

Slow *N*-acetylation as a possible contributor to bladder carcinogenesis

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Funding information

American University of Beirut,
Grant/Award Number: URB Award #103182

Abstract

Bladder cancer (BCa) is an exophytic tumor that presents as either noninvasive confined to the mucosa (NMIBC) or invading the detrusor muscle (MIBC), and was recently further subgrouped into molecular subtypes. Arylamines, major BCa environmental and occupational risk factors, are mainly metabolized by the genetically polymorphic *N*-acetyltransferases 1, *NAT1* and *NAT2*. In this study, we investigated the association between *N*-acetyltransferases genetic polymorphism and key MIBC and NMIBC tumor biomarkers and subtypes. A cohort of 250 males with histologically confirmed urothelial BCa was identified. Tumors were genotyped for *NAT1* and *NAT2* using real-time polymerase chain reaction (PCR), and characterized for mutations in *TP53*, *RB1*, and *FGFR3* by PCR-restriction fragment length polymorphism. Pathology data and patients' smoking status were obtained from medical records. Pearson χ^2 and Fisher exact tests were used to check for associations and interactions. Results show that *NAT1* G⁵⁶⁰A polymorphism is significantly associated with higher muscle-invasiveness (MIBC vs NMIBC; $P = .001$), higher tumor grade (high grade vs low grade; $P = .011$), and higher *FGFR3* mutation frequency within the MIBC subgroup ($P = .042$; $.027$). *NAT2* G⁸⁵⁷A polymorphism is also found to be significantly associated with higher muscle-invasiveness (MIBC vs NMIBC; $P = .041$). Our results indicate that slow *N*-acetylation is a contributor to bladder carcinogenesis and muscle-invasiveness. These findings highlight *NAT1* as a biomarker candidate in BCa and a potential target for drug development.

KEYWORDS

bladder cancer, *FGFR3*, MIBC, *N*-acetyltransferases, *NAT1*, *TP53*

1 | INTRODUCTION

Globally, bladder cancer (BCa) is the tenth most common cancer, the sixth most common among men, and the ninth leading cause of cancer death, with four-fold higher risk in males compared with females.¹ Incidence rates are normally higher in industrialized countries, and are much lower in developing countries, with very few exceptions, such as in Lebanon and Egypt.²

BCa is a particularly heterogeneous tumor, initiated by a clonal expansion of genetically altered urothelial cell populations arising from a broad area of the mucosa lining the bladder wall. The most common type is the urothelial transitional cell carcinoma.³ BCa manifest as either non-muscle invasive confined to the mucosa or lamina propria (non-muscle invasive bladder cancer [NMIBC]) or as a clinically aggressive tumor invading the detrusor muscle (muscle invasive bladder cancer [MIBC]).⁴ Distinct histopathological features characterize these two different

clinical tracks, where NMIBCs develop as recurring papillary projections while MIBCs develop as invasive flat lesions progressing rapidly, with a potential to metastasize into lymph nodes, liver, lungs, bone, brain, and peritoneum.⁵

At the molecular level, all studies concluded that NMIBCs and MIBCs form two broad distinct subsets.⁶ The MIBC-associated subset exhibits higher expression of genes involved in cell cycle progression and mitosis, extracellular matrix, and immune cells, whereas the NMIBC cluster shows upregulation in ribosomal genes.⁷ More specifically, the MIBC subset is distinguished by genomic instability and higher mutation rates with copy number variations in *TP53*, *RB1* and *E2F3*, while NMIBC is characterized by a higher prevalence of *FGFR3* and *PIK3CA* activating mutations.⁸ MIBCs are further sub-grouped into two major intrinsic categories, luminal and basal,⁹ and very recently six MIBC molecular subtypes have been identified based on an international consensus on gene expression and mutation load patterns; these include the luminal papillary, luminal non-specified, luminal unstable, stroma-rich, basal/squamous, and neuroendocrine-like subtypes.¹⁰⁻¹⁵ In particular, the luminal papillary MIBC subtype is found to be distinctly enriched with *FGFR3* activating mutations.^{11,16}

Most BCa cases are attributed to environmental exposures. The urothelial cells lining the mucosal surfaces may be exposed to carcinogens that are either eliminated in urine or bioactivated by drug-metabolizing enzymes.¹⁷ Chemical carcinogenesis is believed to underlie most of the BCa incidence, and is particularly triggered by arylamines from tobacco smoking, diet, hair dyes, fossil fuel emissions, occupational exposures, and other environmental sources.¹⁸ The arylamine *N*-acetyltransferases, NAT1 and NAT2, are Phase II drug-metabolizing enzymes that utilize acetyl coenzyme A (AcCoA) to acetylate a wide range of arylamines including hydrazines, aromatic amines, and heterocyclic amines.¹⁹ The NAT1 isoenzyme is widely expressed in fetal and adult tissues including the urinary bladder, while NAT2 is found primarily in the liver, intestine, and colon.²⁰ In recent years, accumulating evidence showed that NAT1 has an unusual expression in malignancies, and a wide panel of inhibitors, including environmental toxicants and chemotherapeutic agents.²¹⁻²⁴ Both enzymes are genetically polymorphic, with many polymorphisms found to affect their enzymatic activity, expression levels, and stability.²⁵ An individual's phenotype may be classified, based on an international consensus, as either a "Rapid," "Normal," or "Slow" acetylator for NAT1 and NAT2 based on carried haplotypes.²⁶ Recently, NAT1 phenotypic variants nomenclature was slightly modified, where "decreased activity" replaced "Slow", and "increased activity" replaced "Rapid" (Table S1).²⁷ Many human epidemiological investigations, meta-analyses, and genome-wide association studies have shown that *N*-acetylation genetic polymorphism is associated with BCa and may modify susceptibility to arylamine exposures.²⁸⁻³¹ In this study, we investigated an association between NAT1 and NAT2 genetic polymorphisms, independently and in interaction with smoking, with key MIBC and NMIBC molecular tumor markers.

2 | MATERIALS AND METHODS

2.1 | Study population

Two hundred fifty patients with histologically confirmed urothelial BCa diagnosed between 2013 and 2017 were identified from the archives of two major medical centers in the capital city Beirut. Recruited cases included Lebanese patients above the age of 50, starting with the most recently diagnosed. The study excluded non-Lebanese patients, subjects under 50 years of age, patients with additional types of cancer, and those with unavailable archival tumor tissues. Given the low BCa incidence in females, enrollment focused on male patients to maintain statistical power. Information on tumor grade and stage, and patients' smoking status, were obtained from medical records. All collected data was obtained as a deidentified set. Institutional Review Board approvals from the American University of Beirut and collaborating medical centers were obtained before conducting the study.

2.2 | DNA extraction

Multiple sections of 5 μ m-thickness were made from formalin-fixed paraffin-embedded (FFPE) tumor blocks for each of the identified patients. Sections were prepared by a trained pathologist to insure acquiring tumor tissue while avoiding necrotic areas. Prepared sections were deparaffinized by xylene and digested by proteinase K, then DNA was extracted using a QIAamp DNA FFPE Tissue kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Extracted tumor DNA was quantified by a fluorometer and evaluated for quality by agarose gel electrophoresis.

2.3 | *N*-Acetyltransferases genotyping

The NAT1 polymorphisms C⁵⁵⁹T (rs5030839), G⁵⁶⁰A (rs498782), and C¹⁰⁹⁵A (rs15561) were detected by real-time PCR (CFX96; Bio-Rad, Hercules, CA) using a customized TaqMan Drug Metabolism Genotyping Assay (Thermo Fisher Scientific, MA). Briefly, a TaqMan Universal PCR Master Mix was combined with primers and probes, and 40 ng of tumor DNA, making a final volume of 25 μ L. Polymerase chain reaction (PCR) conditions consisted of two initial hold steps (50°C for 2 minutes followed by 95°C for 10 minutes) and 40 cycles of a two-step PCR: 92°C for 15 seconds, 60°C for 90 seconds. The NAT2 polymorphisms T³⁴¹C (rs1801280), G⁵⁹⁰A (rs1799930), G⁸⁵⁷A (rs1799931) were also detected by real-time PCR using a customized TaqMan assay as described.³² Briefly, a TaqMan Universal PCR Master Mix was combined with 500 nM of primers and 150 nM of probes, and 40 ng of tumor DNA, in a final volume of 20 μ L. PCR conditions consisted of two initial hold steps (50°C for 2 minutes followed by 95°C for 10 minutes) then 40 cycles of a two-step PCR (95°C for 15 seconds, 57°C for 60 seconds).

2.4 | Tumors' molecular markers mutation detection

PCR and restriction digestion with optimized conditions were used to detect mutations in the tumors for the following molecular markers: *TP53* at exon 4-codon 72 (rs1042522) and at exon 7-codon 248 (rs121912651), *RB1* non-sense mutation (rs137853293) at exon 23, and *FGFR3* somatic activating mutations at exon 7-codon 248 (rs121913482) and codon 249 (rs121913483). Given the degraded nature of archival tumors due to formalin fixation, primers we designed and evaluated several times to obtain short intact amplicons targeting desired mutations effectively. Briefly, each PCR reaction consisted of 200 ng of tumor DNA, 300 nM of primers, PCR buffer, and HotStarTaq DNA Polymerase (Qiagen, Valencia, CA), in a final volume of 50 μ L. PCR conditions consisted of a 95°C for 5 minutes step, 40 cycles of a three-step PCR (94°C for 15 seconds, suitable annealing temperature for 1 minute, and 72°C for 1 minute) and a 72°C for 10 minutes step. The resulting amplified DNA was incubated with the appropriate restriction enzyme at optimal conditions. Digested DNA fragments were then analyzed by agarose gel electrophoresis. Designed PCR primers, annealing temperatures, and restriction enzymes are detailed in the Supplementary Material (Table S2).

2.5 | Statistical analysis

Descriptive statistics were used to summarize study population characteristics. The associations between *NAT1* and *NAT2* genetic polymorphisms with mutational frequency in molecular tumor markers, tumor grade, and muscle-invasiveness, were examined using Pearson's χ^2 and Fisher exact tests, both independently and in interaction with smoking status. Associations were examined in both the total sample and in subgroups stratified by muscle-invasiveness (MIBC vs NMIBC). Statistical analysis was conducted using Stata data analysis and statistical software (Stata 13. MP). The analysis was adjusted for multiple comparison testing using the Bonferroni correction. Originally, power analysis was carried out to calculate the adequate sample size based on the expected mutations' prevalence and considering a 5% margin of error, and a 90% confidence level.³³ A total sample of 256 BCa cases was targeted on that basis.

3 | RESULTS

3.1 | Patients' smoking status and tumors' characteristics

Out of 263 identified patients with histologically confirmed urothelial BCa, 250 tumor samples yielded sufficient amounts of DNA to run the analyses. Out of 250 samples analyzed, 226 were high grade, 8 were low grade, and the 16 remaining samples were unknown; 143 tumors were NMIBC while 106 were MIBC, and one sample was unknown. In addition, 185 patients were ever smokers, while 52 were non-smokers, and 13 were unknown (Table 1).

3.2 | Frequency distribution of NATs single nucleotide polymorphisms and mutations in molecular tumor markers

The frequency of *N*-acetyltransferases' single nucleotide polymorphisms (SNPs) and molecular markers' mutations are summarized in Table 2. Briefly, *NAT1* C¹⁰⁹⁵A, G⁵⁶⁰A, and C⁵⁵⁹T polymorphisms were found to be prevalent in 88%, 45%, and 2% of the total sample, respectively. On the other hand, *NAT2* G⁵⁹⁰A, T³⁴¹C, and G⁸⁵⁷A polymorphisms were prevalent in 73%, 61%, and 27%, respectively. In addition, homozygous mutants for *TP53* at codon 72 were about 20% of the total sample, while *TP53* codon 248 and *RB1* exon 23 mutations were totally absent (Table 2). In addition, 90% of the tumors tested positive for the *FGFR3*-codon 249 activating mutation, while 96% of the samples tested negative for the *FGFR3*-codon 248 activating mutation (Figure 1).

On the other hand, patient-based molecular characterization showed that 1.2% of samples carry all three *NAT1* SNPs, while only 0.8% do not carry any of the tested SNPs. In addition, 10.8% of the total sample carry all three *NAT2* SNPs, while only 2.4% do not carry any of the tested SNPs. Both *TP53* tested mutations at codons 72 and 248 were absent in 45.6% of the total sample, while both *FGFR3* tested mutations at codons 248 and 249 were absent in only 9.6% of the total sample. *RB1* mutation at exon 23 was not detected in the entire sample (Table 2). The detailed allelic frequency distribution for *NAT1* and *NAT2* and the prevalence of tumor markers' mutations in subgroups stratified by muscle-invasiveness are described in the Supplementary Material (Tables S3 and S4).

In summary, the most prevalent *NAT1* SNPs in this group are C¹⁰⁹⁵A, G⁵⁶⁰A, which is consistent with previous studies in this target population.³⁴ While for *NAT2*, G⁵⁹⁰A is found to be the most prevalent SNP. In addition, among tested tumor markers, the most commonly prevalent tumor drivers are mutations at *FGFR3*-codon 248 and *TP53*-codon 72.

TABLE 1 Frequencies of tumor stage, grade, and muscle-invasiveness, and patients' smoking status in the total sample (N = 250)

Characteristic	Frequency N (%)
Tumor grade	
High grade	226 (90.4)
Low grade	8 (3.2)
Unknown	16 (6.4)
Invasiveness	
MIBC	106 (42.4)
NMIBC	143 (57.2)
Unknown	1 (0.4)
Smoking status	
Smoker	185 (74.0)
Nonsmoker	52 (20.8)
Unknown	13 (5.2)

Abbreviations: MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer.

TABLE 2 Frequency distribution of NAT1 and NAT2 SNPs, and mutations in molecular tumor markers TP53, RB1, and FGFR3 mutations in the total sample (N = 250); the frequencies of NAT1 and NAT2 SNPs in homozygous and heterozygous samples, as well as the mutational frequencies of molecular tumor markers, are summarized for the entire tested sample; the table also shows the patient-based genetic frequency distribution for each examined gene, presenting clustering of the different NAT SNPs and tumor markers' mutations in categories of one- or two- or three-SNP/mutation(s), or no SNP/mutations

SNP or mutation	-/- N (%)	+/- N (%)	-/+ N (%)	Unknown N (%)	Patient-based frequency distribution			
					1 SNP/mutation N (%)	2 SNP/mutations N (%)	3 SNP/mutations N (%)	No SNP/mutations N (%)
NAT1 C ⁵⁵⁹ T	243 (97.2)	0 (0)	6 (2.40)	1 (0.4)	4 (1.6) [G ⁵⁶⁰ A]	3 (1.2) [C ⁵⁵⁹ T & C ¹⁰⁹⁵ A]	3 (1.2)	2 (0.8)
NAT1 G ⁵⁶⁹ A	135 (54)	2 (0.8)	111 (44.4)	2 (0.8)	109 (43.6) [C ¹⁰⁹⁵ A]	106 (42.4) [G ⁵⁶⁰ A&C ¹⁰⁹⁵ A]		
NAT1 C ¹⁰⁹⁵ A	2 (0.8)	103 (41.2)	118 (47.2)	27 (10.8)				
NAT2 T ³⁴¹ C	92 (36.8)	84 (33.6)	70 (28)	4 (1.6)	50 (20) [T ³⁴¹ C]	18 (7.2) [T ³⁴¹ C & G ⁸⁵⁷ A]	27 (10.8)	6 (2.4)
NAT2 G ⁵⁹⁰ A	75 (30)	45 (18)	113 (45.2)	17 (6.8)	57 (22.8) [G ⁵⁹⁰ A]	59 (23.6) [T ³⁴¹ C & G ⁵⁹⁰ A]		
NAT2 G ⁸⁵⁷ A	175 (70)	5 (2)	63 (25.2)	7 (2.8)	8 (3.2) [G ⁸⁵⁷ A]	15 (6) [G ⁵⁹⁰ A & G ⁸⁵⁷ A]		
TP53 C72	119 (47.6)	49 (19.6)	72 (28.8)	10 (4)	121 (48.4) [C72]	0	N/A	114 (45.6)
TP53 C248	239 (95.6)	1 (0.4)	2 (0.8)	8 (3.2)	3 (1.2) [C248]			
RB1 E23	218 (87.2)	0	0	32 (12.8)	0	N/A	N/A	218 (87.2)
FGFR3 C248	240 (96.0)	1 (0.4)	8 (3.2)	1 (0.4)	0 [C248]	9 (3.6)	N/A	24 (9.6)
FGFR3 C249	24 (9.6)	3 (1.2)	222 (88.8)	1 (0.4)	216 (86.4) [C249]			

Note: -/-, homozygous wild-type; +/-, homozygous mutant; -/+ , heterozygous.
Abbreviation: N/A, not available; SNP, single nucleotide polymorphism.

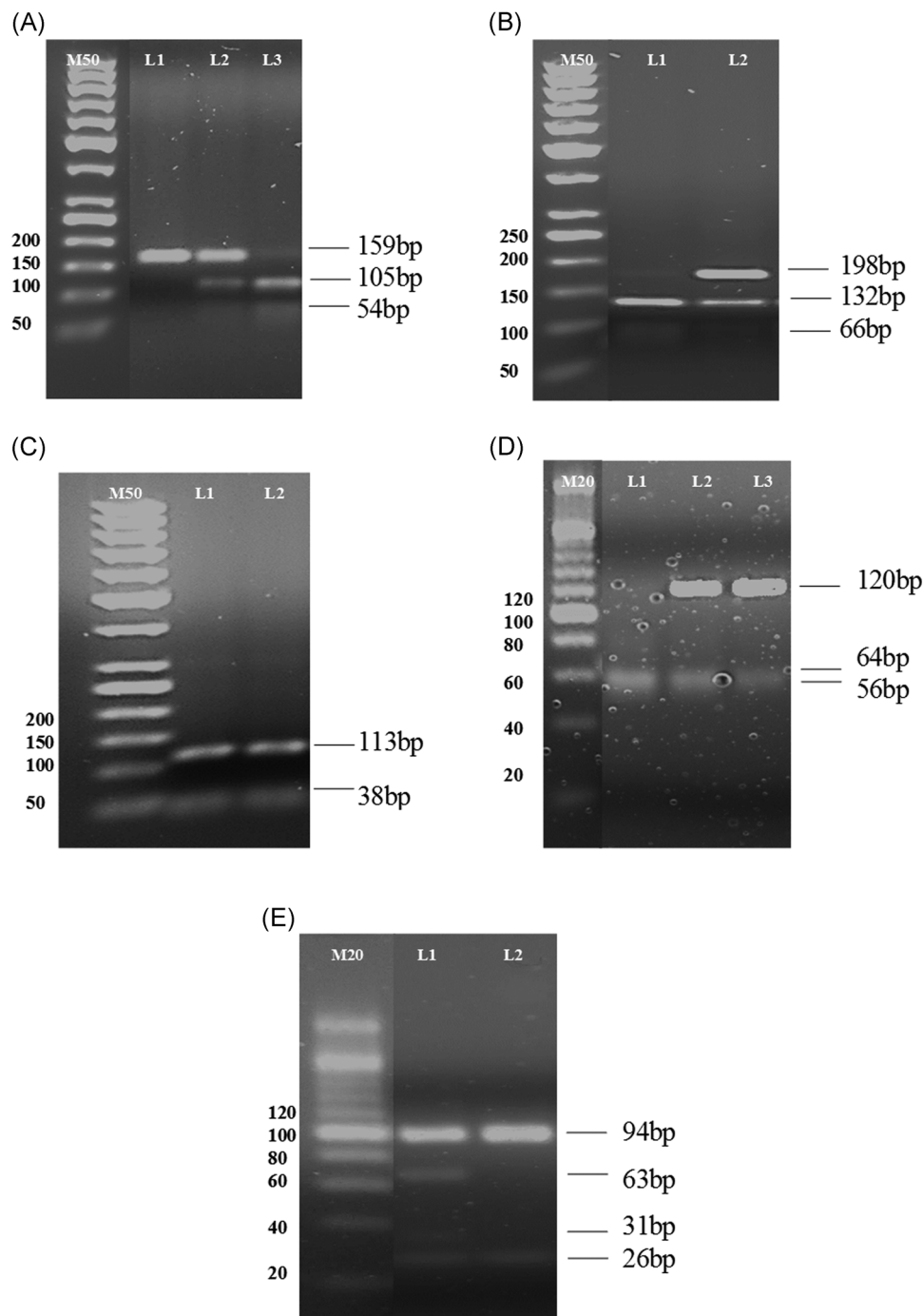


FIGURE 1 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) agarose gels for *TP53*, *RB1*, and *FGFR3*. (A) *TP53* codon 72: M50: 50 base pair (bp) ladder; lane 1: HM; lane 2: HT; lane 3: HW. (B) *TP53* codon 248: M50: 50 bp ladder; lane 1: HW; lane 2: HT. (C) *RB1* E23: M50: 50 bp ladder; lanes 1 and 2: HW. (D) *FGFR3* codon 248: M20: 20 bp ladder; lane 1: HW; lanes 2 and 3: HT. (E) *FGFR3* codon 249: M20: 20 bp ladder, lane 1: HT; lane 2: HW. Abbreviation: bp, base pair; HM, homozygous mutant; HT, heterozygous; HW, homozygous wild-type

3.3 | Association of NATs polymorphism with tumors' characteristics and patients' smoking status

Our results show that *NAT1* G⁵⁶⁰A polymorphism is significantly associated with higher muscle-invasiveness (MIBC vs NMIBC; $P = .001$), and higher tumor grade (high grade vs low grade; $P = .011$)

(Table 3). The percentage of patients carrying one or more copies of *NAT1* G⁵⁶⁰A, is significantly higher among those with MIBC (53%), compared with those who have NMIBC (46%), while the percentage of non-carriers is significantly higher among patients with NMIBC (66%) compared with MIBC (33%) (Table 4). *NAT1* G⁵⁶⁰A polymorphism is also found to be significantly associated with higher

TABLE 3 Pearson's χ^2 test results for the different NAT1 and NAT2 SNPs and patient's smoking status, cross-tabulated with the mutational frequency of molecular tumor makers, tumor grade, and muscle-invasiveness in the total sample (N = 250)

	TP53 C72	FGFR3 C248	FGFR3 C249	Tumor grade	Muscle- invasiveness
NAT1 C⁵⁵⁹T					
χ^2	1.171	0.231	0.343	4.229	0.132
P value	.557	.630	.558	.040*	.716
NAT1 G⁵⁶⁰A					
χ^2	5.094	0.580	6.649	6.388	10.259
P value	.078	.446	.010*	.011*	.001*
NAT1 C¹⁰⁹⁵A					
χ^2	0.269	1.018	4.434	11.942	6.929
P value	.874	.601	.109	.003*	.031*
NAT2 T³⁴¹C					
χ^2	0.593	0.213	0.005	0.000	0.813
P value	.441	.644	.970	.995	.367
NAT2 G⁵⁹⁰A					
χ^2	1.732	0.196	0.633	0.216	0.218
P value	.188	.658	.426	.642	.640
NAT2 G⁸⁵⁷A					
χ^2	1.110	0.039	3.208	0.784	4.1676
P value	.292	.843	.073	.376	.041*
Smoking status					
χ^2	0.918	0.002	2.780	2.868	0.384
P value	.338	.964	.095	.238	.535

Abbreviation: SNP, single nucleotide polymorphism.

*Significant at a $P \leq .05$.

mutation frequency at *FGFR3* codon 249 in the total sample ($P = .01$), where the percentage of patients carrying one or more copies of NAT1 G⁵⁶⁰A SNP, is significantly higher among those having the *FGFR3* codon 249 mutation (95%), compared with patients who do not have the mutation (4%) (Table 4).

NAT2 G⁸⁵⁷A polymorphism was also found to be significantly associated with higher muscle-invasiveness (MIBC vs NMIBC; $P = .041$) (Table 3), where the percentage of patients carrying NAT2 G⁸⁵⁷A, is significantly higher among those with MIBC (52%), compared with NMIBC (47%) (Table 4). In contrast, NAT1 C¹⁰⁹⁵A was found to be significantly clustered among NMIBC (64%) compared with MIBC tumors (35%) ($P = .031$) (Tables 3 and 4). On the other hand, associations between smoking status and tumor markers were all found to be not statistically significant. In addition, all other tested associations between NAT1 and NAT2 polymorphisms and tumor markers and characteristics did not show statistical significance, both independently and in interaction with patient smoking status.

In summary, both NAT1 G⁵⁶⁰A and NAT2 G⁸⁵⁷A SNPs were significantly clustered among the MIBC subgroup, suggesting an association with muscle-invasiveness.

3.4 | Association of NATs polymorphism with molecular tumor markers stratified by muscle-invasiveness

NAT1 G⁵⁶⁰A polymorphism was found to be significantly associated with higher *FGFR3* mutation frequency within the MIBC subgroup both at codon 248 ($P = .042$) and codon 249 ($P = .027$) (Table 5). The percentage of patients with MIBC carrying one or more copies of NAT1 G⁵⁶⁰A is significantly higher among those who have the *FGFR3* C249 mutation (96%), compared with patients who do not have the mutation (3%) (Table 5). However, none of the patients with MIBC carrying NAT1 G⁵⁶⁰A had the *FGFR3* C248 mutation. In summary, in muscle-invasive BCa tumors, NAT1 G⁵⁶⁰A is associated with higher mutational frequency in the molecular tumor marker *FGFR3*.

4 | DISCUSSION

Although NAT1 is a drug-metabolizing enzyme, several studies have recently reported its participation in many other intracellular biochemical pathways.^{19,35,36} Emerging evidence suggests that NAT1 is associated with changes in cell growth and survival, cell morphology, and various intracellular metabolic pathways.^{37,38} The NAT1 gene is reported to be upregulated in several cancer types, and its overexpression may lead to increased survival.³⁹ Here, the association of NAT1 with urinary BCa characteristics and main molecular tumor markers are reported for the first time. Our results suggest a role for NAT1 in bladder carcinogenesis and muscle-invasiveness, with a possible specificity in the luminal papillary subtype. Our findings on the association of NAT1 polymorphism with tumor invasiveness are supported by previous reports for other types of cancer. For instance, NAT1 gene expression levels were found to correlate with epithelial-to-mesenchymal activation in breast-cancer bone metastasis.⁴⁰ In addition, NAT1 expression levels were reported to increase as tumorigenesis progressed from benign to vertical growth to metastasis in melanoma patients, hence suggesting an association with an invasive and a more aggressive clinical outcome.⁴¹ Furthermore, our findings of an association between the "decreased-activity" acetylation phenotype of the NAT1 G⁵⁶⁰A—characterizing the NAT1*14 haplotype—and bladder tumorigenesis is consistent with a previously published report in the same target population, where NAT1*14 was reported to be significantly clustered in BCa cases compared with controls.³⁴ However, the exact mechanisms of action for this enzyme in BCa and the different tumorigenic cellular processes remain unknown.

The role of NAT1 in bladder carcinogenesis may be following either a gene-environment interaction-dependent (GxE)_d or an independent (GxE)_i mechanism; both possible mechanisms deserve to be considered and are worthy of further investigations. Based on our results, carriers of the "decreased-activity" acetylation polymorphism may be at a greater risk of a muscle-invasive disease compared with non-carriers. The NAT1*14 (G⁵⁶⁰A) polymorphism, in particular, confers substantially reduced NAT1 protein levels, a 15-fold decreased

TABLE 4 Pearson's χ^2 detailed cross-tabulations for NAT1 SNPs frequencies, with molecular tumor markers mutational frequencies, tumor grade, and muscle-invasiveness in the total sample (N = 250); this table shows the details of the cross-tabulations for tests that have shown statistically significant results; all NAT1 genotypes were grouped into two categories for analysis: presence of the SNP (+/+ and +/-) or absence (-/-) except for the NAT1 C¹⁰⁹⁵A genotype which was kept in three categories (+/+), (+/-), (-/-) to avoid misrepresentation given the scarcity of the homozygote wild-type

	NAT1 C ⁵⁵⁹ T			NAT1 G ⁵⁶⁰ A			NAT1 C ¹⁰⁹⁵ A			NAT2 G ⁸⁵⁷ A				
	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	Total	P value
Muscle invasiveness														
MIBC	3 (50.0%)	103 (42.5%)	106 (42.7%)	60 (53.5%)	45 (33.3%)	105 (42.5%)	36 (35.3%)	60 (50.8%)	0 (0.00%)	96 (43.2%)	0.031*	67 (38.5%)	103 (42.5%)	.041*
NMIBC	3 (50.0%)	139 (57.2%)	142 (57.2%)	52 (46.4%)	90 (66.6%)	142 (57.5%)	66 (64.7%)	58 (49.1%)	2 (100%)	126 (56.7%)		107 (61.5%)	139 (57.4%)	
Total	6	242	248	112	135	247	102	118	2	222		174	242	
Tumor grade														
HG	4 (80.0%)	221 (96.9%)	225 (96.5%)	101 (100%)	123 (93.8%)	224 (96.5%)	94 (95.9%)	106 (97.2%)	1 (50.0%)	201 (96.1%)	.003*			
LG	1 (20.0%)	7 (3.1%)	8 (3.4%)	0 (0.0%)	8 (6.1%)	8 (3.4%)	4 (4.1%)	3 (2.7%)	1 (50.0%)	8 (3.8%)				
Total	5	228	233	101	131	232	98	109	2	209				
FGFR3 C249														
+/+ and +/-	5 (83.3%)	219 (90.3%)	224 (90.3%)	108 (95.6%)	115 (85.8%)	223 (90.3%)	95 (92.2%)	104 (88.1%)	1 (50.0%)	200 (86.7%)	.109			
-/-	1 (16.6%)	23 (9.5%)	24 (9.6%)	5 (4.4%)	19 (14.2%)	24 (9.7%)	8 (7.7%)	14 (11.8%)	1 (50.0%)	23 (10.3%)				
Total	6	242	248	113	134	247	103	118	2	223				

Note: -/-, homozygous wild-type; +/+, homozygous mutant; +/-, heterozygous.

Abbreviations: HG, high grade; LG, low grade; MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; SNP, single nucleotide polymorphism.

*Significant at a P ≤ .05.

TABLE 5 Pearson's χ^2 test results for NAT1 SNPs with FGFR3 mutation frequencies stratified by muscle invasiveness (N = 250); in the upper part of the table, test results and P values are shown for NAT1 G⁵⁶⁰A, NAT1 C⁵⁵⁹T, and NAT1 C¹⁰⁹⁵A SNPs cross-tabulated with both FGFR3 mutations C248 and C249 stratified by muscle-invasiveness (MIBC and NMIBC subgroups); in the lower part of the table, details of the cross-tab for NAT1 G⁵⁶⁰A with FGFR3 mutations within the MIBC subgroup are shown; NAT1 genotypes were grouped into two categories for analysis: presence of the SNP (+/+ and +/-) or absence (-/-)

	FGFR3 C248		FGFR3 C249					
	MIBC	NMIBC	MIBC	NMIBC				
NAT1 C⁵⁵⁹T								
χ^2	0.089	0.136	0.286	1.660				
P value	.764	.712	.592	.198				
NAT1 G⁵⁶⁰A								
χ^2	4.117	0.463	4.901	2.054				
P value	.042*	.496	.027*	.152				
NAT1 C¹⁰⁹⁵A								
χ^2	1.684	3.538	0.989	3.389				
P value	.194	.170	.320	.184				
MIBC subgroup								
	FGFR3 C248			P value	FGFR3 C249			P value
	+/+ and +/-	-/-	Total		+/+ and +/-	-/-	Total	
NAT1 G⁵⁶⁰A								
+/+ and +/-	0 (0.00%)	60 (100%)	60	.042*	58 (96.67%)	2 (3.33%)	60	.027*
-/-	3 (6.67%)	42 (93.33%)	45		38 (84.44%)	7 (15.56%)	45	
Total	3 (2.86%)	102 (97.14%)	105		96 (91.43%)	9 (8.57%)	105	

Note: -/-, homozygous wild-type; +/+, homozygous mutant; +/-, heterozygous.

Abbreviations: HG, high grade; LG, low grade; MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; SNP, single nucleotide polymorphism.

*Significant at $P \leq .05$.

catalytic activity, and an increased substrate-specific Michaelis-Menten kinetic constant K_m .⁴² At the mechanistic level, the encoded protein in NAT1*14 carriers is rapidly degraded and unable to be acetylated by AcCoA, hence resulting in ubiquitination and rapid degradation by the 26S proteasome.⁴³ In those subjects with decreased acetylation phenotype, the NAT1 acetylation enzymatic pathway may compete poorly with Phase I bioactivation pathways, hence significantly increasing the odds of tumorigenesis in the bladder, upon chronic exposure to carcinogens like arylamines. On the other hand, the fact that NAT1 has evolved while conserving functionality over time,⁴⁴ combined with its wide expression in the body including the urinary bladder, supports another hypothesis suggesting a role for NAT1 in bladder tumorigenesis independently of a gene-environment interaction mechanism. Possibly, alterations in folate homeostasis and other cell metabolic pathways may lead to DNA damage, ultimately resulting in malignant transformation in the bladder.⁴⁵ An alternative mechanism may be a NAT1-mediated increase in gain-of-function p53, and an attenuation in the production of reactive oxygen species. Consequently, a deficiency in NAT1 expression levels and activity, as in the case of the NAT1 G⁵⁶⁰A carriers, would increase intracellular oxidative stress and inhibit apoptotic pathways.¹⁹ However, the exact molecular mechanisms that may underlie such role and its attributed effects, as well as the influence of the interindividual variations in NAT1, remain to be explored and verified.

On the other hand, the association we found between NAT1*14 (G⁵⁶⁰A) and the FGFR3 activating mutation Ser249Cys is another evidence supporting a role for NAT1 in bladder carcinogenesis, especially that FGFR3 activating mutations are drivers of malignancy in several human tissues, including lung, cervix, blood, and most importantly the urinary bladder.⁴⁶ In particular, the observed NAT1*14 (G⁵⁶⁰A) association with FGFR3 mutations in the muscle-invasive subgroup suggests that NAT1 may specifically play a role in the luminal papillary MIBC molecular subtype. This novel finding warrants further investigation. Previous studies have shown that NAT1 has a higher expression in luminal compared with basal-like carcinoma,⁴⁷ hence suggesting that NAT1 may be playing a role in the biology of luminal carcinomas.⁴⁸

Our findings on NAT1 in BCa are novel and may have an important prognostic value in predicting clinical outcomes among patients with BCa. NAT1 tumor acetylation status may be useful in patients' risk stratification for muscle-invasiveness, which may help in the selection of an appropriate postoperative follow-up schedule. In patients with NMIBC, such prognostic biomarkers could have an impact on the decision-making process regarding surveillance schedules and administration of therapy.⁴⁹ For instance, preoperative biomarkers, similar to NAT1*14 in its correlation with muscle-invasiveness and high tumor grade, could help predict more precisely the risk of progression, enabling better selection of clinical T1 high-grade

patients who should undergo radical cystectomy as primary treatment as compared with intravesical Bacillus Calmette-Guerin. This scenario is an example of how such prognostic biomarker could be useful in the daily management of patients with BCa. However, the value of this new prognostic marker warrants further study for its own sensitivity and specificity, and in combination with other biomarkers as part of a multifactorial prediction model. On the other hand, aside from its clinico-pathologic and prognostic significance, the association between NAT1 and muscle invasiveness reported here, as well as with *FGFR3* activating mutations, which are key drivers within the MIBC subgroup, may be useful for treatment. Findings on NAT1 reported in breast cancer were the main driver to develop small-molecule NAT1 inhibitors using short-hairpin RNA directed against the enzyme to control its activity. One such drug is Rhod-o-hp, and which showed promising effects in breast cancer, particularly by inhibiting cell proliferation in the G2/M, and by reducing invasiveness in an invasion assay in vitro.⁵⁰ Moreover, the knockdown of NAT1 in breast cancer cells led to effective attenuation in anchorage-independent growth.⁵¹

At the same time, several limitations could have affected the results of the current study. First, NAT1 and NAT2 may include other functional polymorphisms not tested for in the current study. However, many of these nucleotide substitutions and corresponding NAT haplotypes are reported to be very rare and considered as nucleotide diversity rather than genetic polymorphism, with many of them silent. In addition, our investigation targeted alleles common in this population based on results reported in previous studies.⁵² Another limitation is the fact that we examined NAT1 and NAT2 SNPs in archival tumors and not in blood-based DNA. This may be biased by the possible genetic alterations in the tissue resulting from disease progression and treatment. Given the unavailability of corresponding blood samples, germline profiling was not possible to discriminate from somatic alterations. On the other hand, the deidentified nature of the obtained data precluded us from accessing patients contact info to obtain blood samples. However, it is important to note that examining haplotypes in bladder tumors may be more relevant than that in blood for NAT1 considering that its expression is mainly in the bladder. Another limitation is the possibility of polymorphisms in other drug-metabolizing enzyme coding genes not tested for, that may also be contributing to the tumor's pathological characteristics. On the other hand, several strengths should also be highlighted in this study. First, the quality of the measures used is high based on the use of probe-based sensitive genotyping methods. Second, samples were obtained from two different referral centers to improve representativeness of collected data. Third, all the different statistical models used to test associations yielded similar patterns in the observed results, hence reflecting the high internal validity of the observed findings.

In summary, this is the first report that associates NAT1 decreased acetylation genotype with mutations in *FGFR3* and muscle-invasiveness, and implies a role for NAT1 in a specific MIBC molecular subtype. At the same time, our findings on NAT2 G⁸⁵⁷A polymorphism (NAT2*7A), which also codes for a slow acetylation phenotype, is also in support of a role for slow acetylation in BCa

muscle-invasiveness. These findings may be physiologically relevant given the polymorphic nature of NAT1 combined with its wide interindividual variation, and the high frequency of the NAT1*14 (G⁵⁶⁰A) decreased acetylation haplotype in the targeted population as previously reported.^{52,53} Currently, there are no known mechanisms that can explain our findings. Nevertheless, accumulating evidence indicates that NAT1 has a possible prognostic value in cancer patients and highlights it as a novel target for anticancer drug development. The molecular mechanisms that underlie the role of NAT1 in bladder carcinogenesis require further investigation. It would be important to identify the molecular pathways linking NAT1 activity and expression levels with *FGFR3* oncogenic activity within BCa tumors, and to confirm its association with particular MIBC molecular subtypes using the recent consensus classification.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The authors state that they have followed the principles outlined in the Declaration of Helsinki for all human investigations. Ethical approval was obtained from the American University of Beirut and collaborating medical centers before conducting the study.

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How to cite this article: El Kawak M, Dhaini HR, Jabbour ME, Moussa MA, El Asmar K, Aoun M. Slow N-acetylation as a possible contributor to bladder carcinogenesis. *Molecular Carcinogenesis.* 2020;59:1017–1027. <https://doi.org/10.1002/mc.23232>