T	81	Pro>Ser	Missense
1678	454	C>T	81	Pro>Ser	Missense
1698	479	T>A	89	Leu>His	Missense
1662	483	del10nt	161		Frameshift
1689	512	del18nt	100		Deletion
1661	517	ins56nt	102		Frameshift
1674	535	delC	108		Frameshift
1671	542	ins6nt	110		Insertion
1697	544	del10nt	111		Frameshift
1688	553 + 1	delg	114		Splice
1683	595	delC	128		Frameshift
1687	621	delT	136		Frameshift
1696	632	delTC	140		Frameshift
1680	637	delG	142		Frameshift
1655	644	delG	144		Frameshift
1669	677 – 1	g>c	155		Splice
1660	683	C>T	157	Thr>Ile	Missense

*Abbreviations in this table are defined as follows: nt = nucleotide; del = deletion; ins = insertion.

†Patients whose numbers are underscored also showed the nt 454 C>T mutation in non-neoplastic kidney parenchyma.

‡Exonic nts are capitalized, and intronic nts are in lower case. Alterations in exonic sequences are indicated by either the exact nt or the 5'-most nt. Alterations in intronic sequences are indicated by the distance from the closest exonic nt.

A frequent C>T missense mutation at nt 454, changing proline to serine within the VHL protein at codon 81 (Pro81Ser), contributed to the high percentage of missense mutations in exon 1. Thirteen (39%) of the RCCs with VHL mutations had this nt 454 mutation, which was accompanied by additional mutations at other sites within the VHL gene in nine (69%) of the RCCs (Table 2). The mutation at nt 454 was present in RCCs of both

the clear and papillary (chromophilic) types. Moreover, the nt 454 mutation was also detected in DNA isolated from tumor-adjacent normal kidney parenchyma of four patients (Nos. 1654, 1656, 1658, and 1695; Fig. 2). Four other mutations were detected in more than one RCC: a C>G change at nt 570 (Phe119Leu; n = 3), a G>A change at nt 713 (Arg167Gln; n = 2), an intronic g>c change at a splice acceptor site in exon 3 (n = 2), and a C>T change at nt 811 (Arg200Trp; n = 2).

Eighteen RCCs with intragenic somatic VHL mutations were evaluated for 3p LOH. All 18 RCCs showed LOH at the corresponding 3p allele (Table 3).

Mutational Hot Spot in VHL Exon 1 at nt 454

The question whether the frequent mutation at nt 454 (C>T) was a specific, disease-related change or a polymorphism was addressed by use of an *Acil*-based restriction assay applied to nontumor DNA. Lymphocyte DNA from patients with RCC and TRI exposure (n = 44) and from 97 control individuals without RCC was tested for the presence of the nt 454 mutation. All nontumor DNA samples tested negative.

To elucidate whether the nt 454 mutation is specific for RCCs of TRI-exposed patients, we applied the *Acil*-based restriction assay to tumor DNA from 107 unexposed patients with RCC. None of these tumors displayed the nt 454 mutation.

Synopsis and Statistical Data Analysis

Among 44 patients with RCC, 17 were exposed to high (+++), 24 to medium (++), and three to low (+) levels of TRI. VHL mutations were detected in patients who had high and medium exposure levels but not in patients with a low exposure level. Among 33 RCC patients with VHL mutations, 19 (58%) were smokers and 14 (42%) were nonsmokers.

Among 33 patients whose RCCs bore VHL mutations, 15 (45%) had experienced high (+++), 18 (55%) medium (++), and none (0%) low (+) exposure levels of TRI. Of 14 patients with RCCs that bore multiple VHL mutations, 11 (79%) had been exposed to high and three (21%) to medium exposure levels (Table 3). There was a statistically significant association ($P = .0006$) between the severity of TRI exposure and the multiplicity of the mutations (Table 4).

Neither the correlation between the latency and the TRI exposure level ($r = .19$; $P = .22$) nor the correlation between the latency and the number of VHL mutations ($r = -.03$; $P = .87$) was statistically significant (Table 3). We observed that the mean time from onset of TRI exposure to diagnosis of RCC was shorter when the nt 454 mutation was present as a single VHL mutation (n = 4; mean, 23 years; 95% confidence interval [CI] = 2–44 years) than in patients with additional mutations (n = 9; mean, 36 years; 95% CI = 25–46 years) or in patients with single or multiple VHL mutations different from the nt 454 mutation (n = 20; mean, 37 years; 95% CI = 32–42 years) (Table 3).

DISCUSSION

As excretory organs, kidneys should be a prime target site for carcinogens because they are constantly being exposed to a large number of metabolites. As a result of exposure to exogenous carcinogens, hot-spot mutations in tumor suppressor genes may occur (15,16). RCC of the clear-cell type is mainly caused by somatic mutations of the VHL tumor suppressor gene (1,3–6,17,18). Genetic analysis of sporadic RCC has previously failed

Fig. 1. Somatic mutations in the von Hippel-Lindau (VHL) gene in 33 patients with renal cell carcinoma who had high, cumulative exposure to trichloroethylene. **Shaded boxes** indicate exons 1, 2, and 3 of the VHL gene, including nucleotides (nts) 213 through 852 of the published complementary DNA sequence (1). Patients are identified (ID) with four-digit numbers, and their single or multiple mutations are indicated along **dotted horizontal lines**. **Black circles** represent missense mutations, **open squares** represent frameshift mutations, **black triangles** represent deletions, **X** indicates mutations affecting splice consensus regions, an **inverted open triangle** represents an insertion, an **open circle** represents a silent mutation, and **§** indicates an additional intronic change. Amino acids (aa)/codons of the protein sequence are given at the bottom. Marked codons 81, 119, 167, and 200 indicate the location of frequent missense mutations at specific nts. Patients whose identifiers are **underscored** showed the hot-spot mutation affecting codon 81 in normal kidney parenchyma adjacent to the tumor.

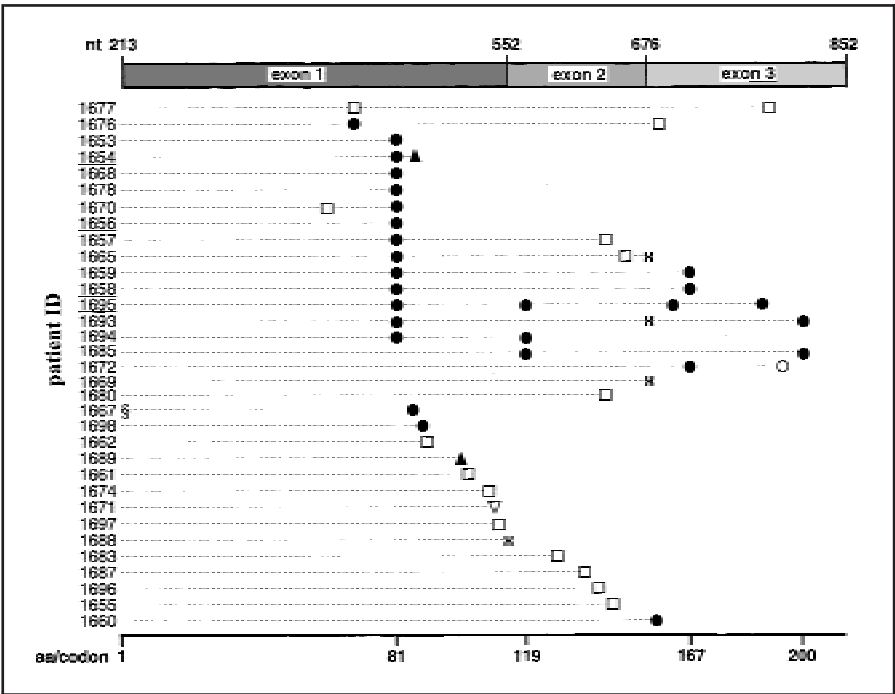


Fig. 2. Detection of the nucleotide 454 C>T mutation in the von Hippel-Lindau (VHL) gene of patients with high, cumulative exposure to trichloroethylene. **A)** Electrophoretic analysis of loss of the *Acil* wild-type restriction site in four patients. **Lanes I through IV** show polymerase chain reaction (PCR) products of control samples: **lane I**, undigested 154-base-pair (bp) PCR product; **lane II**, *Acil*-digested wild-type DNA; **lane III**, *Acil*-digested germline DNA with the nt 454 C>T mutation; and **lane IV**, *Acil*-digested wild-type DNA of archival tissue. The following lanes show the digested PCR products of patients 1695, 1658, 1656, and 1654: **L**, lymphocyte DNA; **N**, normal tumor-adjacent parenchyma; and **T**, tumor tissue. **Arrows** indicate relevant *Acil* fragments: **1**, 104 bp, representing a mutated allele with nt 454 C>T change; **2**, 60 bp; and **3**, 54 bp, the latter two fragments representing wild-type sequences. Fragments smaller than 54 bp result from additional *Acil* restriction sites flanking the main 104-bp fragment. **B)** DNA sequencing of PCR products of normal tumor adjacent tissue and tumor tissue of patients 1654 and 1656 in forward and reverse directions. **Arrows** indicate the C>T change as superimposed blue and red peaks in the forward direction and the G>A change as superimposed black and green peaks in the reverse direction.

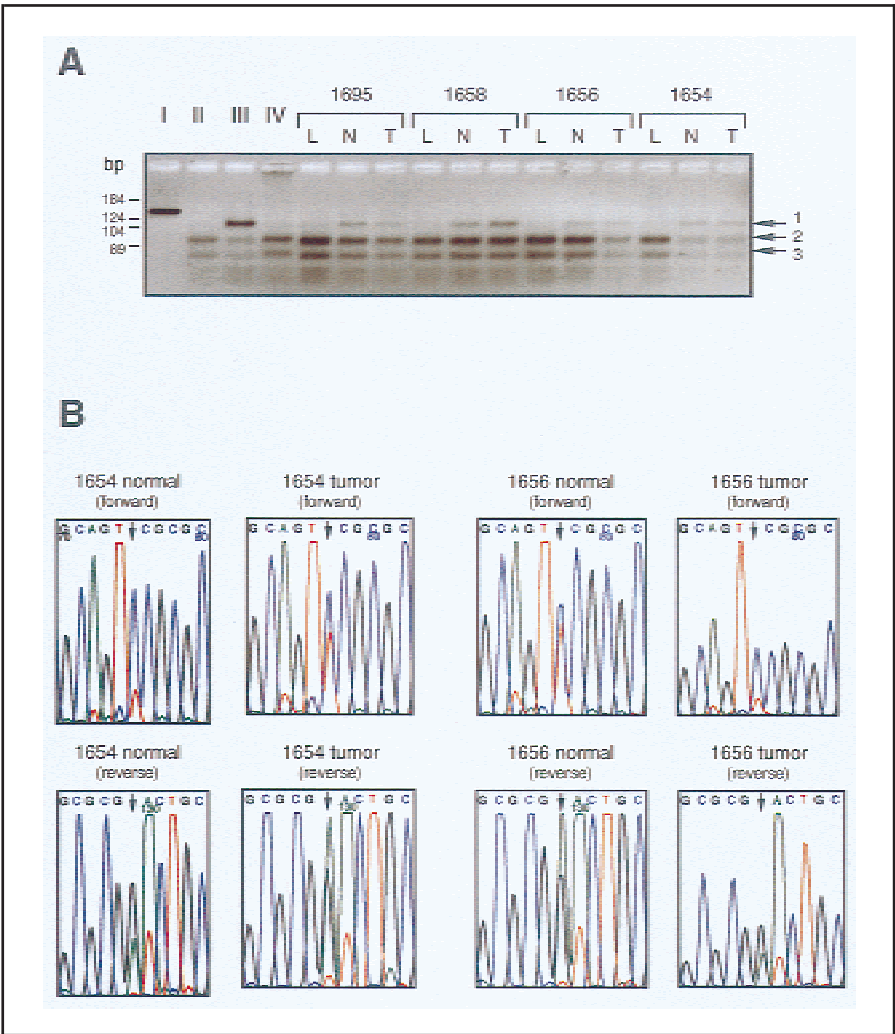


Table 3. Characteristics of patients with renal cell carcinoma who had high, cumulative exposure to trichloroethylene

Patient	Age at first exposure, y	Age at diagnosis, y	Δt^*	Severity of exposure†	No. of VHL mutations‡	Nucleotide 454 mutation‡	3p LOH§
1695	27	38	11	+++	4	yes	yes
1693	38	58	20	+++	3	yes	@
1665	25	67	42	+++	3	yes	@
1685	28	57	29	+++	2	no	yes
1654	25	59	34	+++	2	yes	yes
1667	19	53	34	+++	2	no	yes
1658	22	60	38	+++	2	yes	yes
1672	21	59	38	+++	2	no	@
1670	18	62	44	+++	2	yes	@
1694	16	74	58	+++	2	yes	yes
1676	25	84	59	+++	2	no	@
1696	16	55	39	+++	1	no	yes
1660	16	60	44	+++	1	no	yes
1669	17	61	44	+++	1	no	yes
1687	33	83	50	+++	1	no	x
1684	23	57	34	+++	—	—	n.d.
1691	22	59	37	+++	—	—	n.d.
1659	14	47	33	++	2	yes	yes
1677	24	57	33	++	2	no	x
1657	16	56	40	++	2	yes	yes
1678	29	38	9	++	1	yes	@
1689	43	60	17	++	1	no	x
1668	28	48	20	++	1	yes	x
1662	45	67	22	++	1	no	@
1656	47	70	23	++	1	yes	yes
1661	16	42	26	++	1	no	@
1683	29	55	26	++	1	no	yes
1698	28	61	33	++	1	no	x
1674	16	51	35	++	1	no	@
1655	17	53	36	++	1	no	yes
1688	24	60	36	++	1	no	yes
1697	16	55	39	++	1	no	yes
1653	32	73	41	++	1	yes	yes
1671	37	79	42	++	1	no	@
1680	14	73	59	++	1	no	yes
1692	35	55	20	++	—	—	n.d.
1652	14	39	25	++	—	—	n.d.
1681	18	58	40	++	—	—	n.d.
1701	14	55	41	++	—	—	n.d.
1675	14	63	49	++	—	—	n.d.
1690	26	81	55	++	—	—	n.d.
1664	22	49	27	+	—	—	n.d.
1682	27	82	55	+	—	—	n.d.
1686	30	64	34	+	—	—	n.d.

* Δt = time in years between onset of trichloroethylene exposure and diagnosis of renal cell carcinoma.

†Exposure levels: +++ = high, ++ = medium, + = low.

‡VHL = von Hippel-Lindau; — = no VHL mutation.

§LOH = loss of heterozygosity; @ = amplification of DNA failed at microsatellite loci; x = no paired DNA samples (normal and tumor) available; n.d. = not done.

to identify VHL mutational hot spots. We have reported (19) preliminary molecular findings of frequent VHL gene mutations in RCC patients who had been exposed to high, cumulative doses of a well-defined carcinogenic agent, TRI. The ongoing controversy over an association between TRI exposure and RCC (20) led us to attempt to confirm this association with comprehensive occupational and molecular data from exposed and unexposed subjects. The present study relies on the standardization of TRI exposure levels of RCC patients (10) and methodologic refinements in the VHL analysis of their single tumors.

We established several lines of evidence for unique mutational events in RCCs of patients who had been exposed to TRI. First, we detected multiple mutations of the VHL gene in the same tumor from 42% of patients exposed to the carcinogen who exhibited any VHL mutation. Of these tumors, 57% showed additional loss of the homologous VHL allele. Any number of

mutations greater than two implies that at least two of them affect the same allele. Moreover, these cases of RCC may present a variation of the two-hit hypothesis (21) because they show more than two homologous tumor suppressor gene mutations. These interpretations are based on the assumption that a tumor with multiple mutations represents a single clone of cells, which is in agreement with the observed morphologic homogeneity. However, genetic heterogeneity may have occurred during carcinogenesis. Further experiments will have to elucidate whether multiple VHL mutations affected the same cell, leading to a homogenous tumor cell population, or whether VHL mutations accumulated independently in different tumor cells.

Second, we detected five cluster regions in which the VHL gene was affected more than once. Among these regions, the nt 454 change (C>T) of codon 81 (Pro>Ser) was the most common and affected 39% of RCCs in this patient sample with any VHL

Table 4. Association between trichloroethylene (TRI) exposure levels and the frequency of von Hippel–Lindau (VHL) gene mutations*

Severity of exposure†	Total No. of patients	No. of patients (%) with			
		Nucleotide 454 mutation	No mutation	1 mutation	≥2 mutations
+++	17	7 (41)	2 (11)	4 (24)	11 (65)
++	24	6 (25)	6 (25)	15 (63)	3 (13)
+	3	0 (0)	3 (100)	0 (0)	0 (0)
–	107‡	0 (0)	31/73‡ (42)	42/73‡ (58)	0/73‡ (0)

*Statistical analysis of the association between TRI exposure level and number of VHL mutations yielded a value of 11.28 with one degree of freedom in chi-square testing according to Mantel–Haenszel (two-sided $P < .001$).

†See footnotes to Table 3 for exposure classifications.

‡Indicates that among patient control subjects all were analyzed for presence or absence of the nucleotide 454 mutation, and 73 patients were completely analyzed for all VHL exons (our unpublished data).

mutation. Absence of this mutation in lymphocyte DNA of patients and control subjects ruled out the mutation as representing a polymorphism or a germline change. Moreover, the nt 454 mutation was not present in RCCs of unexposed patients, indicating specificity. Patients whose tumors carried a single intragenic mutation at nt 454 exhibited an earlier age at disease onset. This preliminary observation may require an explanation for the mutation's role in pathogenesis. In four patients, we detected the nt 454 change in apparently normal kidney parenchyma adjacent to the tumor. This observation is highly suggestive that the nt 454 mutation alone may not suffice to induce cellular transformation. This parenchyma may represent TRI-insulted tissue that has not yet been affected by a second hit. Alternatively, it is tempting to speculate that the C>T change at nt 454 might characterize a precancerous condition in which the tumor has not yet become morphologically detectable.

Third, most VHL mutations were missense mutations with 70% C>T transitions. C>T transitions are known to be mutational changes frequently associated with deamination of 5-methylcytosine at CpG dinucleotides (22) or with alkylation and DNA adduct formation of O^6 -alkylguanine involved in G>A transitions of the opposite DNA strand (23). Because CpG dinucleotides are abundant especially in exon 1 and exon 3 of the VHL gene (6,24), this may render those exons particularly susceptible to mutation. The overall frequency of 38% C>T transitions in RCCs of TRI-exposed patients identified in this study, compared with 6% in RCCs of non-TRI-exposed patients from combined studies [(3–5,25,26); Brauch H, Weirich G, Brieger J, Glavac D, Rödl H, Eichinger M, et al.: unpublished data], may be explained by carcinogen-induced hypermutability.

In humans TRI is taken up by direct contact through the skin and by inhalation (27). To understand the chemical's mutagenic impact on the VHL gene, it is important to put TRI into its proper metabolic context and to consider the bioreactivity of its metabolites. Evidence for a possible role of TRI in renal carcinogenesis was gathered originally from animal studies (28,29). In rats, TRI is processed in the liver either by oxidation or by glutathione binding (30). S-(1,2-Dichlorovinyl)-L-cysteine (DCVC), an amino acid analogue derived from the glutathione–TRI conjugate, is enriched in renal epithelial cells and acts as a

substrate for *N*-acetyltransferases and β -lyase (31). Enzymatic degradation of DCVC results in intermediates, i.e., chlorinated thioketenes, and specific mercapturic acids, measurable end-stage products used as flag compounds (32,33). These mercapturic acids were detected in the urine of TRI-exposed individuals and in a patient who swallowed TRI as part of a suicide attempt (34,35), pointing to an analogous glutathione-dependent pathway in humans.

DCVC is mutagenic in bacteria (36–38), and electrophilic halothioketenes readily react with DNA *in vitro* (39,40). Recently, another *in vitro* study (41) established the DNA-damaging effects of reactive TRI metabolites. Incubation of renal epithelial cells with DCVC induced mutations in the TP53 gene (also known as p53) (41). Furthermore, it was demonstrated that TRI metabolites preferentially interact with exocyclic amino groups of cytosine and adenine that may result in spontaneous depurination (40). Ester formation at the sugar/phosphate backbone and cross-linking of DNA strands are additional effects of these metabolites, both of which may lead to DNA strand break. Compelling evidence for cytosine targeting of chlorothioketene, the ultimate reactive intermediate of β -lyase-mediated cleaved DCVC, has come most recently from the detection of cytosine adducts in reaction mixtures containing chlorothioketene and cytosine (42). Our findings of a unique VHL mutation spectrum in RCCs of TRI-exposed patients may reflect a nonrandom affinity of mutagenic TRI metabolites for the VHL gene. In particular, the frequent C>T transitions identified are in agreement with the presence of DNA-reactive metabolites.

In human cancer, few examples are known of a relationship between mutation of a tumor suppressor gene and exogenous carcinogen exposure. Our work extends the series of exogenous mutagens involved in cancer-specific tumor suppressor mutations. Such relationships were first established by findings of TP53 mutations in hepatocellular carcinoma due to dietary aflatoxin B₁ consumption (15,16) and liver angiosarcoma resulting from vinyl chloride exposure (43). These studies differed from ours in that the target organ in those studies was the liver and primary substances could be used for *in vitro* assays to demonstrate mutagenicity. The renal epithelium is a downstream target site in the body for water-soluble metabolites, which may be a reason why specific mutagenic compounds have been difficult to find, even when primary substances were long suspected to cause kidney cancer. Our findings of unique and frequent VHL mutations in RCCs of TRI-exposed patients present, to our knowledge, the first molecular evidence for a relationship between exposure to a defined carcinogen, gene damage, and kidney cancer.

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NOTES

Present addresses: G. Weirich, Laboratory of Immunobiology, National Cancer Institute-Frederick Cancer Research and Development Center, National Can-

cer Institute, Frederick, MD; T. Wöhl, Wilex Biotechnology GmbH, Munich, Germany.

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