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Key terms: [acetylation phenotype](#); [aromatic amines](#); [bladder cancer](#)

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Occupational history and genetic N-acetyltransferase polymorphism in urothelial cancer patients of Leverkusen, Germany

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Objectives The study was designed to realize possible shifts in the ratio of slow to fast acetylators within a group of 196 urothelial cancer patients in an area with earlier benzidine production.

Methods The subjects were interviewed for occupational and nonoccupational risk factors. The patients were phenotyped for N-acetyltransferase 2 (NAT2) by Grant's caffeine test. A subgroup of 54 patients was additionally genotyped for NAT2.

Results The antimode in the NAT2 phenotyping with the caffeine test (AFMU:1X ratio) was 1.0, as evidenced by additional genotyping of the subgroup of 54 patients. The prevalence of slow acetylators in the entire group of bladder cancer patients was 55%, in accordance with published figures for European populations. In a subgroup of 40 patients with occupational histories as workers in chemical or rubber industries 65% were slow acetylators. In a further subgroup of 28 cases having specifically worked at chemical production sites of the local chemical industry, 68% were slow acetylators.

Conclusions In contrast to earlier studies, this study shows no increased prevalence of slow acetylators among urothelial cancer patients in comparison with the normal population. However, in subgroups of cases with a likelihood of past occupational contacts with aromatic amines, there was a trend towards a higher representation of slow acetylators. This finding is in accordance with observations of others that the percentage of slow acetylators in urothelial cancer patients is generally decreasing, possibly because the production of benzidine and benzidine-based dyes ceased in the early 1970s.

Key terms acetylation phenotype, aromatic amines, bladder cancer.

The role of N-acetyltransferase (NAT2) polymorphism in the pathogenesis of human bladder cancer has been investigated in several studies using the phenotyping of "slow" and "rapid" acetylators. A key study was published by Cartwright et al in 1982 (1). In this hospital-based case-referent study of 111 bladder cancer cases and 95 referents, the proportion of slow acetylators was 57% among the referents but 67% among the cases. In a subgroup of 23 cases with past occupational exposure to benzidine or 2-naphthylamine, as many as 22 slow acetylators were found.

In general, case-referent studies performed in Western countries on this subject have shown an excess of slow acetylators in patients suffering from bladder can-

cer, compared with reference populations (2–13). This finding has been interpreted as being compatible with a generally increased risk of some 30% for bladder cancer among slow acetylators, compared with rapid acetylators (14).

However, it is important to note that similar studies in non-European populations (15–17) show results that differ from those of Western studies. Therefore the study of ethnic differences in this specific field is important. In particular, a recent study performed in China showed a prevalence of rapid acetylators in bladder cancer patients with past occupational contact to benzidine (17). In this study only 23.3% of the referents were slow acetylators. This finding is in marked contrast to, for example, obser-

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vations of Lewalter & Miksche (vide infra) at the benzidine manufacturing Bayer plant in Leverkusen (18) and points to ethnic differences in the pathways of the metabolic activation of benzidine and related aromatic amines. Quantitative differences among the results of Western studies (2–13) might be explained by differences in environmental or occupational exposures to aromatic amines, in addition to possible differences in the particular genetic or ethnic background.

In view of the debated inconsistencies in the previous studies (19) further research in this field is needed. In a recent study based on the NAT2 genotyping of 160 bladder cancer patients and 460 reference subjects in Berlin, Germany, 61.1% of the referents and 65.6% of the bladder cancer cases were slow acetylators genotypes. This very slight difference was not statistically significant (13). In general, it appears that the differences in acetylator genotypes or phenotypes between referents and unselected bladder cancer cases in Western populations are decreasing with time, probably indicating a decreasing impact of classical aromatic amines (eg, benzidine, 4-aminodiphenyl, 2-naphthylamine) on the general population.

The largest group of bladder cancer patients with a proved previous occupational exposure to benzidine has been studied at Bayer, Leverkusen, Germany, by Lewalter & Miksche (18). Phenotyping of 331 persons having worked between 1951 and 1967 in the production of benzidine revealed 160 slow acetylators (48%) and 171 rapid acetylators (52%). The entire group of workers had been under medical surveillance for 16 years at the time of publication. As many as 92 persons of this group were finally found to suffer from urothelial neoplasms, among them 75 being slow acetylators (82%) and 17 being rapid acetylators (18%). This unique local background has now promoted further investigations. The importance of benzidine and related aromatic amines for the manufacture of dyes, especially in Germany, had been a matter of a previous publication (20).

Our study was designed to realize shifts in the ratio of slow to fast acetylators within a group of urothelial cancer patients in an area with a specific chemical industry, along with past employment in this local industry, and to compare the circumstances of these patients with the situations described in earlier publications (2–13).

Subjects and methods

Bladder cancer cases

Our study was performed in 1991–1993 with patients with urothelial cancer at the Department of Urology of the Leverkusen Hospital. We used NAT2 phenotyping by the caffeine method (21). Part of the patients were

also genotyped by allele-specific polymerase chain reaction (PCR) and restriction fragment length polymorphism studies. The genotype analyses were used to calibrate the antimode of the urinary caffeine metabolite ratio (AFMU:1X) in the phenotyping assay. Genotyping was done with the same methodology and in the same laboratory of a preceding study by Cascorbi et al (13).

The hospital-based investigation was performed between June 1991 and June 1993 on 129 in-patients and 67 out-patients of the Department of Urology of the Leverkusen Hospital [160 men, 36 women, total 196, mean age 69 (SD 11.5) years]. These patients were first histologically diagnosed between 1977 and 1993 to suffer from a tumor of the urothelium and were treated using different therapeutic regimens.

Written consent was obtained from all the patients who participated in the study, which included NAT2 phenotyping or genotyping. Tumors of the urogenital tract other than those of the urothelium (eg, squamous-cell carcinomas, adenocarcinomas, sarcomas) were not included in the study. All the persons participating in the study were of European origin. The same physician obtained their history using a questionnaire in a standardized interview. (For details, see reference 22.) The detailed medical histories, smoking habits, drug intake and occupational histories, and, particularly, any type of work in chemical and related industries were recorded.

Memberships of particular health insurance funds were recorded. Within the group of patients, a first subgroup was formed of all patients who were members of the BKK Bayer (BKK = Betriebskrankenkasse = health insurance fund). This subgroup (I) included all those presently employed at Bayer, their dependents and the retired former employees with their dependents (N = 66).

The classification of occupations followed the official list of occupations given by the German Federal Office of Statistics (23).

The interview of the 196 patients with respect to occupational activities led to a total of 379 occupations or occupational functions which lasted for at least one year. According to these occupations, a second subgroup (II) of patients (39 men and 1 woman) was formed of blue-collar workers in the chemical or rubber industry (including classification numbers 141x “Chemiebetriebswerker” (chemical production worker); 142x “Chemielaborwerker” (laboratory worker); 633x “Chemielaborant” (laboratory assistant); 145x “Gummihersteller, -verarbeiter, Vulkaniseur” (rubber manufacturing, processing, curing). Out of this subgroup, the patients who had worked at chemical production sites (141x “Chemiebetriebswerker”) were also separately evaluated as a further subgroup (III) containing 27 men and 1 woman.

Eleven patients had been involved in the production of dyes; among them, six had been compensated for

occupationally induced bladder cancer (BK 1301 of the German list of occupational diseases). Dye application was reported by 16 patients; one of them having received compensation. One patient had had contact with dyes in a research laboratory. Two maintenance workers employed at Bayer had been in contact with a large number of chemicals; they had been compensated for bladder cancer induced by aromatic amines. Some patients reported occupational contacts with specific aromatic amines, aniline contact being reported by eight patients (two being compensated), benzidine by six patients (three compensated), 2-naphthylamine by two patients (both compensated), 1-naphthylamine by one patient, and aromatic amines, in general, by three patients (one compensated). It should be noted that the term "aniline" had also been practically used in former times for aromatic amines in general.

N-acetyltransferase genotyping

Genotyping for "rapid" and "slow" alleles of the NAT2 gene was performed on a subgroup of 54 patients randomly selected from a total of 196 bladder cancer patients, according to the method of Cascorbi et al (24).

Genomic DNA (deoxyribonucleic acid) was prepared from leucocytes of anticoagulated blood samples by phenol extraction according to standard methods. Frequent mutations of the human NAT2 gene were detected according to the fundamentals given by Grant (25). The mutation T341C was identified by allele-specific PCR, and the mutations C282T, C481T, G590A, A803G, and G857A by PCR amplification of parts of the NAT2 gene and subsequent digestion with restriction enzymes Fok I, Kpn I, Taq I, Dde I, and Bam HI, respectively. Distinct alleles were defined by the constellation of mutations (table 1). With the use of six known "slow" and one "rapid" allele all combinations of slow alleles only were addressed as slow acetylator genotypes; the remainder were considered rapid acetylator genotypes according to the new nomenclature of N-acetyltransferases (26).

N-acetyltransferase phenotyping

The caffeine test method of Grant et al (21) was used for the NAT2 phenotyping. The subjects drank one to two cups of a caffeine-containing beverage (mostly coffee,

sometimes tea or a soft drink). In all cases, urine was obtained 2–3 h after the caffeine intake. A second urine sample was obtained after 4 h from 42 bladder cancer patients (out of 196). No differences were observed in the individual phenotypes, based on these parallel samples, and both values (AFMU:1X ratios) of the same subject were always very similar. Hence, for persons from whom two urine samples were analyzed, a mean value of the two samples was used in the further evaluation.

The urine was immediately adjusted to pH 3.5 by the addition of small quantities of 1 M hydrochloric acid, and then stored in 10-ml "monovettes" (plastic tubes) at –20°C until processing. The individual storage times did not exceed six weeks. Quantitation of the caffeine metabolites AFMU (5-acetylamin-6-formylamino-3-methyluracil; acetylated metabolite) and 1X (1-methyl-xanthine; non-acetylated metabolite) was done by high-pressure liquid chromatography (HPLC), using the standard addition procedure. Standard 1X was a commercial product of Sigma (Deisenhofen, Germany); AFMU was synthesized by Röhrkasten et al (27), according to a modification of the method of Fink et al (28). The final product contained 6% AAMU (5-acetylamin-6-amino-3-methyluracil), which was appropriately considered in the calculations based on the standard solution. Stock solutions of AFMU (2.5 mg AFMU in 10 ml of 0.05% acetic acid) were stored frozen in 1-ml fractions and thawed before use. Stock solutions of 1X (1.5 mg 1X in 10 ml of 0.05% acetic acid) were prepared weekly and stored at 4°C until use.

The HPLC procedure followed the method of Grant et al (21). By use of the standard addition method, the molar ratio of AFMU over 1X was calculated for each individual urine sample. The validity of use of the AFMU:1X molar ratio, even for alkaline urines, for NAT2 phenotyping has recently been documented by Butler et al (29).

In previous studies using the caffeine test to study acetylator phenotypes, probit transformations were used to determine the distribution of slow versus rapid acetylators (30). They clearly showed a bimodal distribution of NAT2 phenotypes. Based on probit transformation of individual molar AFMU:1X ratios, an antimode of 0.6 between slow and rapid acetylators had been inferred by Butler et al (29), both for smokers and nonsmokers. In other laboratories, different values for the antimode had been in use, for example, 0.48 by Grant et al (21) and 0.85 by Braz Vieira de Silva Pontes et al (31). The numerical value of this antimode is obviously laboratory-dependent (see references 21 and 32); the instability of AFMU standards is probably an important point in this respect. In an early stage of investigation (43 cases which had been both genotyped and phenotyped at that time), an interim assessment of genotypes and corre-

Table 1. Alleles of N-acetyltransferase according to the nomenclature of Vatsis et al (26).

Allele	Synonym	Mutation	Phenotype
NAT2*4	R, F1	.	Rapid
NAT2*5B	S1a, r3	T341C, C481T, A803G	Slow
NAT2*5A	S1b, M1	T341C, C481T	Slow
NAT2*5C	S1c	C481T, A803G	Slow
NAT2*6A	S2, M2, r2	C282T, G590A	Slow
NAT2*7B	S3	C282T, G857A	Slow

sponding phenotypes was made based on an antimode (AFMU:1X) of 0.48 (21). It appeared at that time that 8 out of 29 rapid acetylator phenotypes did not correspond with the genotype, while only 1 out of 14 slow acetylator phenotypes differed from the corresponding genotype. Hence the critical question of the AFMU:1X antimode was evaluated in detail. (See the Results section.) Using this evaluation we finally confirmed an antimode of the molar AFMU:1X ratio of 1.0 to distinguish between slow and rapid acetylator phenotypes.

Results

Coherence between *N*-acetyltransferase genotypes and phenotypes (caffeine test)

The introduction of the caffeine test by Grant et al (21, 30) for NAT2 phenotyping has particularly facilitated studies of diseased patients. However, this test requires careful consideration of the antimode of the urinary caffeine metabolite ratio (AFMU:1X) that distinguishes between slow and rapid acetylators. Fifty-four of the bladder cancer patients studied were also genotyped in a blind manner, in addition to the phenotyping by the caffeine test.

The nomenclature and analysis of genotypic variants used were identical with those of the preceding study of Cascorbi et al (13) and followed the proposals of Grant (25). Figure 1 shows the relation between the results of the independent NAT2 genotyping (abscissa) and phenotyping (ordinate) assays. A clear antimode in the AFMU:1X ratio (ordinate) between the slow and rapid acetylator genotypes appeared in this study at a value of 1.0.

Distribution of *N*-acetyltransferase phenotypes

The prevalence of 55% slow acetylators (107 persons) of 196 bladder cancer patients in the entire group (men and women) and of 58% slow acetylators (92 persons) among the men ($N = 160$) of this group was, in general, consistent with the finding of other studies with a similar design

(2–13). These prevalence rates are somewhat smaller than in the previous study of Cascorbi et al (13), however, where 66% slow acetylators had been found among 160 bladder cancer patients.

There was no significant difference in the prevalence of NAT2 phenotypes with age or smoking habits or between in- and out-patients of the hospital. In addition, a careful examination of drugs used or prescribed did not point to influences of drug intake on NAT2 phenotyping. The differentiation of patients according to smoking histories and histopathological tumor gradings did not indi-

Figure 1. Correlation of NAT2 genotyping and phenotyping of a subgroup of 54 patients with urinary bladder carcinoma. For the nomenclature of the NAT2 alleles, see table 1. [open circle = phenotyping 2 h after the drinking of 1–2 cups of caffeine-containing beverage, closed circle = phenotyping 2 and 4 h after the drinking of 1–2 cups of caffeine-containing beverage (mean value)]

Table 2. Synopsis of the results obtained from 196 patients with urothelial cancer studied at the Leverkusen hospital. (OR = odds ratio, 95% CI = 95% confidence interval)

Classification by exposure	Cases (N)	Slow acetylators among the cases (%)	OR	95% CI	P-value
Members of the Bayer health insurance fund (subgroup I)	66	62	1.53	0.84–2.83	0.167
Blue-collar workers in the chemical or rubber industry (subgroup II)	40	65	1.74	0.83–3.65	0.141
Workers from chemical production ^a (subgroup III)	28	68	1.98	0.83–4.71	0.123
Blue-collar workers from the chemical or rubber industry and also members of the Bayer health insurance fund ^a	34	71	2.25	0.99–5.10	0.052
Patients not belonging to the above categories	124	52	1.00		

^a Included also in subgroup II.

cate any shifts in the results of the phenotyping. (Details not shown; see references 22 and 33.)

Because of the geographic location of the hospital where the study was performed, close to a chemical enterprise with a major history in the manufacturing and processing of benzidine and other aromatic amines, it

was of interest to concentrate on subgroups of diseased persons with an occupational history related to the local chemical industry. In this context, it is important that the production of benzidine at Bayer had ceased in 1967 (18) and that the production of benzidine-based dyes, in general, had ended in Germany in 1971, according to an

Table 3. Prevalence of "slow acetylators" (phenotype, if not otherwise stated) among bladder cancer patients, according to published studies performed in Europe and North America. (NS = not significant)

Reference	Number	Slow acetylators (%)	Odds ratio	95% confidence intervals	P-value
Lower et al, 1979 (2)					
Referents	74	51	.	.	.
Bladder cancer patients	71	65	1.74	0.89—3.40	NS
Lower et al, 1979 (2)					
Referents	118	67	.	.	.
Bladder cancer patients	115	70	1.18	0.68—2.05	NS
Lower and Bryan, 1979 (3)					
Referents ^a	41	49	.	.	.
Bladder cancer patients ^a	34	59	1.50	0.60—3.75	NS
Wolf et al, 1980 (4)					
Referents	74	51	.	.	.
Bladder cancer patients	71	65	1.74	0.89—3.40	NS
Cartwright et al, 1982 (1)					
Referents	95	57	.	.	.
Bladder cancer patients	111	67	1.51	0.86—2.68	NS
Bladder cancer patients and benzidine- or arylamine-exposed patients	23	96	16.7	2.16—129.07	0.007
Woodhouse et al, 1982 (5)					
Referents ^a	27	59	.	.	.
Bladder cancer patients ^a	30	70	1.60	0.54—4.80	NS
Evans et al, 1983 (6)					
Referents	852	60	.	.	.
Bladder cancer patients	100	66	1.30	0.84—2.00	NS
Miller & Cosgriff, 1983 (7)					
Referents ^a	26	69	.	.	.
Bladder cancer patients ^a	26	46	0.38	0.12—1.19	NS
Mommsen et al, 1985 (8)					
Referents	100	51	.	.	.
Bladder cancer patients	228	65	1.78	1.10—2.86	0.018
Hanssen et al, 1985 (9)					
Referents ^a	42	43	.	.	.
Bladder cancer patients ^b	105	62	2.17	1.04—4.48	0.037
Ladero et al, 1985 (10)					
Referents	157	57	.	.	.
Bladder cancer patients	130	64	1.35	0.84—2.17	NS
Bladder cancer patients and benzidine- or arylamine-exposed patients	15	73	2.10	0.64—6.89	NS
Hanke & Krajewska, 1990 (11)					
Referents ^a	22	45	.	.	.
Bladder cancer patients ^a	43	61	1.84	0.65—5.18	NS
Bladder cancer patients and benzidine- or arylamine-exposed patients	24	88	8.40	1.92—36.62	0.0046
Roots et al, 1992 (12)					
Referents	101	52	.	.	.
Bladder cancer patients	101	66	1.78	1.01—3.15	0.046
Cascorbi et al, 1994 (13)					
Referents ^c	460	61	.	.	.
Bladder cancer patients ^c	160	66	1.25	0.86—1.82	NS

^a Small number of persons studied.

^b Slow acetylators more frequent among the former dye workers.

^c Genotyping by polymerase chain reaction.

industrial agreement. (See reference 20.) However, the long latency time of 30 years or more for urinary bladder cancer caused by occupational exposure (34) must be considered. In other words, bladder cancer cases now diagnosed must be related to past exposures in the 1950s and 1960s.

When the subjects were examined according to their subgroups, 41 persons (62%) were found to be slow metabolizers.

In subgroup II, of 40 persons with 41 occupational categories, 26 (65%) were slow acetylators. Finally, 19 (68%) out of 28 subjects in subgroup III were slow acetylators.

Although these differences, owing to the number of available cases, were not statistically significant, they followed the expected trend towards a higher representation of slow acetylators in groups of cases with a likelihood of past occupational contact with aromatic amines. (See table 2 on page 335.)

Discussion

Previous hospital-based investigations in different Western countries have uniformly demonstrated a small excess of slow acetylators in groups of bladder cancer patients, in comparison with healthy referents. However, differences appear between studies (table 3) with regard to the percentage of both slow acetylators in "normal" reference populations and in bladder cancer patients. Moreover, owing to methodological factors, the positioning of the "antimode" in NAT2-phenotyping studies using the caffeine test (21) is of paramount methodological importance.

The present data and those of an earlier (13) study, in principle, are in line with the results of others (table 3). However, due to the unique geographic location of the group of patients in our study, close to a major chemical enterprise with a special history in the manufacture of aromatic amines, the behavior of the subgroups of patients with an occupational background in the local chemical industry was of particular interest. The results (table 2) suggest that the probability of a past occupational contact with aromatic amines for individuals within the group of bladder cancer patients corresponds with a tendency towards an overrepresentation of slow acetylators. In this respect, the extreme is marked by the group of workers from former benzidine production at Bayer (until 1967) who later suffered from bladder cancer, as published by Lewalter & Miksche (18).

Small shifts towards a higher representation of slow acetylators in studies of bladder cancer patients without a special occupational history have mostly been assigned to the effects of aromatic amines in tobacco smoke. In

this context, environmental tobacco smoke should also be considered (35) in that it contains at least 17 different mono- and bicyclic aromatic amines, including 2-naphthylamine and 4-aminodiphenyl (36). On the other hand, recent studies on the polymorphism of the human glutathione transferase GSTM1 (37) and its relevance to human lung (38) and bladder (39) cancer have introduced the concept that the GSTM1 gene ought to have a protective role in tobacco-induced bladder cancer, possibly through increased detoxification of biologically reactive intermediates of polycyclic aromatic hydrocarbons.

Hence, further study is required to elucidate the relative roles of aromatic amines and of polycyclic aromatic hydrocarbons in the origin of human bladder cancer, both in "normal" populations and in groups with different types of occupational exposures.

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