GSTM1 and NAT2 Polymorphisms and Colon, Lung and Bladder Cancer Risk: A Case-control Study

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Abstract. *Background: Glutathione S-transferase M1 (GSTM1) and N-acetyltransferase-2 (NAT2) are phase II enzymes involved in the metabolism of xenobiotics and whose polymorphisms have been related to individual cancer risks. Patients and Methods: A case-control study was performed including 92 colon, 75 lung and 23 bladder cancer patients and 121 corresponding controls to verify the existence of an association between the main genetic polymorphisms of GSTM1 and NAT2 and the risk to develop cancer. Genomic DNA, isolated from 5 mL whole blood, was used to study GSTM1 and NAT2 polymorphisms using multiplex PCR and a PCR-RFLP technique, respectively. Results: GSTM1 homozygous null genotype was associated with an increased risk of colon cancer, especially in females and in younger patients. For NAT2 gene, the results suggest a role for the low acetylator phenotype in the development of colon and lung cancer, especially in females. In bladder cancer patients two rare NAT2 genotypes were found at a higher frequency compared with all the other groups. Conclusion: The results do not suggest a different distribution of GSTM1 and NAT2 polymorphisms in the studied population compared to those reported for other Caucasian populations and warrant further studies in order to evaluate their potential relationship with individual cancer risks.*

Humans are continuously exposed to chemical agents, whether introduced exogenously or produced endogenously, and a number of different enzymes are involved in their metabolism and subsequent excretion. Two major enzyme systems, Phase I and Phase II enzymes, have evolved as an adaptive response to environmental aggression and include the cytochrome P450s, the Glutathione S-Transferases and the N-Acetyltransferases (1). Many of these enzymes are genetically polymorphic and are responsible for wide inter-individual variation in the ability to metabolize xenobiotics, including drugs and carcinogens (2). Polymorphisms derive from genetic mutations which have survived and passed through generations and which have a prevalence in a population of at least 1%. These genetic variations may also result in different patterns of susceptibility to the effects of carcinogenic agents and several studies have attempted to link genetic polymorphisms of xenobiotic metabolizing enzymes with an altered risk for some cancers, particularly those with an important environmental contribution such as lung, bladder and colon cancers (3-5).

Glutathione S-transferases (GSTs) are a family of enzymes that detoxify activated carcinogen metabolites by conjugating them with reduced glutathione (6). These phase II enzymes play a significant role in the detoxification of exogenous carcinogens, including activated metabolites of tobacco smoke compounds such as benzo-pyrene (7). The cytosolic GST enzyme family consists of six gene classes, classified according to their primary structure, termed alpha, mu, pi, sigma, theta and zeta. The GSTM1 locus has at least three allelic variants, GSTM1*A and GSTM1*B which differ by a single base in exon 7, and GSTM1*0, in which the entire gene is deleted. GSTM1 enzyme activity is absent in about 45-50% of Caucasian populations. The absence of GSTM1 activity is caused by the inheritance of two GSTM1*0 alleles (8).

The N-acetyltransferases locus comprises two functional genes (NAT1 and NAT2) and a pseudogene (NATP). NAT2 is involved in the detoxification of several carcinogenic arylamines, including β-naphthylamine and 4aminobiphenyl,

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and in the bioactivation of food mutagens such as 2-amino-3-methylimidazo[4,5f]quindine (9). The most frequent NAT2 alleles consist of the wild-type fast allele NAT2*4 and five slow alleles NAT2*5A, 5B, 5C, 6 and 7 (10). The presence of at least one wild-type allele confers a fast acetylator phenotype, whereas the presence of two mutant alleles is associated with the slow acetylator phenotype, according to the intensity of acetylation. About 50-60% of Caucasians have the slow-acetylator phenotype but the distribution of this phenotype varies greatly, from 5% among Canadian Eskimos to 90% among Northen Africans (11, 12). A simple PCR-RFLP technique allows the identification of a small subset of NAT2 alleles identified as WT, M1, M2 and M3. The M1 alleles are due to the presence of the C481T substitution that is found in many but not all of the NAT2*5 alleles. The M2 allele is characterized by the G590A missense substitution, which is found in NAT2*5 and NAT2*6 allelic clusters. The M3 allele is characterized by the G857A missense substitution which thus far has been identified only on NAT2*7A and NAT2*7B alleles.

Human epidemiological studies have investigated the role of GSTM1 and NAT2 polymorphisms in different types of cancer with conflicting results probably due to the fact that human populations are genetically heterogeneous and exposure to carcinogens are difficult to estimate (13-18). In this study the distribution of GSTM1 and NAT2 polymorphisms in healthy individuals and colorectal, lung and bladder cancer patients was analysed in order to identify genotypical and phenotypical differences among the analysed populations and to determine whether any association exists between the main polymorphisms of these genes and the development of neoplastic diseases.

Patients and Methods

Subjects. The subjects studied were 190 cancer patients (92 colon, 75 lung and 23 bladder cancer cases) and 121 control patients who were enrolled in the Centro di Riferimento Oncologico of Basilicata in Southern Italy. Colon cancer cases included 51 males and 41 females and the mean age at diagnosis was 63 (range: 44-89). Lung cancer cases included 63 males and 12 females and the mean age at diagnosis was 67 (range: 30-90). Bladder cancer cases included 19 males and 4 females and the mean age was 70 (range: 53-83). Overall, cancer cases included 133 males and 57 females with a mean age at diagnosis of 67 years (range 30-90). All colon cancers were adenocarcinoma. The lung cancer cases showed three predominant histological forms: adenocarcinoma, small cells carcinoma and squamous cell carcinoma. The bladder cancer cases included all transitional cell carcinomas. The controls were healthy subjects visiting the hospital for routine blood tests who had no personal and/or family history of cancer. They included 56 males and 65 females and the mean age was 64 (range: 50-82). After written informed consent, all subjects involved in the study provided a sample of blood (5 mL). Genomic DNA was isolated from peripheral white blood cells by proteinase K digestion and extracted using a salting-out method (19).

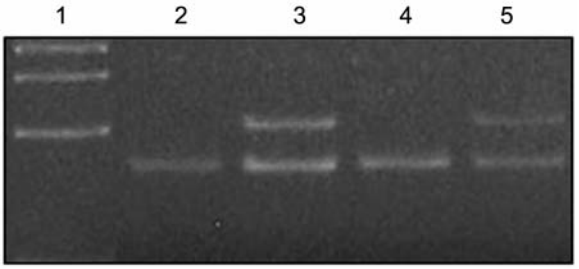

Figure 1. *Analysis of GSTM1 polymorphisms by Duplex PCR. Lane 1: DNA molecular weight standards; Lanes 2 and 4: GSTM1 Null genotype samples (only the Interferon-β product is detected); Lanes 3 and 5: GSTM1 positive genotypes (GSTM1 product=218 bp and Interferon-β=180 bp).*

Analysis of GSTM1 polymorphisms. Analysis of GSTM1 polymorphisms was performed essentially as previously described (20). Briefly, 100 ng of isolated DNA was used for a duplex polymerase chain reaction (PCR). This PCR method uses gene specific primer pairs together with a second primer pair used as a control in the same amplification mixture. The GSTM1 specific primers sequences were: forward 5'-GAACTCCCTGAAAA GCTAAAGC-3' and reverse 5'-GTTGGGCTCAAATATACGGTGG-3' and the specific amplified fragment was 218 bp long. The interferon-β (INF-β) gene was also analyzed using the following primers: forward 5'-GGCACAACAGGTAGTAGGCG-3' and reverse 5'-GCCACAGGAG CTTCTGACAC-3' and the specific amplified fragment was 180 bp long. The INF-β gene was always amplified as an internal positive control in the PCR reaction to detect failure of the amplification reaction. PCR reaction was carried out in a 50 μL total volume of a mixture containing the following components: 30 pM of each primer, 0.2 mM of each dNTP, 1U of Taq polymerase and 3.3 mM $MgCl_2$. After an initial melting temperature of 94˚C for 5 minutes, the reaction mixture was subjected to 25 cycles of 94˚C for 10 seconds, 57˚C for 20 seconds and 72˚C for 45 seconds followed by a final 72˚C extension step for 5 minutes. The PCR products from coamplification of GSTM1 and INF-β genes were separated on a 3% agarose gel and visualized by ethidium bromide staining on a UV transilluminator. GSTM1 genotype was determined by the presence (positive genotype) or absence (null genotype) of the specific PCR product (Figure 1).

Analysis of NAT2 polymorphisms. NAT2 polymorphisms were analyzed by PCR-RFLP technique. This analysis allows the identification of different genotypes corresponding to the two phenotypes, high and low acetylator.

Briefly, a PCR amplification was followed by digestion of the amplified fragment with Kpn1, Taq1 and BamH1 restriction enzymes in order to identify three known slow-acetylator alleles (M1, M2, M3) due to the different combination of wild-type (WT) and mutant (M) alleles. The M1, M2 and M3 alleles are due to the substitutions C481T, G590A and G857A, respectively. The WT allele is formally defined as the absence of C481T, G590A and G857A mutations.

The high acetylator phenotype was determined by the presence of at least one WT allele (genotypes WT/WT or WT/M1, WT/M2, WT/M3) while the low acetylator phenotype was determined by the presence of two copies of NAT2 mutant alleles (Mx/Mx where x=1, 2 or 3).

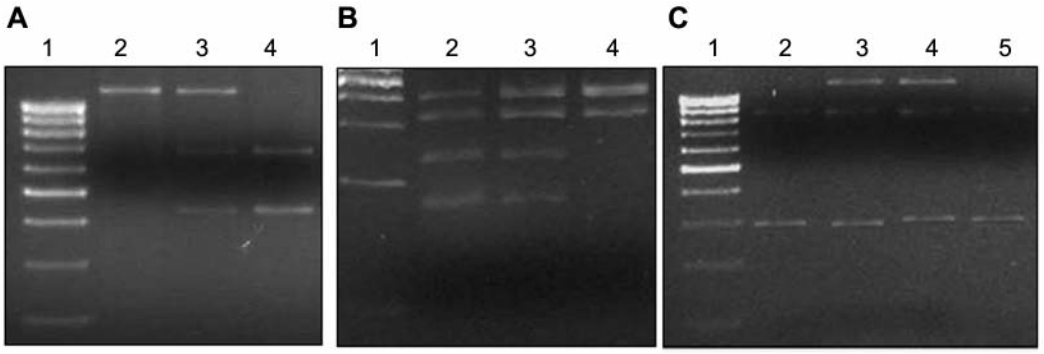

Figure 2. *Analysis of NAT2 polymorphisms by PCR-RFLP analysis. A) Kpn1 digestion products. Lane 1: DNA molecular weight marker (MW); Lane 2: mutant homozygote M1/M1 (1093 bp); Lane 3: heterozygote WT/M1 (660, 433 nd 1093 bp); Lane 4: wild-type homozygote (660 and 433 bp). B) Taq1 digestion products. Lane 1: MW; Lane 2: wild-type homozygote WT/WT (380, 317, 226 and 170 bp); Lane 3: mutant heterozygote WT/M2 (380, 317, 226, 170 and 396 bp); Lane 4: mutant homozygote M2/M2 (317, 380 and 396 bp). C) BamH1 digestion products. Lanes 1: MW; Lanes 2 and 5: wild-type homozygote (811 and 282 bp); Lanes 3 and 4: mutant heterozygotes WT/M3 (1093, 811 and 282 bp).*

A single PCR was carried out using specific primers: forward 5'-GGAACAAATTGGACTTGG-3' and reverse 5'-TCTAGCAT GAATCACTCTGC) (21). Genomic DNA (200 ng) was added to a PCR mix composed of 50 pM of each primer, 0.2 mM of each dNTP, 1U of Taq polymerase, PCR buffer and 2.0 mM $MgCl_2$ in a volume of 80 μL. The reaction was denatured at 94˚C for 4 minutes and subjected to 30 cycles of 94˚C for 30 seconds, 57˚C for 30 seconds and 72˚C for 90 seconds. A final 72˚C extension for 5 minutes was performed and the specific amplified fragment was 1093 bp long. Following PCR, 20 μL aliquots of the PCR reaction were subjected to restriction digestion with KpnI (37˚C for 1h; M1 allele), TaqI (65˚C for 1h; M2 allele) or BamHI (30˚C for 1h; M3 allele). The restriction digests were electrophoresed on 2% (M1 and M3) or 3% (M2) agarose gels and visualized by ethidium bromide staining on a UV transilluminator (Figure 2).

Statistical analysis. X^2 tests were used to examine differences of genotypic and phenotypic frequencies between patients and controls and the results were considered statistically significant when the *p*-value was ≤0.05.

Results

One-hundred and twenty-one controls and 190 cancer patients (including 92 colon, 75 lung and 23 bladder patients) were included in this study.

The null GSTM1 genotype was detected in 61 (66%) colon cancer patients, 42 (56%) lung cancer patients and 13 (57%) bladder cancer patients and in 68 (56%) of the controls (Table I). Thus, the frequency of GSTM1 null genotype was higher in colon cancer compared with control subjects and with lung and bladder cancer cases but these differences were not significant. In colon and bladder cancer cases, but not in lung cancer cases and in controls, null genotype was more frequent (p=0.01 and p<0.05, respectively) than positive genotype. The distribution of GSTM1 null genotype in females was 52% in the control group, 71% in colon cancer, 50% in bladder cancer and only 25% in lung cancer (Table II). Thus, null genotype frequency in females was higher in colon cancer patients compared to all other groups but reached significance only compared with the group of female lung cancer patients (p=0.01). The frequency of GSTM1 null genotype in the control group and cases according to age were also evaluated. The cut-off value for young versus old subjects was set at 65 years. The frequencies of GSTM1 null and positive genotypes in >65 year old subjects were similar in controls, colon, lung and bladder cancer patients (Table II). On the other hand, the frequency of null genotype in ≤65 year old subjects was significantly higher in colon cancer (77%) compared with controls (50%, p=0.01) and with lung cancer (48%; p<0.05) but not with bladder cancer patients which were a limited number of subjects.

Table I. *GSTM1 phenotypes in cancer patients and in healthy controls*

	Colon Cancer N (%)	Lung Cancer N (%)	Bladder Cancer N (%)	Controls N (%)
Total	92	75	23	121
Null	61 (66)	42 (56)	13 (57)	68 (56)
Positive	31 (34)	33 (44)	10 (43)	53 (44)

The NAT2 low acetylator phenotype, determined by the presence of two mutant alleles, was found in fifty (41%) controls and in fifty-one colon (55%), thirteen bladder (57%) and 44 lung (59%) cancer patients (Table III). Thus, frequency of the low acetylator phenotype was higher in colon and lung cancer patients compared with control group and the difference was significant (p<0.05). In the control group, but not in cancer groups, the high acetylator phenotype was more represented than the low acetylator phenotype

Table II. *GSTM1 phenotypes in cancer patients and healthy controls stratified by sex and age.*

	Colon Cancer N (%)	Lung Cancer N (%)	Bladder Cancer N (%)	Controls N (%)
Males				
Total	51	63	19	56
Null	31 (61)	40 (64)	11 (58)	35 (62)
Positive	20 (39)	23 (36)	8 (42)	21 (38)
Females				
Total	41	12	4	65
Null	29 (71)	3 (25)	2 (50)	34 (52)
Positive	12 (29)	9 (75)	2 (50)	31 (48)
≤65 years				
Total	35	33	5	66
Null	27 (77)	16 (48)	2 (40)	33 (50)
Positive	8 (23)	17 (51)	3 (60)	33 (50)
>65 years				
Total	57	42	18	55
Null	34 (60)	27 (64)	11 (62)	35 (64)
Positive	23 (40)	15 (36)	7 (38)	20 (36)

Table III. *NAT2 phenotypes in cancer patients and in healthy controls*

	Colon Cancer N (%)	Lung Cancer N (%)	Bladder Cancer N (%)	Controls N (%)
Total	92	75	23	121
Low	51 (55)	44 (59)	13 (57)	50 (41)
High	41 (45)	31 (41)	10 (43)	71 (59)

Table IV. *NAT2 phenotypes in cancer patients and healthy controls stratified by sex and age.*

	Colon Cancer N (%)	Lung Cancer N (%)	Bladder Cancer N (%)	Controls N (%)
Males				
Total	51	63	19	56
Low	25 (49)	35 (56)	12 (63)	23 (41)
High	26 (51)	28 (44)	7 (37)	33 (59)
Females				
Total	41	12	4	65
Low	25 (61)	8 (67)	1 (25)	27 (42)
High	16 (39)	4 (33)	3 (75)	38 (58)
≤65 years				
Total	35	33	5	66
Low	14 (40)	22 (67)	1 (20)	25 (38)
High	21 (60)	11 (33)	4 (80)	41 (62)
> 65 Years				
Total	57	42	18	55
Low	36 (63)	20 (48)	12 (67)	25 (45)
High	21 (37)	22 (52)	6 (33)	30 (55)

Table V. *Distribution of NAT2 Genotypes in cancer patients and healthy controls.*

	Colon cancer N (%)	Lung cancer N (%)	Bladder cancer N (%)	Controls N (%)
Total	92	75	23	121
WT/WT	6 (6.5)	4 (5.3)	1 (4.3)	11 (9)
WT/M1	23 (25)	18 (24)	6 (26)	41 (33.9)
WT/M2	12 (13)	10 (13.3)	2 (8.7)	16 (13.2)
WT/M3	1 (1)	0 (0)	1 (4.3)	3 (2.5)
M1/M1	16 (17.4)	17 (22.7)	4 (17.4)	19 (15.7)
M1/M2	19 (20.6)	20 (26.7)	5 (21.7)	20 (16.5)
M1/M3	7 (7.6)	1 (1.3)	1 (4.3)	4 (3.3)
M2/M2	6 (6.5)	5 (6.7)	2 (8.7)	7 (5.8)
M2/M3	2 (2.2)	0 (0)	1 (4.3)	0 (0)

(p=0.01). The distribution of the low acetylator phenotype in female and male controls was 42% and 41%, respectively (Table IV). In colon and lung cancers, the frequency of the low acetylator phenotype was higher in females (61% and 67%, respectively) than in males (49% and 56%, respectively) while in bladder cancer the frequency of low acetylators was higher in males than females (63% and 25%, respectively) but these differences were not significant, probably because of a different number of females and males in the control group and in the three groups of cancer patients. The frequency of low acetylator phenotype in colon cancer patients was higher (63%) in >65 year old subjects compared with ≤65 year old subjects (40%) (p<0.05). Conversely, in lung cancer cases the frequency of low acetylators was higher in ≤65 year old subjects (67%) than in >65 year old subjects (48%) and this difference (p=0.01) was significant. Moreover, the percentage of low acetylators observed in ≤ 65 year old lung cancer cases was higher than the one observed in ≤65 year old subjects with colon (p<0.05) and bladder (p<0.05) cancer and in ≤ 65 year old controls (p=0.01). In the bladder cancer groups, 80% of ≤65 year old subjects displayed the high acetylator phenotype, but because of the too small number of cases, the differences were not significant (Table IV).

The NAT2 genotypes associated with a high acetylator phenotype more frequently detected in controls and cases were WT/M1 and WT/M2, while the genotypes most frequently associated with a low acetylator phenotype were M1/M1 and M1/M2 (Table V). Although the differences were not significant, the percentage of the two NAT2

genotypes that determined a high acetylator phenotype was higher in controls compared with colon, lung and bladder cancer groups. M3/M3 genotype was not found in cases neither in controls; M2/M3 genotype was found in cancer cases but not in the control group. Although the bladder cancer cases were only 23, in this type of cancer two rare genotypes were identified, namely WT/M3 (4.3% in bladder cancer, 1% in colon cancer, 0% in lung cancer, 2.5% in controls) and M2/M3 (4.3% in bladder cancer, 2.2% in colon cancer, 0% in lung cancer and controls) which displayed in this group of patients a higher frequency than in controls and in the other cancer cases.

Discussion

Numerous studies have investigated the relationship between GSTM1 and NAT2 genotypes and cancer susceptibility (22-25). The results vary widely and are often discordant likely because of ethnic and geographic differences of the enrolled subjects. This study has been carried out on subjects resident in Basilicata, a small region in Southern Italy which, because of its mountainous nature and cultural customs, has remained isolated for centuries, maintaining a rather homogeneous genetic background within its population.

Considering the distribution of the GSTM1 polymorphisms among the types of tumours (colon, lung and bladder cancers) analyzed in this study, colon cancer patients displayed the highest percentage of null genotype (66%), while the percentage in lung (56%) and bladder (57%) cancer cases was not different from that observed in the healthy controls (56%). This finding suggests that GSTM1 null genotype might be a risk factor for colon cancer and particularly for women (71% of the females with colon cancer showed a GSTM1 null phenotype against 52% of healthy females). On the contrary, only 25% of females with lung cancer showed the null phenotype. Thus, this polymorphism might not have influence on the development of lung cancer in females. These data also suggest an association between the null GSTM1 polymorphism and age of onset of colon cancer. In fact, this polymorphism was more frequent in ≤65 year old colon cancer patients compared with controls and lung cancer patients ($p<0.05$) of the same age group. In conclusion, the findings regarding GSTM1 confirm and extend previous data suggesting that the null genotype has an important role in the development of colon cancer especially in females and younger (<65 year olds) subjects (20). On the contrary, it does not appear to have an equally important role in the development of other types of tumours, such as lung and bladder cancer in which environmental factors and/or other genetic factors might be more relevant.

The data regarding NAT2 gene suggest a role for the low acetylator phenotype in the development of colon and lung cancer since it was more frequent in these two types of cases than in healthy controls ($p<0.05$). In bladder cancer cases the percentage of low acetylator phenotype was similar to that observed in colon and lung cancer patients and higher compared with the controls, but the difference was not significant probably due to the small number of subjects analysed.

Analysing data in relation to the different types of tumour, these findings suggest a clear trend for an association between the low acetylator phenotype and female gender in colon and lung cancer cases. On the contrary, the low acetylator phenotype appeared to be associated with the male gender in bladder cancer cases. In fact, nearly all females with bladder cancer showed the high acetylator phenotype, but the small number of samples does not allow definitive conclusions to be drawn. The results also suggest an association between NAT2 low acetylator phenotype and age of onset of lung cancer. In fact, the frequency of low acetylator polymorphism was significantly higher in ≤65 year old subjects with lung cancer compared with ≤65 year old controls ($p=0.01$) as well as colon ($p<0.05$) and bladder ($p<0.05$) cancer cases. On the contrary, the frequency of high acetylator phenotype was higher in ≤65 year old subjects with colon and bladder cancer compared with controls and with older (>65 years old) patients.

The different distribution of NAT2 phenotypes in the different types of cancers could be explained with the double role of the NAT2 enzyme which is involved in the detoxification of numerous xenobiotics but is also able to produce reactive molecules capable of interfere with cellular components such as DNA. Consequently, the low acetylator phenotype can be, in some cases, more protective then a high acetylator phenotype since, depending on the type of environmental exposure, it might be associated with the production of a reduced amount of reactive molecules. The results of the present study suggest that the presence of a low acetylator phenotype is associated with a greater risk to develop colon and lung cancer at an younger age, especially in women. The results obtained in bladder cancer cases seem to disagree with the ones obtained for other types of tumours. Moreover, in bladder cancer two rare NAT2 genotypes were found at a greater percentage compared with controls as well as lung and colon cancer cases. Further studies on a larger cohort of bladder cancer cases are warranted to fully investigate the role of NAT2 polymorphisms and the occurrence of the above mentioned polymorphisms in bladder cancer.

In conclusion, the results of the present study do not suggest a different distribution of GSTM1 and NAT2 polymorphisms in the population of the Basilicata region compared to those reported for other Caucasian populations. Moreover, they suggest a potential important relationship between polymorphisms in these two genes and tumor development but further studies are warranted to confirm this relationship and to evaluate its importance and role in the development of different types of cancers.

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