

The Genetic and Environmental Factors Involved in Benzidine Metabolism and Bladder Carcinogenesis in Exposed Workers

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1. ABSTRACT

Genetic susceptibility to bladder cancer in individuals exposed to arylamines may be explained by interindividual metabolic differences that lead to arylamine bioactivation or detoxification. In this article, occupational bladder cancer risk factors and the evidence that links benzidine exposure to bladder cancer are reviewed. Benzidine metabolism is described and compared with that of other aromatic amines. Metabolic polymorphisms and bladder cancer in the context of occupational exposure to aromatic amines are also reviewed, and the environmental and genetic relationships of benzidine exposure and genetic susceptibility are outlined. Only a few studies of bladder cancer genetic susceptibility in populations exposed occupationally to arylamines have been published. The results of these case-control studies show conflicting results, reflecting metabolic differences between monoarylamines and diarylamines such as benzidine. Additional studies and pooled analyses of existing data are needed to establish if individuals are at higher risk of bladder cancer given the presence of certain alleles that make them more susceptible to this disease.

2. INTRODUCTION

Bladder cancer risk factors, including exposures to aromatic amines (arylamines) in occupational settings and to cigarette smoke, have been known for more than 40 years. There is now realization that most cancers result from a complex interaction of environmental and genetic

factors. It is possible that genetic susceptibility to bladder cancer is explained by interindividual metabolic differences that lead to the bioactivation or detoxification of arylamines. In this article, bladder cancer risk factors and benzidine metabolism as a key factor in bladder carcinogenesis are reviewed. Existing evidence showing that some individuals may be genetically susceptible to the effects of benzidine is summarized. In addition, benzidine metabolism is compared with the metabolism of other aromatic amines and an integrated understanding of metabolic polymorphisms and bladder cancer in the context of exposure to aromatic amines is provided.

3. WORKPLACE BLADDER CANCER RISK FACTORS

Bladder cancer is the ninth most common neoplasm worldwide. An estimated 357,000 new cases of bladder cancer occur each year, with 77% of the cases occurring in men. Sixty-three percent of all incident cases occur in developed countries (1).

The World Health Organization estimates that bladder cancer cases can be subdivided into two broad categories based on etiology: those caused by tobacco and industrial carcinogens, and those caused by schistosomiasis. The former are predominantly transitional cell carcinomas and are common in industrialized countries. The latter are more likely to be squamous cell

carcinomas and are found chiefly in Mediterranean and African countries. Thus, both types are in principle, preventable (2).

Bladder cancer is historically the neoplastic disease most strongly linked to occupational exposure to chemicals. Several occupations have been suspected to increase the risk of bladder cancer, but strong associations only exist for dye workers, aromatic amine manufacturing workers, leather workers, rubber workers, painters, truck drivers, and aluminum workers (3). Occupational exposures to chemicals such as arylamines, polycyclic aromatic hydrocarbons and other industry-related agents may explain some of the risk associated with these occupations (4).

A relationship between bladder cancer and exposure to chemical dyes was first established in 1895 (5). In the late 1930's, Hueper *et al.* showed that oral administration of the industrial arylamine 2-naphthylamine could induce bladder cancer in dogs (6). In 1954, Case *et al.* reported a 20-fold excess of bladder cancer in arylamine-exposed individuals, compared to the general population of England and Wales (7). Since then, the most investigated bladder carcinogens have been 2-naphthylamine, benzidine, and 4-aminobiphenyl. Several studies have reported increased risks of bladder cancer in workers exposed to 2-naphthylamine and 4-aminobiphenyl, and have been reviewed elsewhere (8).

Many synthetic organic dyes contain benzidine or congeners structurally similar to benzidine, and have been shown experimentally to cause bladder cancer (9). Yoshida *et al.* observed an increase of six to eight times the expected rate of bladder cancer in 200 kimono painters who ingested dyes by licking their brushes to obtain fine points. Four specific benzidine-based dyes were frequently used: Direct Black-38, Direct Green-1, Direct Red-17 and Direct Red-28 (10). Two structural analogs of benzidine, MDA (4,4'-methylene-dianiline) and MBOCA (4,4'-methylene-bis(2-chloroaniline)) are carcinogenic in animals and possibly in humans (9). MDA, a curing agent for resins, was associated with a statistically significant threefold elevation of the proportional mortality ratio for bladder cancer (11), and was suggested as the agent responsible for non-invasive papillary bladder tumors in two young, nonsmoking workers in a MBOCA production plant (12).

o-Toluidine, a precursor of an antioxidant used in tire manufacturing with mixed exposures, has also been suggested as a bladder carcinogen in three published studies (13-15). In a cohort of chemical workers exposed to both *o*-toluidine and aniline, a statistically significant elevation in the risk of bladder cancer, likely to be attributed to *o*-toluidine, was reported (15). In a cohort of 49 workers exposed to 4-chloro-*o*-toluidine, another arylamine, seven developed bladder cancer, a 50-fold increase over the expected number of cases in that region (16).

In addition to the production and use of dyes and the manufacture of arylamines, occupations with exposure to combustion gases and soot from coal involve exposures

to polycyclic aromatic hydrocarbons and arylamines. A study in coal carbonizing workers reported a 2.4-fold increased risk of bladder cancer, and found that a sample of tar volatiles included 2-naphthylamine. Excesses of bladder cancer also have been reported among machinists and engineers, possibly exposed to cutting oils and cutting fluids containing arylamines and additives (17).

Occupational bladder cancer continues to be a public health concern in many parts of the world. The estimated population attributable risk for bladder cancer from occupational exposures ranges from 0% to 20% (18). As bladder carcinogenic chemicals have been eliminated from the workplace, new ones are emerging, generating a dynamic shift in high-risk occupations (19). Examples include diminished risks among rubber and leather workers, and emerging risks among truck drivers and aluminum smelter workers. Furthermore, there is a long latency period between contact with a carcinogen(s) and the eventual development of bladder cancer. It has been estimated that the latency for chemically induced bladder cancer ranges from 4 to 45 years (9, 20). Therefore, even workers exposed during the 1960s are still at risk of developing bladder cancer.

4. BENZIDINE EXPOSURE AND BLADDER CANCER

Benzidine is an aromatic amine produced commercially, and predominantly used in the production of dyes, especially azo dyes in the leather, textile and paper industries (21). Production of benzidine was banned in the U.S. in 1973 (22). In recent years, no imports of benzidine itself have been reported, although benzidine-based dyes may still be imported into the U.S. (21). Some developing countries reportedly still produce and use benzidine in dye production (23), and the transfer of benzidine production from other European countries to the former Yugoslavia and to Korea has been reported (24). Exposure to benzidine occurs mainly via dermal contact or by inhalation.

Benzidine has been causally associated with an increase of bladder cancer in humans and induces malignant neoplasms in experimental animals (8). In this section, a select number of studies conducted in workers exposed to benzidine is reviewed. The evidence has been thoroughly reviewed elsewhere (8, 21, 25).

In the 1950s, Case *et al.* studied workers from 21 factories in England and Wales (7). Bladder cancer occurred approximately 15-20 years after exposure and rates were 30 times highest among workers exposed to 2-naphthylamine, 1-naphthylamine, benzidine, or a mixture of these chemicals. Subsequent studies reported a high proportion of bladder cancer cases among subjects exposed occupationally to benzidine and other arylamines (9, 26-29).

In a cohort of 25 workers exposed to benzidine during its manufacture, more than half developed transitional cell bladder cancer in about 13.6 years. No cases were seen in men exposed for fewer than six years

(30). Meigs *et al.* reported a statistically significant excess of bladder tumors in a cohort of workers at a benzidine manufacturing facility in Connecticut [standardized incidence ratio (SIR) = 343, 95% confidence interval (CI) = 148, 676] (31). Risk was greatest among those in the highest exposure category (SIR=1303) with a 21 year latency; those in medium or low exposure categories showed little or no excess risk. A decline in the overall bladder cancer incidence was observed among those employed after 1950, coincident with the implementation of major preventive exposure measures.

In Japanese workers engaged in manufacturing and handling of intermediate products of dyes, Shinka *et al.* observed that of 874 men exposed to benzidine, 105 developed urothelial cancer (more than 85% in the bladder) (32). The mean interval from initial exposure to diagnosis was approximately 24 years, and as other investigators had noted, tended to be shorter as the age at initial exposure increased.

In a cohort of 1,972 benzidine-exposed subjects in China, Bi *et al.* reported an overall SIR of 25, with risks ranging from 4.8 to 158.4 for low to high exposures (33). Benzidine-exposed workers who also smoked cigarettes had a 31-fold risk of bladder cancer, compared to an 11-fold risk observed in non-smoking workers, suggesting a synergistic effect. Wu reported a similar synergistic effect in a retrospective cohort study of 2,525 workers at benzidine manufacturing plants in China (34).

These and other studies have demonstrated that benzidine exposure is associated with an elevated risk of developing bladder cancer. Although some of these studies had small sample sizes and low power, and despite the difficulty in measuring exposure to benzidine and other chemicals present in the plants, there are consistent positive associations with some indication of dose-response relationships. Important evidence of the strength of the association comes from the observation that major preventive measures to reduce exposure to benzidine may have reduced bladder cancer risk. Based on the analysis of the published literature, IARC has classified benzidine as a Group 1 carcinogen and three benzidine-based dyes as Group 2A carcinogens (35-37), and U.S. federal agencies have concurred (22).

5. BENZIDINE METABOLISM

Knowledge of benzidine metabolism is critical in understanding the genetic and environmental risks related to bladder carcinogenesis. As with most chemical carcinogens, aromatic amines need to be metabolized into reactive electrophiles to exert their carcinogenic effect. Hepatic *N*-oxidation of aromatic amines is considered a necessary step for activation to occur (38). The *N*-hydroxy derivatives, once formed, must reach the bladder (39). It has been proposed that *N*-glucuronide conjugates are formed and excreted from the liver. These conjugates then accumulate in urine in the bladder lumen and are

hydrolyzed to hydroxy amines forming arylnitrenium ions that bind DNA (38).

N-Acetylation also occurs in the liver, is considered a detoxification mechanism for aromatic amines and is thought to compete with *N*-oxidation. Primary amines are considered easier to oxidize than their acetylated amide products (40). If this is true, slow acetylators will generate higher levels of *N*-hydroxyarylamines than rapid acetylators. Support for this hypothesis comes from studies of workers exposed to 4-aminobiphenyl, a monoarylamine, demonstrating that slow acetylators are at highest risk for bladder cancer (41, 42), and have a greater formation of adducts (43-44).

Benzidine is a diarylamine whose metabolic pathway leading to carcinogenesis appears to be different from that of aromatic amines having one amine substituent. Zenser *et al.* conducted numerous experimental studies in animals *in vivo* and *in vitro* and in human tissues, to elucidate benzidine metabolism (39, 40, 45-56). Figure 1 shows a comparison of key pathways in the metabolism of benzidine and monoarylamines.

Human benzidine metabolism occurs in the liver and bladder. In the liver, benzidine is acetylated to *N*-acetylbenzidine, which in turn can be acetylated to *N*, *N'*-diacetylbenzidine. *N*-acetyltransferase enzymes (NAT) reportedly catalyze these reactions (49). Two distinct NAT isozymes (NAT1 and NAT2) have been reported (57). Kinetic studies using human liver slices and human recombinant NAT1 and NAT2 suggest that benzidine is a preferred substrate for NAT1 (51). When *N*,*N'*-diacetylbenzidine is formed, it rapidly deacetylates to *N*-acetylbenzidine, and therefore does not accumulate (49). In contrast to monoarylamines, *N*-acetylbenzidine has another free amine group that is susceptible to *N'*-oxidation and *N'*-glucuronidation (51). Consequently, *N*-acetylation is not a detoxifying mechanism for benzidine, but likely an important activation step. In a recent report, Degen *et al.* (58) conclude that *N*-acetylbenzidine plays a key role in bladder carcinogenesis, as it was very efficient in producing DNA adducts in a urothelial cell culture system, even though benzidine was not. Also, the system evidenced that benzidine is easily converted within the urothelium to *N*-acetylbenzidine.

In rats, the cytochrome P-4501A family (CYP1A1 and CYP1A2) plays a major role in the metabolism of *N*-acetylbenzidine and *N*,*N'*-diacetylbenzidine in liver microsomes (53). In humans, benzidine is not thought to be a cytochrome P-450 substrate. However, its mono- and di-acetylated products are substrates for CYP enzymes (49).

More than one glucuronidase transferase is likely to be metabolizing benzidine in humans (40, 47). It has been suggested that all gene products of the human *UGT1* gene family glucuronidate primary amines (59). Extensive *N*-glucuronidation of benzidine and *N*-acetylbenzidine occurs in humans (55). *N*-Glucuronide conjugates are transported by the blood and filtered by the kidneys, and

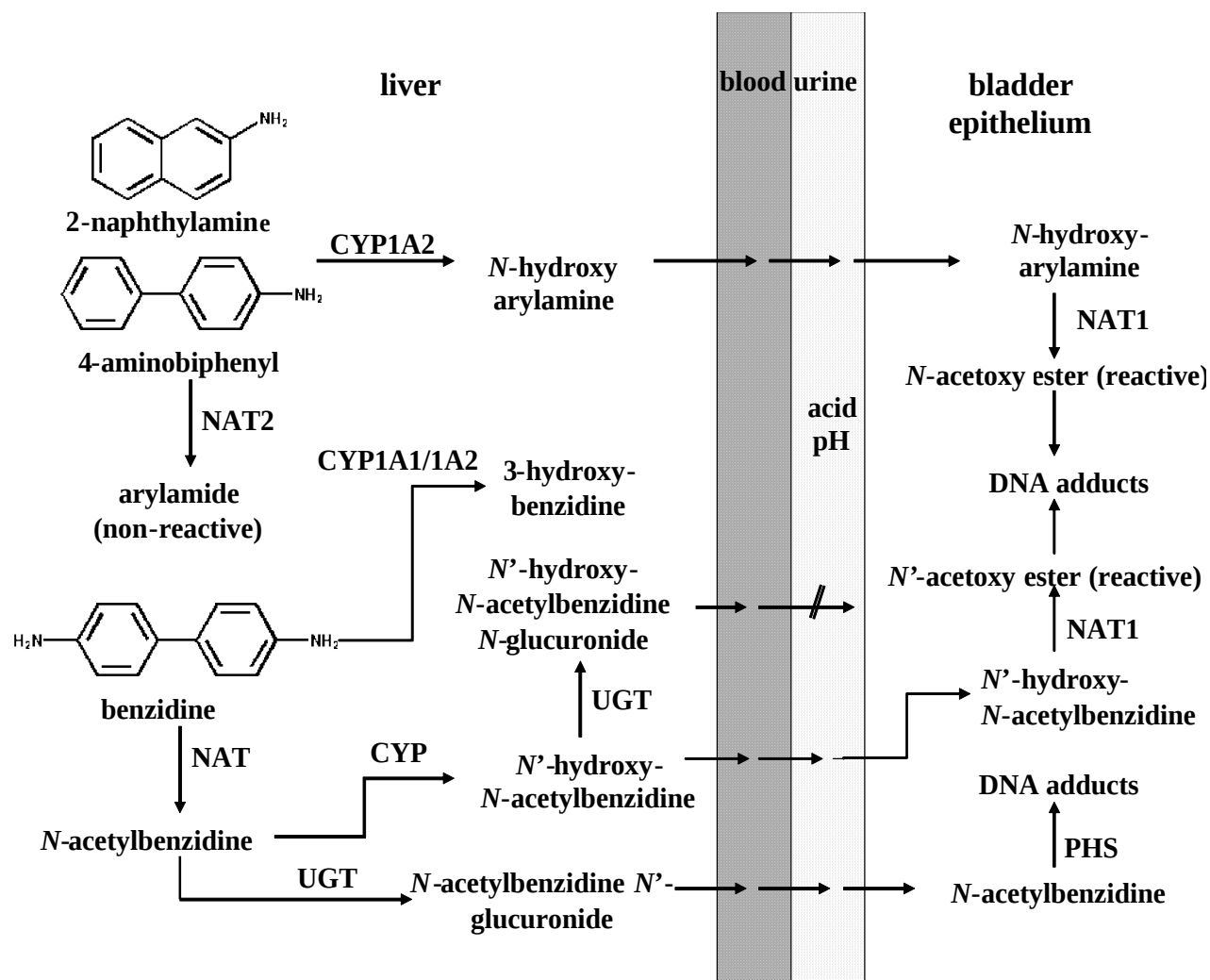


Figure 1. Comparison of key pathways in the metabolism of monoarylamines and diarylamines. Abbreviations: CYP1A1/CYP1A2, cytochrome P-450 1A1/1A2; NAT1/NAT2, *N*-acetyltransferase 1/2; UGT, UDP-glucuronosyltransferase; PHS, prostaglandin H-synthase (78).

accumulated in urine within the lumen of the bladder. *N*-Glucuronidation provides a mechanism for hepatic excretion, transport by plasma, filtration by the kidney, and accumulation in urine (55). Because glucuronidation results in inactivation and excretion, *N*-glucuronidation competes with *N*-oxidation (39).

N-Glucuronides accumulate in urine, where they are acid hydrolyzed to their corresponding arylamines (45). Urinary pH ranges from 4.6 to 8 (38), and urine pH from post-workshift benzidine exposed workers showed an inverse relationship with the proportions of benzidine and *N*-acetylbenzidine present as free (unconjugated) compounds (60).

The O-glucuronides of N-hydroxy-N-acetylbenzidine and N-hydroxy-N,N'-diacetylbenzidine are not acid-labile. The N-glucuronide of N'-hydroxy-N-acetylbenzidine is acid-labile and approximately 30 times more stable at pH 5.5 than N-acetylbenzidine N'-

glucuronide. Similarly, *N*-hydroxy-*N*-acetylbenzidine *N*-glucuronide is 14 times more stable at pH 5.5 than *N*-acetylbenzidine *N'*-glucuronide (39). Therefore, the *N*-glucuronide of *N*-acetylbenzidine is much more likely to be involved in acidic urine-catalyzed hydrolysis than its *N*-hydroxy metabolites (55).

Substantial amounts of prostaglandin H synthase have been detected in the bladder epithelium. Due to its peroxidatic activity, this enzyme activates a variety of aromatic amine carcinogens, including benzidine and *N*-acetylbenzidine (55). In animal studies *in vitro*, prostaglandin H synthase has been used to activate *N*-acetylbenzidine to form a specific DNA adduct, N'-(3'-monophospho-deoxyguanosin-8-yl)-*N*-acetylbenzidine (54). This result is consistent with studies of workers exposed to benzidine showing that this is the major adduct found in exfoliated urothelial cells (61). In addition, subjects with urine pH values <6 exposed to benzidine had tenfold higher levels of this adduct than did subjects with pH ≥ 7 (60). This adduct causes mutations in

bacterial and mammalian test systems and in oncogenes of tumors (62-66). Thus, the peroxidatic activity of bladder cells is capable of activating *N*-acetylbenzidine to form DNA adducts. These adducts may initiate carcinogenesis by producing mutations that become fixed in the genome and eventually contribute to tumor formation (55). Recent evidence suggests that a local peroxidative metabolism of benzidine could also be related with processes of inflammatory response to benzidine (67). These processes might be relevant in promotion of urothelial carcinogenesis.

In summary, the metabolic model suggests that benzidine is *N*-acetylated in the liver. Both benzidine and *N*-acetylbenzidine can be either oxidized or glucuronidated. *N*-Glucuronidation is the favored pathway in humans. *N*-Glucuronides are transported by the blood and filtered by the kidneys, with the resulting accumulation in urine within the lumen of the bladder. These *N*-glucuronides are acid-labile and are converted to their carcinogenic aromatic amines in acidic urine. Prostaglandin H synthase represents a potential peroxidatic pathway for activation of aromatic amines by the bladder to form DNA adducts.

6. METABOLIC POLYMORPHISMS AND BLADDER CANCER

Interindividual variation in activity is characteristic for many metabolic enzymes. In some cases, the variability has been attributed to inherited polymorphisms (68). Phenotypic and genotypic tests have shown that variation in xenobiotic metabolizing enzymes is associated with cancer risk and may have an influence in human susceptibility to genotoxic agents.

In the following section, a review of occupational studies that show associations between bladder cancer and metabolic polymorphisms is presented. Table 1 summarizes the design and power for each study. Crude or adjusted odds ratios (OR) reported in the articles are included in the table; when not reported, crude odds ratios were calculated.

6.1. *N*-Acetyltransferases

Both rapid and slow *N*-acetyltransferase phenotypes and genotypes have been associated with the risk of several cancers. The phenotype is usually determined by administering the subject a drug metabolized by NAT2 and measuring the ratio of acetylated to non-acetylated metabolites in plasma and urine. For the determination of genetic polymorphisms, DNA is isolated from blood or tissues and amplified by a polymerase chain reaction (PCR), generally followed by restriction digests with defined restriction endonucleases (restriction fragment length polymorphisms or RFLP) (69). These procedures have been used to identify the most common alleles and have been shown to predict acetylation status.

For NAT2, nine studies that included bladder cancer cases with documented exposure to aromatic amines in occupational settings were identified (70-78). Of those, only five included exposed controls. Power in these studies is very low—only one had power above 80% to detect a two-fold risk (OR=2.0) and most were below 50%.

Cartwright *et al.* published the first study, with English subjects formerly employed in dye manufacturing (70). Twenty two of 23 subjects with bladder cancer were slow acetylators (OR 16.7, 95% CI 2.2-129.1). Results of this magnitude, however, have not been replicated. Cartwright *et al.* indicated that workers were exposed to benzidine, but that other exposures, such as 2-naphthylamine, were likely and that acetylation metabolism is only relevant for some but not all *N*-substituted arylamines.

Four studies showed slow acetylation as a risk factor for bladder cancer. Of these, the studies of Hanke and Krajewska (73), Lewalter and Mischke (74), and Risch *et al.* (76) reached statistical significance. The Lewalter and Mischke study reported exposures to benzidine, but there is no indication that workers were also exposed to other compounds. Furthermore, analytical procedures were not fully described, and this precluded further evaluation of the reliability of the methods employed.

The studies of Sone *et al.* (72), Hayes *et al.* (75) and Carreón *et al.* (78) reported that slow acetylation is a protective factor for bladder cancer. Golka *et al.* (79) have suggested that metabolic differences between Chinese and Caucasian populations might explain these discrepancies. Alternatively, Carreón *et al.* (78) have proposed that variations in the metabolism of mono- and diarylamines might explain the differences (Figure 1).

The strong positive associations by studies such as those conducted by Cartwright *et al.* and Hanke and Krajewska occurred in populations exposed to monoarylamines. In both studies, exposure was not characterized, but exposure to a number of different aromatic amines or mixtures of chemicals was suspected. The associations are consistent with monoaryamine metabolism data, where slow acetylators are at higher risk because *N*-acetylation appears to be a detoxification pathway.

In contrast to the studies where workers were exposed to a number of aromatic amines, the exposure documented by Hayes *et al.* and Carreón *et al.* is believed to be only to benzidine. The negative association reported by Hayes *et al.* is consistent with data published by Golka *et al.* in a case series of bladder cancer patients (80). A higher but not statistically significant proportion of slow acetylators was found among subjects working in benzidine production, compared to individuals with other occupations (80). In a cross-sectional study in Indian workers exposed to benzidine, the NAT2 slow acetylator genotype was not associated with production of acetylated benzidine metabolites or DNA adducts (61), demonstrating consistency with Zenzer's model of benzidine metabolism.

Only two studies reporting an association between NAT1 polymorphisms and bladder cancer were identified (78, 81). Higher risks of bladder cancer were

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Table 1. Summary of case-control studies of bladder cancer and metabolic polymorphisms in populations occupationally exposed to arylamines

Area of study; (reference)	Cases	N	Controls	N	Phenotype (substrate)/ Genotype; % presumed at risk	OR Adjustment	Subgroup analysis reported	Exposure	Exposure assessment	Power to detect a RR=2.0 ¹	OR (95% CI)
NAT2 (slow vs. fast acetylators or non NAT2*4 or NAT2*13 vs. heterozygous or homozygous for NAT2*4 or NAT2*13)											
UK (70)	Bladder cancer; excluded treated with sulphonamides, isoniazid, procainamide or hydralazine	23	Unexposed urological patients or patients undergoing surgery for other conditions	95	Phenotype (dapsone); cases: 67% controls: 57%	None	Non-occupational group: 1.5 (0.9-2.7) ² ; cigarette smoking; tumor classification	Benzidine and 2-naphthylamine	Dye-working status by interview	27.8%	16.7 (2.2-129.1) ²
Spain (71)	Bladder cancer; cardiac or hepatic insufficiency were excluded	15	Healthy and unexposed from general Spanish population	157	Phenotype (sulfamethazine); cases: 73% controls: 57%	None	Smoking, consumption of coffee, tumor classification, occupational group (ORs not reported)	Jobs with high risk of urothelial cancer	Occupation status by interview	21.5%	2.1 (0.6-6.9) ²
Japan (72)	Bladder cancer	20	Healthy, arylamine-exposed and unexposed from Wakayama prefecture	82	Phenotype (isoniazid); cases: 10% controls: 13%	None	None	Benzidine and 2-naphthylamine	Dye-working status	20.2%	0.7 (0.1-3.5) ²
Poland (73)	Bladder cancer divided by exposure to aromatic amines	67	Unexposed representing general Polish population	22	Phenotype (sulfamethazine); cases: 70% controls: 45%	None	Total & unexposed cases	Benzidine	Occupation status by interview	29.1%	8.4 (1.9-36.6) ²
Germany (74)	Bladder cancer, workers from plant in Leverkusen	239	Healthy workers from plant	92	Phenotype (sulfamethazine); cases: 82% controls: 48%	None	None	Benzidine	Occupation status by interview	80.1%	5.0 (2.9-8.4) ²
China (75)	Bladder cancer, from cohort of workers exposed to benzidine	38	Negative urine cytology, from cohort of benzidine-exposed workers, matched by age (5-years) and city	43	Genotype; cases: 13% controls: 30%	None	Phenotype (dapsone), benzidine exposure	Benzidine only	Benzidine (years exposed & cumulative exposure)	32.3%	0.5 (0.1-1.8)
UK (76)	Bladder cancer, Caucasian	62	Caucasian patients from same hospital with non-malignant urological complaints	59	Genotype; cases: 71% controls: 44%	None	Occupational exposure risk, age, smoking (ORs not reported)	Arylamines	Occupation status by interview	47.0%	3.1 (1.5-6.6) ²
China (77)	Bladder cancer, from cohort of benzidine-exposed workers	29	Healthy and unexposed, from rural area	112	Genotype; cases: 14% controls: 13%	None	Unexposed cases	Benzidine	Dye-working status	26.9%	1.1 (0.3-3.7) ²
China (78)	Bladder cancer, from cohort of workers exposed to benzidine	68	Negative urine cytology, from cohort of benzidine-exposed workers, matched by age (5-years) and city	107	Genotype; cases: 9% controls: 24%	Cumulative benzidine exposure and lifetime smoking	Phenotype (caffeine), benzidine exposure, lifetime smoking, NAT1, GSTM1	Benzidine only	Benzidine (years exposed & cumulative exposure)	54.6%	0.3 (0.1-1.0)
NAT1 (non NAT1*10 vs. heterozygous or homozygous for NAT1*10)											
China (81)	Bladder cancer, benzidine-exposed	38	Healthy, from cohort of benzidine-exposed workers	214	Genotype; cases: wt/*10: 34%, 10/*10: 26%; controls: wt/*10: 71%, *10/*10: 48%	None	Meles/females, cytological grading, smoking status	Benzidine	Dye-working status	wt/*10: 33.6% *10/*10: 48.2%	wt/*10: 0.8 (0.4-1.3) *10/*10: 1.6 (0.6-4.0)
China (78)	See above	28	See above	58	Genotype; cases: wt/*10: 42%, 10/*10: 25%; controls: wt/*10: 47%, *10/*10: 9%	See above	See above	See above	See above	wt/*10: 31.4% *10/*10: 17.5%	wt/*10: 2.8 (0.6-8.3) *10/*10: 2.2 (0.8-7.0)

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Area of study; (reference)	Cases	N	Controls	N	Phenotype (substrate)/ Genotype; % presumed at risk	OR Adjustment	Subgroup analysis reported	Exposure	Exposure assessment	Power to detect a RR=2.0 ¹	OR (95% CI)
GSTM1 (null vs. combined homozygous and heterozygous wild type)											
China (61)	Bladder cancer, from cohort of workers exposed to benzidine	38	Negative urine cytology, from cohort of benzidine-exposed workers, matched by age (5-years) and city	43	Genotype: cases: 61% controls: 60%	Factorv size, duration of exposure	Combined risk of benzidine exposure and GSTM1 null	Benzidine only	Benzidine (cumulative & peak exposures)	33.7%	1.0 (0.4-2.7)
Japan (84)	Urothelial cancer, from cohort with prior exposure to dye-stuff intermediates	36	From cohort without urothelial cancer	101	Genotype: cases: 58% controls: 47%	None. No change after adjustment for age, city, tobacco use, and NAT2 genotype	Work factors	Mainly benzidine and 2-naphthylamine	Occupation data from labor office records	41.7%	2.3 (0.9-5.6)
China (83)	Bladder cancer, from cohort of benzidine-exposed workers	29	Healthy and unexposed, from rural area	182	Genotype: cases: 59% controls: 54%	None	Unexposed cases	Benzidine	Dye-working status	36.9%	1.2 (0.5-2.7) ²
China (78)	See above	68	See above	105	Genotype: cases: 57% controls: 53%	See above	See above	See above	See above	56.7%	1.1 (0.6-2.3)
GSTT1 (null vs. combined homozygous and heterozygous wild type)											
China (83)	See above	29	See above	182	Genotype: cases: 48% controls: 48%	See above	See above	See above	See above	39.2%	1.0 (0.5-2.2) ²
GSTP1 (homozygous wild A/A vs. heterozygous A/G or homozygous mutant G/G)											
China (83)	See above	29	See above	179	Genotype: cases: A/G: 55%, G/G: 0%; controls: A/G:33%, G/G: 6%	See above	See above	See above	See above	A/G: 47.0% G/G: 18.4%	A/G: 2.1 (1.0-4.4) ² G/G: Not computed
UGT2B7 (homozygous wild C/C vs. heterozygous C/T or homozygous mutant T/T)											
China (88)	Bladder cancer, from cohort of workers exposed to benzidine	36	Healthy cohort members, some with cytological abnormalities on Pap smear	218	Genotype: cases: C/T: 42%, T/T: 25%; controls: C/T: 47%, T/T: 9%	None	Papanicolaou's gradings	Benzidine	Dye-working status	C/T: 41.2% T/T: 28.4%	C/T: 2.5 (1.1-5.5) ² T/T: 8.8 (4.0-19.7) ²

¹Power was calculated using the formula by Schlesselman (113) for an unequal number of cases and controls, using a spreadsheet program developed by the authors of this review ($\alpha=0.05$, two-sided test), ²OR non-reported. OR and 95% CI were calculated by the authors of this review using SAS V.8 (SAS Institute, Cary, NC).

observed among study participants homozygous for the NAT1 allele 10. For the heterozygous carriers, results are inconsistent between both studies. Both analyses had limited power to detect an odds ratio of 2.0 ($\alpha=0.05$).

6.2. Glutathione S-Transferases

Four studies have been conducted for GSTM1 polymorphisms in workers exposed to aromatic amines. Rothman *et al.* (82), Ma *et al.* (83) and Carreón *et al.* (78) did not observe an association, while Shinka *et al.* (84) reported a higher risk among those subjects carrying the GSTM1 null genotype, but the association did not reach statistical significance.

These studies may not be comparable however, because exposures are different. Shinka's study involves workers exposed to benzidine and 2-naphthylamine, while in the other studies benzidine was the sole exposure. Power in all studies was low (below 50%). Rothman *et al.* supported their findings by reporting the results of a cross-sectional study of workers who manufactured benzidine-based dyes and benzidine dihydrochloride in India, where GSTM1 genotype had no effect on urine mutagenicity or DNA adduct levels. In addition, in an *in vitro* analysis, these authors found that human GSTM1 did not metabolize benzidine and several benzidine metabolites (82). It was therefore concluded that GSTM1

polymorphisms do not have an effect on benzidine-induced bladder cancer susceptibility. These results are also consistent with the proposed metabolism of benzidine described above and with a study in Chinese workers exposed to benzidine, where the GSTM1 0/0 genotype was not associated with a high cytological grading in a Papanicolaou test (85).

Only one study has reported results for GSTT1 and GSTP1 polymorphisms, occupational exposure to arylamines, and bladder cancer risk (83). The study suggests that the GSTT1 deletion genotype does not have an effect on the risk of bladder cancer, while GSTP1 might be associated. These findings are consistent with results obtained in exfoliated urothelial cells (86). Since power in these studies is low, no statistical significance was attained.

6.3. UDP Transferases

The metabolism of benzidine involves N-glucuronidation of benzidine and N-acetylbenzidine and has been hypothesized to play an important role in benzidine-induced bladder cancer (55). Genetic mutations and polymorphisms have been identified in several UDP transferases (UGTs) genes (87). One study evaluated the association between UGT2B7 and bladder cancer in workers exposed to benzidine (88). Both mutant variants analyzed increased the risk of bladder cancer, particularly among those carrying the homozygous mutant allele.

These results suggest the involvement of UGT polymorphisms in the risk of bladder cancer among benzidine-exposed subjects, and suggest the need to evaluate the effect of other UGT polymorphic genes on bladder cancer.

7. DISCUSSION AND PERSPECTIVES

Only nine published case-control studies have investigated the association between *NAT2* metabolic polymorphisms and bladder cancer risk in populations exposed occupationally to arylamines. This is rather unexpected considering that numerous case-control studies investigating *NAT2* and bladder cancer in the general population have been conducted worldwide (89, 90). The difficulty in finding and having access to adequate occupational groups and the gradual elimination of arylamines from the workplace may explain the low number of published studies.

In five studies, slow acetylators were identified using phenotype information. Phenotype data, however, may be affected by a number of situations, including impaired liver or renal function, alcohol consumption, and cancer chemotherapy (91), subject compliance, dose of the substance administered, sample collection and storage methods, and measurement errors (92).

Four studies used genotypic analysis of *NAT2* polymorphisms to identify slow acetylators. While genotyping is not affected by environmental conditions or disease status, errors related to DNA analysis methods and false detection of a pseudogene may affect the results (92). In addition, exclusion of the analysis of rare alleles could result in misclassification of acetylation status (93). The study by Hayes *et al.* showed a 97% correlation between acetylation status determined biochemically, and that predicted by *NAT2* genotyping (75). This finding is consistent with other studies that have shown high correlation between phenotype and genotype data (94-96). These results suggest that technical factors or differences in study design cannot account for the discrepancy in the results between the three studies, and support the hypothesis that slow acetylators exposed to benzidine are at lower risk of bladder cancer than fast acetylators.

Four studies were identified for the association between *GSTM1* polymorphisms and bladder cancer, and showed conflicting results. GST enzymes do not appear to be involved in the metabolism of benzidine, however, studies in the general population have found that the *GSTM1* null genotype is associated with elevated bladder cancer risk. This genotype may be a general risk factor for bladder cancer, or may be interacting directly or indirectly with specific bladder carcinogens. A role of GST enzymes in the metabolism of benzidine has been suggested (58).

In an epidemiologic study, appropriate power is necessary to detect any difference of importance and to have precise estimates of the association. In all the studies reviewed, only one exceeded 80% power and most were below 50% (for an OR=2 and $\alpha=0.05$, two tails). Because

the power of a study depends on the prevalence of the metabolic polymorphism under study and the magnitude of the risk, larger sample sizes are required for polymorphisms with low prevalence or low relative risk. Furthermore, interethnic differences cause a further reduction in power. For *NAT2*, the slow acetylator polymorphism occurs in about 50% of Caucasian populations, but in less than 10% of Asian populations (97), requiring a higher number of cases in studies of Asian populations. The pooling of comparable data in the Chinese studies would increase the limited power of individual studies.

Other factors that need to be considered in such studies include the selection of cases and controls. None of the studies reviewed indicated if bladder cancer cases were incident or prevalent, but it can be assumed that all included prevalent cases. When prevalent cases are used and an association is observed, it is difficult to differentiate whether the gene affects the susceptibility or the survival of the cases. Brennan (98) has provided an alternative explanation for studies that show an association between the *GSTM1* null genotype and bladder cancer. He has suggested that this genotype may be associated positively with survival, and would therefore be overrepresented in a group of prevalent cases when compared to the background population.

The selection of controls was different among studies. Urological patients or patients undergoing surgery for other conditions were selected as controls in the study by Cartwright *et al.* (70). The inclusion of hospital controls is not desirable, particularly in phenotypic studies, where a medical condition may affect the rate of metabolism of the substrate administered. For those studies that included healthy controls, the possibility that bias was introduced by population stratification has been suggested (98). Population stratification occurs when any factor—genetic or environmental—whose frequency varies between ethnic groups with different risks of disease, can appear to be related to disease even if there is no causal relationship. Wacholder *et al.* (99) have shown that population stratification can cause a spurious allele-disease association only when allele frequency and disease rates differ substantially across ethnic groups and strongly correlate with each other and the true risk factor (e.g., arylamine exposure) that is responsible for the disease rate difference. Furthermore, the true risk factor must be unknown or the investigators must fail to account for it. Since that was not the case in these studies, bias due to population stratification is minimal.

Tobacco use is a known cause of bladder cancer, with 2- to 3-fold increased risk among individuals who have ever smoked (100). Furthermore, tobacco smoke has been reported to contain 4-aminobiphenyl and 2-naphthylamine (101). Some of the studies reviewed did not report the effect of smoking. Studies evaluating the effect of polymorphisms of metabolic enzymes on bladder cancer should control for the effect of smoking in the analysis; otherwise, their conclusions would be limited.

Human disease is caused by interactions among genetic and environmental factors, and bladder cancer is not an exception. Interaction has been defined as the coparticipation of two or more agents in the same causal mechanism leading to disease development (102). Thus in the design and analysis of epidemiologic studies, such interactions need to be explicitly considered. Examining only the association between a genotype and disease may mask the effect of biologic interaction between the genotype and other risk factors (103). Therefore, epidemiologists need to measure all relevant risk factors, which may pose a problem when records are used as the source of information. Similarly, as more than one enzyme is involved in the metabolism of xenobiotics, interactions are likely to be occurring between exposure and two or more genes. Evidence for a three-way interaction has been provided by Taylor *et al.* for *NAT2* and *NAT1* genotypes and smoking as risk factors for bladder cancer in the general population (104). Statistical models commonly used to assess interaction assume a multiplicative effect between the factors studied, but departures from additivity or other relationships may also need to be explored (105). Power and sample size are critical in the statistical analysis of these models of interaction. When both the exposure frequency and the proportion of susceptibles are close to 50%, a moderate number of cases and controls is required to detect an interaction. However, when the exposure frequency is very low or very high, or when the proportion of risk-increasing alleles at the susceptibility locus is very rare or very common, a large sample size will be required but may be unattainable (106). A more efficient study design, the case-only study, has been used in the evaluation of gene-environment and gene-gene interactions (107, 108). This method assumes independence between exposure and genotype in the population, and requires fewer case subjects than a case-control design. While this method is used to assess departures from multiplicative effects, it does not allow the investigation of the independent effects of the exposure alone or the genotype alone. Alternatively, frequency matching for known environmental risk factors with a low prevalence in the population has been proposed to gain power in the study of gene-environment interactions (109).

Furthermore, genotyping of individual single-nucleotide polymorphisms (SNPs) alone may not always provide enough information to evaluate associations. It is important to link SNPs in terms of haplotypes (the determination of the status of SNP variants on a single chromosome) which carry more information about the genotype-phenotype relationship. Special analytical techniques have been designed to determine the allocation of mutations to either DNA strand, and have been used in the analysis of *NAT2* polymorphisms and conditions such as Parkinson's disease, Crohn's disease and macular degeneration (110-112), but not bladder cancer.

In summary, a limited number of studies of bladder cancer genetic susceptibility in populations exposed occupationally to arylamines has been published. Additional studies are needed to establish if individuals are at higher risk of bladder cancer given the presence of

certain alleles that make them more susceptible. The results of meta-analyses of studies of *NAT2* slow acetylation or *GSTM1* null genotype and bladder cancer in the general population confirm the increased risk associated with these genotypes (90). In the workplace, various metabolic polymorphisms could be acting in combination with occupational toxicants to produce risk. For occupational bladder cancer, polymorphic genotypes in members of the *NAT*, *GST* and *UGT* families of genes have been explored. Their joint effect, together with the effect of other genotypes needs to be investigated. Moreover, the metabolic differences between monoarylamines and diarylamines such as benzidine warrant careful attention to the specific compounds to which each worker is exposed.

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