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Discovery of a novel genetic variant in the *N-acetyltransferase2* (*NAT2*) gene that is associated with bladder cancer risk

Lina Elsalem[1]✉, Ahmad Al Shatnawi[1,2], Mahmoud A Alfaqih[3,4], Ayat Alshoh[1], Saddam Al Demour[5], Ali Al-Daghmin[6,7], Omar Halalsheh[8], Khalid Kheirallah[9] and Mamoun Ahram[10]

[1]Jordan University of Science and Technology, Faculty of Medicine, Department of Pharmacology, Irbid, Jordan; [2]Royal Medical Services, Department of Clinical Pharmacy, Irbid, Jordan; [3]Department of Biochemistry, College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Bahrain; [4]Department of Physiology and Biochemistry, Faculty of Medicine, Jordan University of Science and Technology, Irbid, Jordan; [5]The University of Jordan, School of Medicine, Department of Special Surgery, Division of Urology, Amman, Jordan; [6]King Hussein Cancer Center, Department of Surgery, Amman, Jordan; [7]Abdali Medical Center, Amman, Jordan; [8]Jordan University of Science and Technology, Faculty of Medicine, Department of General Surgery and Urology, Irbid, Jordan; [9]Jordan University of Science and Technology, Faculty of Medicine, Department of Public Health and Community Medicine, Irbid, Jordan; [10]The University of Jordan, School of Medicine, Department of Physiology and Biochemistry, Amman, Jordan

Smoking is a main risk factor for bladder cancer (BC). NAT2 is a drug-metabolizing enzyme that catalyses the detoxification of many xenobiotics and carcinogens. Single nucleotide polymorphism (SNP) in *NAT2* results in different acetylation phenotypes (fast, intermediate or slow). Certain *NAT2* SNPs were associated with BC and/or modified the association of BC with smoking. However, limited evidence is available among BC patients or smokers from Jordan. This study aimed to discover novel SNPs in *NAT2* and to assess the association with BC. This was a case-control study among 120 BC patients and 120 controls. Amplification of a 446 bp fragment of *NAT2* encoding the N-catalytic domain was conducted using a polymerase chain reaction. Gene sequencing was done using Sanger-based technology. A total of 40 SNPs were detected. Two variants were significantly associated with BC ($p<0.05$); namely a novel c.87G>A and the reported c.341T>C. Regarding c.87G>A, genotype distribution was significantly associated with BC and subgroup analysis confirmed that this was significant in both smokers ($p=0.007$) and non-smokers ($p=0.001$). Regression subgroup analysis suggested GA as a risk factor among smokers (AOR= 2.356). The frequencies of TC and CC genotypes of c.341T>C were significantly higher in BC ($p<0.05$). This was statistically significant among smokers only ($p=0.044$), upon subgroup analysis. Multivariate analysis showed that subjects with TC genotype are 6.15 more likely to develop BC and regression subgroup analysis revealed TC as a risk factor among smokers (AOR=5.47). This is the first study from Jordan to report the association of smoking and two *NAT2* variants with BC. The data supports the use of GA and TC genotypes of the novel c.87G>A and the reported c.341T>C SNPs, respectively as potential biomarkers of BC, particularly among smokers. Future investigations with a larger population are required to support our findings.

Keywords: *NAT2*, single nucleotide polymorphism, smoking, bladder cancer, c.87G>A, c.341T>C

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✉e-mail: lmelsalem@just.edu.jo
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Abbreviations: **Abbreviations**: AOR, adjusted odds ratio; BC, Bladder cancer; BMI, body mass index; CI, confidence interval; EDTA, Ethylene-Diamine-Tetra-Acetic acid; *NAT2*, *N-acetyltransferase 2*; OR, odds ratio; PCR, Polymerase chain reaction; SNPs, single nucleotide polymorphisms

INTRODUCTION

Globally, bladder cancer (BC) ranks as the 10th most common malignancy (Bray *et al*., 2018) with tobacco smoking being a main risk factor for the disease development (Freedman *et al*., 2011, Saginala *et al*., 2020). Studies reported a 4-folds increased risk for BC among smoker individuals in comparison to non-smokers (Colombel *et al*., 2008). In addition, more invasive phenotypes of BC were described in smoker patients compared with non-smokers (Jiang *et al*., 2012). It is well known that tobacco contains more than 4000 chemicals, 60 of which are listed as carcinogens (Richter *et al*., 2008). Examples of carcinogenic chemicals that are found in cigarette smoking are aromatic and heterocyclic amines (Turesky and Le Marchand 2011). These chemicals are detoxified by different drug-metabolizing enzymes including *N*-acetyltransferase 2 (NAT2) (Sanderson *et al*., 2007).

Human NAT2 is a Phase II drug metabolizing enzyme that is involved in the detoxification of many xenobiotics and carcinogens by catalyzing the transfer of an acetyl group from acetyl CoA as a donor to the acceptor compound (Grant *et al*., 1992). Many studies reported inter-individual variations in the rate of acetylation, with subjects classified as fast, intermediate or slow acetylators (Werely *et al*., 2007; Garcia-Martin 2008; Sabbagh *et al*., 2011; Al-Ahmad *et al*., 2017; Aklillu *et al*., 2018). Phenotypic differences in acetylation rate explain some of the variation observed in the therapeutic/side effect profile of drugs metabolized by NAT2 such as isoniazid, sulfonamides and others (Werely *et al*., 2007; Ladero 2008; Sim *et al*., 2014; Adole *et al*., 2016). Moreover, many reports suggested that the above differences may also modify the risk of developing BC (Hein 2006) and/or modify the association of BC with smoking (Tao *et al*., 2010; Moore *et al*., 2011; Ribouh-Arras *et al*., 2019). Tao *et al.,* showed that environmental tobacco smoke increased the risk of BC among slow NAT2 acetylators (Tao *et al*., 2010). In addition, a large population-based study from the US revealed that exposure intensity to tobacco smoking was correlated with BC risk in patients with slow acetylation (Moore *et al*., 2011). A recent study among the Lebanese population has shown that patients

with high BC risk were mainly males, current smokers, alcohol drinkers and those with occupational history of exposure to aromatic amines (Nasr *et al*., 2017). Further investigations have shown a strong association between NAT2 slow acetylator phenotype and smoking which is attributed to higher BC risk among the Algerian population (Ribouh-Arras *et al*., 2019).

The NAT2 acetylation phenotype is largely determined by genetic variations in the sequence of the gene encoding the NAT2 enzyme. This gene, also known as *NAT2*, is located on chromosome 2 and has two exons separated by one intron (Blum *et al*., 1990). Transient heterologous transfection of constructs that contain only the second exon of *NAT2* is sufficient to produce a fully functional enzyme upon its translation (Boukouvala *et al*., 2003). Not surprisingly, most of the sequence variations in *NAT2* that result in phenotypic differences in acetylation are located within the second exon which is 873 bps in length (Boukouvala *et al*., 2003; Ribouh-Arras *et al*., 2019).

Jordan is a Third World Middle Eastern country with one of the highest numbers of cigarette smokers per capita worldwide (Jaghbir *et al*., 2014). BC is considered the third most cancer in male patients and accounts for almost 5% of all cancer cases diagnosed in Jordan (Directorate-MOH 2014). In addition, BC is a foremost cause of cancer related death in Jordan. Up to our knowledge, no research group in Jordan has ever sequenced the coding region of *NAT2* to search for novel population specific polymorphisms, despite the importance of having such a database from a pharmacogenetic and personalized medicine standpoint. In addition, no studies to date examined the association between genetic variants of *NAT2* and BC risk in Jordan.

The N-catalytic domain of the NAT2 enzyme is found within the second exon of *NAT2*. Several reports found that most of the genetic variation in the *NAT2* gene that leads to changes in acetylation activity is located within the above domain (Boukouvala *et al*., 2003; Ribouh-Arras *et al*., 2019). Given the above gaps, we sequenced a stretch of the second exon of the *NAT2* gene from subjects of a BC case-control study to discover novel sequence polymorphisms in *NAT2*, test their association with BC in Jordan, and examine if these polymorphisms modify the link between BC and smoking.

MATERIALS AND METHODS

Ethical approval

This study has the approval of the Deanship of Research and the Institutional Review Board committees of (Jordan University of Science and Technology, King Abdullah University Hospital (KAUH)), (University of Jordan, Jordan University Hospital (JUH)) and (King Hussein Cancer Center (KHCC)), (IRB numbers 25/112/2018, 2018/127 and 30/2018, respectively). Subjects were enrolled in this study after providing an informed consent form.

Study Settings and Subjects Enrolment

This was a case-control study among 120 BC patients from Jordan who attended the Urology clinics of KAUH located in the North of Jordan. Patients were also enrolled from the Urology clinics of JUH and the Oncology clinics of KHCC, both located in the capital city.

Patients were included if they were diagnosed with transitional cell carcinoma (TCC) of BC as their primary cancer and were being treated for BC at the time of enrolment at each respective hospital. On the other hand, patients were excluded if they have BC of other subtypes rather than TCC or if BC was a secondary tumor.

Patients were interviewed by a clinical research coordinator who described the study's objectives and clarified that the study will include the collection of demographic, anthropometric measurements and clinical data as well as a blood sample.

Demographic data included the patient age, gender and smoking status, while anthropometric data included the patient height and weight needed to calculate the Body Mass Index (BMI). Clinical data including the pathological stage of the tumor at the time of its diagnosis was obtained from the electronic medical records.

Regarding the control group, it included 120 subjects who attended Family Medicine clinics of KAUH and JUH without a medical history of bladder disease. Control subjects were matched with cases by gender, BMI and smoking status. Subjects in the control group were excluded if they had a medical history of any urology-related symptoms (difficulty upon urinating, blood and other discharges in the urine, burning upon urination).

Of note, all interviews of both groups were conducted by the same coordinator for the duration of patient recruitment.

Blood sample collection

A single venous blood sample (3 ml) was collected from each subject using ethylenediamine tetraacetic acid (EDTA) tubes (AFCO, Amman, Jordan). Tubes were mixed properly to prevent clotting. Blood samples were stored at 4°C for the subsequent DNA extraction.

DNA extraction, PCR and sequencing

Genomic DNA extraction was carried out using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following the final step of the protocol, DNA concentration was measured using an ND-2000 Nanodrop (Thermo Scientific, Waltham, MA, USA). DNA samples were stored at –80°C for further processing (i.e. amplification followed by sequencing).

Using the Ensembl genome browser 98 (http://www.ensembl.org/index.html), the location of the N-catalytic domain was mapped on the nucleotide sequence of the second exon. A primer pair that flanks the N-catalytic domain was then designed using Primer 3 software (http://primer3.ut.ee). Primers were designed to amplify a 446 bp fragment. The sequence of the forward primer was (5' to 3'): CTTGCTTAGGGGATCATGGA while the sequence of the reverse primer was (5' to 3'): GGCTGATCCTTCCCAGAAAT.

Conventional polymerase chain reaction (PCR) was then used to amplify the aforementioned 446 bp fragment. The details of the PCR reaction mixture were as Alfaqih and others (Alfaqih *et al*., 2018). The PCR reaction mixture was then incubated in a T100 thermal cycler (Biorad, Berkeley, CA, USA) under the following reaction conditions; (i) initial denaturation step (95°C, 3 minutes), (ii) 35 denaturation cycles (95°C, 3 minutes), (iii) annealing (65°C, 30 seconds) and (iv) extension (72°C, one minute) and (v) a final extension (72°C, 5 minutes).

Prior to sequencing the PCR products, 5 µls of the PCR mixture were loaded into a 2% agarose gel stained with ethidium bromide. The electrophoresis was run at

Table 1. Distribution of study subjects by independent variables and by disease status

Variable	Controls (n=120)	Bladder cancer (n=120)	*p*-value
Gender (n) (%) Males Females	 106 (88.3%) 14 (11.7%)	 104 (86.7%) 16 (13.3%)	 0.423
Smoking (n) (%) Yes No	 68 (56.7%) 52 (43.3%)	 79 (65.8%) 41 (34.2%)	 0.093
Age (years)α	60.46 (12.02)	64.80 (12.32)	0.006
BMI (kg/m2) α	28.50 (5.00)	28.60 (7.20)	0.906

[α]mean ± standard deviation; BMI, body mass index. The *p*-values were calculated by Pearson Chi-square test for gender, and smoking (%), while the student's *t*-test was used for Age, BMI

140 volts for 40 minutes. Ultraviolet light was used to visualize the products. PCR products of the previous step were sent to (Center Name) for sequencing using Sanger=based technology. Sequencing was performed from both ends of the fragment using the forward and reverse primers of the original PCR reaction.

The Chromas Pro Software (http://technelysium.com.au/wp/chromas) was used to visualize sequence chromatograms. The above software was also used for sequence alignment with a reference sequence of the second exon of *NAT2* to identify genetic variants in the sequence. The NCBI (http://www.ncbi.nlm.nih.gov) and Ensemble genome browsers were used to indicate if any of the identified genetic variants are new or previously reported.

Of note, the alleles and genotypes were successfully determined for all SNPs among all enrolled subjects. The allele and genotype frequencies of each SNP were calculated using standard methods. Deviations from Hardy Weinberg equilibrium were tested using a chi-square test.

In-silico predictions for the effect of genetic SNPs on NAT2 protein

Four web-based applications; PolyPhen 2.0 (http://genetics.bwh.harvard.edu/pph2/), SIFT (https://sift.bii.a-star.edu.sg/), PROVEAN (http://provean.jcvi.org/index.php) and Mutation Taster (http://www.mutationtaster.org/) were used to predict the effect of NAT2 SNPs on protein structure and function.

Statistical analysis

The minimum sample size required to meet the study objective was estimated based on Pourhoseingholi and others (Pourhoseingholi *et al.*, 2013): BC prevalence in Jordan (5%), a margin of error which was set to be ±5% and, a confidence level that was set to be 95%, with 5% alpha level, and power level of 80%. Based on the above information, the minimum sample size required was 73. The number of BC patients who enrolled was 120.

Statistical analysis was carried out using the Statistical Package for Social Studies (SPSS) software (version 23, IBM, NY). Student *t*-test was used to evaluate if significant differences exist between cases and controls in terms of age and BMI, while Pearson Chi-square was used for gender and smoking status. Pearson's Chi-square was also used to evaluate the association of allele or genotype distributions with BC risk. Subgroup analysis was also done according to smoking status. Results were considered significant if $p \leq 0.05$.

Multivariate logistic regression analysis was done to define the association of c.341T>C and c.87G>A SNPs with BC risk in the presence of potential confounders including age, gender, smoking, and BMI. Results were considered significant if $p \leq 0.05$ with a confidence limit of 95%. Regression subgroup analysis was also conducted according to smoking status.

RESULTS

Subject characteristics

A total of 240 subjects were recruited in this study; 120 BC cases and 120 controls. The characteristics are summarized in Table 1. Among cases, the majority (86.7%) were males and 65.8% were smokers. The majority of the cases were in earlier stages of BC; non-muscle-invasive (Ta=48.33% and T1=36.67%) and muscle-invasive (T2=7.5%, T3=5% and T4=2.5%).

Among controls, 88.3% were males and 56.7% were smokers. While significant differences in case status by gender, smoking status, and BMI were not statistically significant, the mean age of cases (64.80 (12.32)) was significantly higher than that for controls (60.46 (12.02)) ($p=0.006$).

Mutational analyses of a 446 bp fragment of the second exon of the *NAT2* gene among study subjects

A total number of 40 variants were detected in our sample. These were a substitution in one single nucleotide (SNPs) and we did not detect any insertions or deletions (Fig. 1 Supplementary at https://ojs.ptbioch.edu.pl/index.php/abp/). Out of the 40 SNPs detected in our population, 12 SNPs were previously reported while 28 SNPs were not reported in any of the publicly available databases (GenBank or Ensembl) (Table A Supplementary at https://ojs.ptbioch.edu.pl/index.php/abp/). None of the SNPs detected in this study had a minor allele frequency of less than 0.2. All of the SNPs were in agreement with Hardy Weinberg Equilibrium except for the two novel SNPs (c.147G>C) and (c.162T>C) ($P< 0.05$). These two SNPs were thus excluded from further analysis. All of the remaining SNPs were brought forward for further evaluation of their association with the risk of BC.

The association of genotypic and allelic frequencies of *NAT2* SNPs with bladder cancer

The relationship between 38 SNPs discovered in the sequenced region of the *NAT2* gene and BC was evaluated. Out of 38 SNPs that fit these criteria, only two SNPs showed a significant relationship with BC case status ($p<0.05$); the reported SNP (c.341T>C) and the novel SNP (c.87G>A).

Table 2. Genotype frequencies of *NAT2* SNPs in study subjects.

SNP ID	Genotype	Control n (%)	Bladder cancer n (%)	*p*-value
c.341T>C	TT TC CC Total	15 (12.5%) 105 (87.5%) 0 (0.0%) 120 (100%)	3 (2.5%) 113 (94.2%) 4 (3.3%) 120 (100%)	0.0097
c.87G>A	GG GA AA Total	66 (55.0%) 54 (45.0%) 0 (0.0%) 120 (100%)	53 (44.2%) 51 (42.5%) 16(13.3%) 120 (100%)	0.001

The *p*-values were calculated by the Pearson Chi-square test

Table 3. Allele frequencies of *NAT2* SNPs in study subjects.

SNP ID	Allele	Control n (%)	Bladder cancer n (%)	*p*-value
c.341T>C	T C	135 (56.2%) 105 (43.8%)	119 (49.6%) 121 (50.4%)	0.143
c.87G>A	G A	186 (77.5%) 54 (22.5%)	157 (65.4%) 83 (34.6%)	0.003

The *p*-values were calculated by the Pearson Chi-square test

Genotype distribution of c.341T>C by case status was statistically significant (p=0.0097). The frequency of the heterozygous TC and the homozygous CC genotypes was higher among BC cases than their control counterparts (Table 2). Similarly, the genotype distribution of c.87G>A was significantly different by case status (p=0.001). The homozygous GG and the heterozygous GA were higher among controls compared to cases, while the homozygous AA genotype was higher in BC cases (Table 2).

Regarding the analysis of alleles association with BC (Table 3), it was found that for c.341T>C, the frequency of the T allele was higher among controls, compared to BC cases, while the frequency of the C allele was higher in cases. For c.87G>A, the frequency of the G allele was significantly higher among controls, while the frequency of the A allele was significantly higher in BC cases (p=0.003).

Upon subgroup analysis of genotype frequency among smokers and non-smokers, separately (Table 4), the genotype distribution of c.341T>C showed a significant association with disease status, cases vs. controls, among smokers only (p=0.044). Among non-smokers, the distribution of c.341T>C by disease status, cases vs. controls, was not statistically significant (p=0.063) (Table 4). Regarding the genotype distribution of the novel SNP c.87G>A by case status among smokers and non-smokers, statistically significant differences were detected between genotype and case status among both smokers and non-smokers.

Multivariate regression analysis of *NAT2* SNPs with bladder cancer

BC patients in this study were significantly older than controls and the percentage of smokers was higher in BC patients than in controls but not statistically significant. Accordingly, a multivariate regression analysis was done to assess if any of the *NAT2* SNPs significantly modulates BC risk upon adjustment of potential confounders. Table 5 represents all variables in the model to assess the effect of genotypes on BC after controlling the effects of age, gender, BMI, and smoking.

It was found that smoking and age increased the risk of BC (p<0.05). Upon controlling for the effect of gender, age, BMI, and smoking status, the genotype distribution of c.341T>C was significantly involved in BC

Table 4. The Genotyping frequencies and smoking status

Smoking status	SNP ID	Genotype	Control n (%)	Bladder cancer n (%)	*p*-value
Smokers	c.341T>C	TT TC CC Total	7 (10.3%) 61 (89.7%) 0 (0.0%) 68 (100%)	2 (2.5%) 74 (93.7%) 3 (3.8%) 79 (100%)	0.044
	c.87G>A	GG GA AA Total	44 (64.7%) 24 (35.3%) 0 (0.0%) 68 (100.0%)	35 (44.3%) 37 (46.8%) 7 (8.9%) 79 (100%)	0.007
Non-Smokers	c.341T>C	TT TC CC Total	8 (15.4%) 44 (84.6%) 0 (0.0%) 52 (100.0%)	1 (2.4%) 39 (95.1%) 1 (2.4%) 41 (100.0%)	0.063
	c.87G>A	GG GA AA Total	22 (42.3%) 30 (57.7%) 0 (0.0%) 52 (100%)	18 (43.9%) 14 (34.1%) 9 (22.0%) 41 (100.0%)	0.001

The *p*-values were calculated by the Pearson Chi-square test

Table 5. Adjusted effects of genotypes on Bladder Cancer

	p-value	AOR (95% C.I)
BMI	0.762	0.992 (0.943-1.044)
Age	0.002	1.042 (1.015-1.068)
Gender Females *vs* Male	0.319	0.63 (0.254-1.563)
Smoker *vs* non-smoker	0.037	1.908 (1.038-3.507)
c.341T>C TT TC CC	 0.007 0.999	 Ref 6.158 (1.652-22.953) –*
c.87G>A GG GA AA	 0.261 0.998	 Ref 1.394 (0.781-2.49) –*

AOR, adjusted odds ratio; CI, confidence interval; *Distorted AOR.

development. Subjects with the TC genotype were 6.15 more likely to develop BC compared to TT (p=0.007 and 95% C.I.=1.6 to 22.9). Regarding the c.87G>A genotype distribution, the p-value indicated no significant difference (p>0.05).

Regression subgroup analysis with smoking status

We further conducted the analysis by smoking status Table 6. Regarding the c.341T>C SNP, the TC was a risk factor among smokers (5.47 times), (p=0.044 and 95% C.I.=1.044 to 28.679). Regarding c.87G>A, GA was a risk factor only among smoker patients (AOR= 2.356, p=0.019 and 95% C.I.=1.152to 4.819).

Prediction of the effect of *NAT2* SNPs on protein function and phenotype

All SNPs effects were predicted depending on different in-silico predictions websites which are Mutation Taster, PROVEAN, Polyphen-2 and Sift. Table 7 represents the prediction of the reported SNP c.341T>C and the novel SNP c.87G>A. For c.341T>C, Isoleucine amino acid was changed to Threonine at position 114 of the amino acids sequence. It was predicted as being polymorphism, deleterious, affecting the protein function and reflecting slow acetylator phenotype. Regarding c.87G>A, no change was detected in the amino acid sequence (Glutamine amino acid), however, it was predicted as a disease-causing, neutral and tolerated.

DISCUSSION

Globally, BC is considered the 10th most common malignancy, and the 13th most fatal cancer, accounting for 2.1% of all cancer fatalities (Halaseh *et al*., 2022). In Jordan, BC was found to be the fourth most prevalent cancer and accounting for 4.1% of all cancer deaths (Abdo *et al*., 2021). NAT2 is an important enzyme that metabolizes xenobiotics, via acetylation reactions, hence,

Table 6. Regression subgroup analysis with smoking status

Genotype	*p*-value	AOR (95% C.I.)
Smokers		
c.341T>C TT TC CC	 Ref 0.044 0.999	 Ref 5.472 (1.044-28.679) –*
c.87G>A GG GA AA	 Ref 0.019 0.999	 2.356 (1.152-4.819) –*
Non- Smokers		
c.341T>C TT TC CC	 Ref 0.061 1	 Ref 9.106 (0.901-92.043) –*
c.87G>A GG GA AA	 Ref 0.286 0.999	 Ref 0.559 (0.192-1.626) –*

AOR, adjusted odds ratio; CI, confidence interval; *Distorted AOR.

modulating susceptibility to environmental carcinogens and toxins that arise from tobacco products, diet, or other environmental exposures (Mittal *et al*., 2004).

NAT2 gene is a polymorphic gene that encodes for NAT2 enzyme (Magalon *et al*., 2008). Genetic variations in this gene result in three different phenotypes; rapid, intermediate, and slow acetylators affecting the drug metabolism, therapeutic effects and adverse effects, as well as the vulnerability of the individual to several carcinogens (Garcia-Martin, 2008; Ladero, 2008; Sabbagh *et al*., 2011). Genetic polymorphisms of *NAT2* have been found to be associated with susceptibility to develop different cancer types with various degrees (Avirmed *et al*., 2021; Zhu *et al*., 2021). It has been hypothesized that the increased susceptibility to BC among smokers is caused by genetic polymorphisms in the *NAT2* gene that results in slow acetylation of the aromatic molecules that exist in tobacco (Tao *et al*., 2010; Ribouh-Arras *et al*., 2019), thus we aimed to test this hypothesis in our study.

To the extent of our knowledge, this study was the first to explore *NAT2* polymorphism and its association with BC among the Jordanian population. Moreover, in our study, the effect of single nucleotide polymorphism of the *NAT2* gene was stratified by smoking status to identify the effect of genetic variations regardless of smoking status.

Our study revealed – as expected – that the majority of BC patients were males and smokers. BC patients were found to be overweight, which is a previously known risk factor for BC in addition to the male gender and smoking (Kirkali *et al*., 2005; Nasr *et al*., 2017; Rezaei *et al*., 2019).

We have investigated the association of several SNPs in the N-terminal catalytic domain in the *NAT2* gene with the risk of BC. The results of our genetic analysis

Table 7. Prediction of novel *NAT2* SNPs on NAT2 protein function and phenotype

SNP ID	NP	Mutation taster	PROVEAN	Polyphen-2	Sift
c.341T>C	p.Ile114Thr	Polymorphism	Deleterious	Benign	Affect protein function
c.87G>A	P.Glu29=	Disease causing	Neutral	Benign	Tolerated

showed that the frequency of the A allele and the AA genotype of the novel SNP c.87G>A was significantly higher in BC. Since tobacco smoking is a major risk factor for the development of BC we performed a stratified analysis. Interestingly, the analysis showed that these results were significant in both smokers and non-smokers, Regression subgroup analysis, however, showed that GA genotype was a risk factor for BC among smokers only. This may indicate that smoking modifies the association between BC and GA genotype of this SNP.

Individuals with the TC genotype of c.341T<C SNP are 6 times more likely to have BC compared to those with the TT genotype. This is consistent with findings of previous studies that revealed c.341T>C SNP with its slow acetylation phenotype to increase the risk of BC especially among smokers (El Desoky *et al*., 2005; García-Closas *et al*., 2005; Quan *et al*., 2016; Song *et al*., 2020). Furthermore, our results found that only smoking individuals carrying the C allele in our population could be at a higher risk of BC in view of their slower acetylation of tobacco smoking confirming the results of others (Marcus *et al*., 2000), while contrasting other studies that indicate no difference in BC risk between different genotypes (Mittal *et al*., 2004; Saleh *et al*., 2019).

The reported SNP c.341T>C was predicted as polymorphism, benign, and affecting protein function with deleterious effects. In addition, this SNP is linked to slow acetylator phenotype. As mentioned earlier, this SNP increased the risk of BC among smokers. This is in accordance with other studies, where SNPs with slow acetylation are associated with a higher risk for different cancer types including BC, and mainly among smokers (Zhu *et al*., 2015; Marcus *et al*., 2000; Kabir & Rehman, 2018).

Our sequence analysis was limited to a short stretch (446 bp) of the second exon of the coding region of the *NAT2* gene; an area that only encompassed the N- catalytic functional domain of the enzyme. Sequence analysis of the entire coding region of the gene is required in future studies. This is especially important since previous studies from other populations showed that genetic areas outside the area sequenced in this investigation may contain genetic variants that affect enzyme function and acetylation phenotype (Boukouvala *et al*., 2003; Ribouh-Arras *et al*., 2019).

A high degree of polymorphism was discovered in this gene across a short stretch of the coding sequence. Previous studies also reported the *NAT2* gene as being highly polymorphic upon gene sequencing (Sekine *et al*., 2001; Matimba *et al*., 2009). Considering that more than half of the genetic variants discovered in this investigation were not previously reported, a more comprehensive evaluation of the *NAT2* gene polymorphism should be conducted on a larger number of individuals representing different geographic areas and ethnic backgrounds present in Jordan.

This report highlights the discovery of a novel SNP in the *NAT2* gene and its association with BC. Studies that examine the effect of the above SNP on enzyme function and/or structure should be conducted using purified enzyme preparations or mammalian cells transfected with vectors carrying the coding region of the enzyme. The above investigations could be coupled with in *vivo* assays which examine the acetylation kinetics of individuals that carry the different genotypes using safe and well tolerated substrates (for example, caffeine).

Considering that *NAT2* polymorphisms may affect the therapeutic dose and safety profile of many of the most commonly prescribed medications and the high degree of polymorphism discovered in *NAT2* (at least among the Jordanian population), we suggest to build a national database of the most frequent alleles of the *NAT2* gene in Jordan including their effect on the metabolism of various drugs and medications. This database can then be used as a tool to promote *NAT2* pharmacogenetic profiling of patients as part of their treatment protocol.

The major strengths of this study include being the first study in Jordan in which a gene of pharmacogenetic importance was sequenced. This population-based study could represent the first step toward establishing a database of *NAT2* gene variants in Jordan – this gene is known to have wide variations across ethnicities (Yee *et al*., 2020). Moreover, our study was not limited to investigating the effects of already reported polymorphisms but rather attempted to discover novel – not previously reported – variants. This might set up the stage for future population-based studies in the region. Moreover, stratifying our analyses according to smoking status enabled us to control smoking as a potential confounder. Our sample was retrieved from three major centers, one of them is the only specialized cancer center in Jordan, in which cancer patients are treated from all over the country. This makes our results generalizable to all Jordanians with BC.

Yet, this investigation is subject to several limitations. First, the relatively small sample size limits our ability to attain sufficient statistical power and increases the risk of type II error. Second, we acknowledge that we were not able to quantitatively assess the smoking status of participants in order to assess its dose-response relationship with BC, compared to what was done by Moore and others (Moore *et al*., 2011). Third, this study did not investigate the acetylation activity of the enzyme nor evaluated the acetylation phenotype (by measuring N-acetylated metabolites after administration of drugs like isoniazid, or caffeine) (Grant *et al*., 1984) distribution among study participants. Since this information is also lacking in Jordan, future studies investigating NAT2 phenotype and genotype associated with BC in the Jordanian population are needed. The lack of enzyme modelling studies assessing the effects of newly discovered variants was another limitation. Finally, despite controlling for smoking status as a confounder, causal inferences still cannot be made due to the nature of observational studies, such as case-control studies, where a temporal sequence is lost.

CONCLUSION

This case-control study demonstrated that smoking and genetic variation in the *NAT2* gene are associated with BC risk. Although several investigations across multiple populations explored the association of *NAT2* polymorphism with the risk of BC, this is the first study from Jordan. Furthermore, this is the first report from the region to present genomic sequence data on the N-catalytic domain of the *NAT2* gene. This genomic-based approach allowed us to identify a novel variant c.87G>A that was not reported before and was found here to be associated with BC risk. In addition, we detected the previously reported SNP (c.341T>C) that was related to slow acetylation phenotype and was also found to increase BC risk. Our data supports the use of TC and GA genotypes of c.341T>C and c.87G>A SNPs, respectively as potential biomarkers of BC, particularly among smokers. However, further investigations with a larger

population are needed to validate our findings. Given the high prevalence of tobacco smoking in Jordan, the initiation of awareness campaigns that explain the implications of cigarette smoking on health and disease is strongly recommended.

Declarations

Authors' contributions. Conception: LE and MAA; Funding acquisition: LE; Methodology: LE, MAA, AAS, and AA; Interpretation or analysis of data: AAS, AA, and KK; Writing original draft: LE and MAA; Revision for important intellectual content: OH, SAD, AAD, KK and MA; Supervision: LE, MAA, OH, SAD, AAD, and MA. All authors have critically reviewed and approved the final draft and are responsible for the content.

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Availability of data and materials. Data is available from the corresponding author upon reasonable request.

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