ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 3/2023
503–507
https://doi.org/10.18388/abp.2020_6235

Regular paper

A case-control study in *NAT2* gene polymorphism studies in patients diagnosed with acute myeloid leukemia

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Introduction: Acute myeloid leukemia (AML) is a clinically defined heterogeneous disease whose pathophysiology is currently unknown. The association of NAT2 acetylation profiles with human cancer risks, particularly with AML, was investigated in molecular epidemiological studies. Additionally, the *NAT2* gene was carried out with acute lymphoid leukemia and other cancers. Aim: In this case-control study, C481T (rs1799929) and G857A (rs1799931) polymorphism studies were investigated in diagnosed AML patients in the Saudi population. Methods: This case-control study included 100 AML patients and 100 control subjects recruited in Saudi Arabia. The C481T and G857A polymorphisms were genotyped using specific primers and restriction enzymes. Statistical analysis was performed on the AML patients and controls using chi-square tests, genotyping, and allele frequencies (odds ratios, 95% of confidence intervals, and *P*-values). Results: Hardy Weinberg Equilibrium was determined to be both within and outside of the G857A and C481T polymorphisms. The allele and genotyping frequencies in AML and control subjects were analyzed, and the results corroborated the unfavorable connection with C481T (CC *vs* CT+TT; OR-1.12; (95% CIs: 0.64–1.96); *P*=0.67 and T *vs* C; OR-0.89; (95% CIs: 0.59–1.35) and *P*=0.60) and G857A polymorphisms (GG *vs* GA+AA; OR-1.50; (95% CIs: 0.83–2.71); *P*=0.17 and A *vs* G; OR-0.71; (95%CIs: 0.43–1.19) and *P*=0.19) in the *NAT2* gene. Conclusion: The study results revealed a negative correlation as well as a protective factor for AML with the C481T and G857A polymorphisms in the *NAT2* gene.

Keywords: Acute myeloid leukemia, C481T, G857A and *NAT2* gene

Received: 24 February, 2022; revised: 08 August, 2022; accepted: 06 August, 2023; available on-line: 16 September, 2023

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Acknowledgements of Financial Support: The author extends their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this Research work through the project number ISPP22-21.
Abbreviations: AML, Acute myeloid leukemia; HWE, Hardy-Weinberg Equilibrium; MDS, myelodysplastic syndromes; NAT2, N-acetyl transferase 2; SNP, Single nucleotide polymorphisms; PCR, polymerase chain reaction

INTRODUCTION

Acute myeloid leukemia (AML) is classified as an aggressive condition in which too many immature, nonlymphoblastic, white blood cells in the bone marrow and blood are identified. AML is also known as acute myelogenous leukemia or acute nonlymphocytic leukemia (Cucchi *et al*., 2021). Myeloid malignancies are a subset of hematopoietic stem/progenitor cell tumors that include AML and myelodysplastic syndromes (MDS) (Döhner *et al*., 2015). An estimated 21 450 cases were reported in 2019, representing between 15–20% of leukemias in the United States (Sasaki *et al*., 2021). The prognosis for AML patients beyond the age of sixty has not changed significantly in decades and remains bleak (Docking *et al*., 2021). AML is a clonal hematopoiesis condition defined by genetic and epigenetic changes that lead to a block of myeloid progenitor development in the bone marrow and blood and the accumulation of leukemia. Although the overall survival rate of AML patients is poor, around 20% to 30% of them never achieve complete remission, and 50% of them relapse beyond complete remission, often within 2-3 years after diagnosis (Bhatnagar *et al*., 2021). AML can also be caused by a growing MDS, such as a clonal condition of hematopoietic stem cells (Chamseddine *et al*., 2016). Despite efforts to enhance the clinical result of AML, current medications fail to eradicate the leading leukemic stem cells (Pegoraro *et al*., 2020). Based on the French and American classification, AML is classified into eight subtypes, M0-M7, depedning on the type of leukemia that develops and the stage of leukemia maturity (Lafuente *et al*., 1993).

AML is the most frequent acute leukemia in adults, which causes many deaths from malignancy. Fatigue owing to anemia, easier bleeding due to thrombocytopenia, increasing leukopenia and bone soreness are further symptoms of AML. Acute leukemia may be myeloid or lymphoblastic depending on the type of cells impacted. The specific etiology of AML is not fully understood, although risk factors for the development of AML are also present. In the great majority of AML cases, genetic mutations are discovered. Some AML-associated chromosomal translocations include t(15:17) and t (8:21). Risk factors include patients with a hematologic condition underlying AML, people who underwent alkylating or radiation chemotherapy in previous cancer treatments, genetic disorders such as Down's syndrome and increased age (Kaser *et al*., 2021). New predictors of diseases like cancer and indications of efficacy of chemotherapy response were developed in single nucleotide polymorphisms (SNPs). To date, most SNPs in AML have been identified with oncological therapy responses (Castro *et al*., 2021). Over the last three decades, genomic aberrations have dominated the pathogenesis of AML, and diagnostic and prognostic indications of cytogenesis have emerged. The Cancer Genome Atlas project has reported a small number of mutations in often disrupted pathways including *NPM1, FLT3, CEBPA, DNMT3A, IDH1,* and *IDH2,* as well as genes recently implicated in leukemogenesis like *EZH2, U2AF1, SMC1A*, and *SMC3. NPM1, CEPBA*, and *RUNX1* mutations are frequent in AML (Bullinger *et al*., 2017). Apart from this multi-

Table 1. *NAT2* gene SNP details

Gene	NC	Rsnumber	Forward Primers	Reverse Primers	PCR	RE	NB	MB
NAT2	C481T	rs1799929	GGAACAAATTGGACTTGG	TAACGTGAGGGTAGAGAGGAT	920bp	KpnI	486/434bp	920bp
NAT2	G857A	rs1799931	GGAACAAATTGGACTTGG	TAACGTGAGGGTAGAGAGGAT	920bp	BamHI	810/110bp	920bp

Table 2. Anthropometric information on the patients in this study

Anthropometric measurements	AML cases (n=100)	Controls (n=100)	*P*-Value
Age (Years)	38.9±15.1	39.9±12.06	0.60
Minimum and maximum ages	19–82	18–63	–
Males (Gender)	61 (61%)	54 (54%)	–
Females (Gender)	39 (39%)	46 (46%)	–

ple single nucleotide polymorphisms were documented in the AML. N-acetyl transferase 2 (NAT2) is a phase II metabolizing enzymes that are vital in the acetylation and detoxification of various hazardous metabolites and carcinogenic agents such as aryl or aromatic amines and hydrazines, which are implicated in the development of carcinogenesis (Yarosh *et al.*, 2014). Several molecular epidemiological studies have investigated the link between NAT2 acetylation profiles and the risk of human cancer. In addition, a significant study showed that persons with the NAT2 phenotype are at greater risk of developing AML. Furthermore, SNPs in the *NAT2* gene also control human sensitivity to different cancers such as pulmonary cancer, bladder cancer, gastric cancer etc (Zou *et al.*, 2017). Growing evidence revealed that *NAT2* polymorphic diseases were combined with higher vulnerability to other diseases, notably acute lymphoblastic leukemia and lung squamous carcinoma. In addition, various studies have shown the relationship of distinct NAT2 polymorphisms with a high risk for acute development of leukaemia, notably AML, with contradictory results (AbdelGhafar *et al.*, 2019).

Leukemia is the third and sixth most common malignancy in Saudi men and women, respectively. In 2013, 12% of newly reported leukemia cases were AML patients. AML is an age-related disease in Saudi Arabia, with a median age of 70 years. AML is the most common adult leukemia, with an increased frequency with age. In 2013, 684 new cases of adult leukemia were reported in the most recent cancer-incidence report in Saudi Arabia in every 100 000 persons and this represents 5.9% of all new adult malignancies (Gaafar *et al.*, 2018). Presently, limited studies have been carried out in the Saudi population and unfortunately there are no documented studies on the C481T and G857A polymorphism in the NAT gene in patients diagnosed with AML in the Saudi population. Therefore, the present study aimed to investigate the case-control study in C481T and G857A polymorphism with *NAT* gene in Saudi patients diagnosed with AML.

MATERIALS AND METHODS

The Ministry of Health provided an ethical grant for this study, which was carried out in accordance with the Helsinki Declaration. In this case-control study, 200 Saudi individuals were recruited as 100 patients were diagnosed with AML and 100 were non-AML (healthy subjects). All of the recruited samples were obtained from the Riyadh regional laboratory in Saudi Arabia's capital city. Both cases and controls were chosen based on the inclusion and exclusion criteria discussed in the earlier publication (Farasani, 2019). The inclusion criteria for AML subjects were recruited based on the diagnosis of the AML with histopathological and cytogenetic confirmation, signed informed consent and Saudi adults. The exclusion criteria for AML cases were patients diagnosed with other cancers, unsigned consent form with non-Saudi subjects. The AML cases were diagnosed with bone-marrow examination, complete blood count and flow cytometry. Additionally, cytogenetics and FISH tests were implemented for reconfirmation. The inclusion criteria forhealthy controls did not include any type of cancer or other diseases. The exclusion criteria were non-Saudi subjects.

Sample selection

2 ml of EDTA blood was obtained from each patient (n=200) and utilized for DNA extraction and molecular analysis (Alshammary *et al.*, 2023).

Molecular analysis

Genomic DNA extraction was done with the specific kits used for separation of the DNA and nanodrop was used to measure the DNA quantification (Alshammary *et al.*, 2023). Genotyping was performed with C481T and G857A polymorphism in the *NAT2* gene using the precise primers as described in the Table 1. Genotyping was carried out with polymerase chain reaction (PCR) which was performed with a total reaction mix of 50 µl microliters consisting of 4 µl genomic DNA and 30 µl PCR mixes, which contain 10X, $MgCl_2$, dNTPs and 10× Taq DNA polymerases. The Master Mix was complemented by 10pmoles of 2 µL of forward and reverse primers followed by 12 µL of distilled water. For the final amount of 50 µL, the PCR reaction has been standardized (Alshammary & Khan, 2021). Both the C481T and G857A primers from earlier studies have been used. After PCR 35 cycles were performed starting with initial denaturation running 5 min at 95°C, 30 s at 95°C for denaturation, various annealing temperatures for both the polymorphisms at 65/60°C – 30 s, extension at 72°C – 45 s and final extension at 72°C – 5 min. The 910bp of PCR products were digested with *KpnI and BamHI* enzymes at 37°C for 18 hours and further samples were loaded on 3% agarose gel stained with ethidium bromide. Further details were shown in the Table 1.

Statistical analysis

SPSS software (version 20) was used to examine the clinical data. Hardy-Weinberg Equilibrium (HWE) was

Table 3. The studies distribution genotype frequencies of C481T and G857A polymorphisms

NAT2 (rs1799929)	Cases (n=100)	Controls (n=100)	OR (95%CI)	*P*-Value
CC	51 (51%)	48 (48%)	Position	Position
CT	30 (30%)	31 (31%)	0.91 (0.48–1.72)	0.77
TT	19 (19%)	21 (21%)	0.85 (0.40–1.77)	0.66
CC *vs* CT+TT	51 (51%)	48 (48%)	1.12 (0.64–1.96)	0.67
C	132 (0.66)	127 (0.635)	Position	Position
T	68 (0.34)	73 (0.365)	0.89 (0.59–1.35)	0.60
NAT2 (rs1799931)	Cases (n=100)	Controls (n=100)	OR (95%CI)	P-Value
GG	71 (71%)	62 (62%)	Position	Position
GA	26 (26%)	34 (34%)	0.66 (0.36–1.23)	0.19
AA	03 (03%)	04 (04%)	0.65 (0.14–3.04)	0.58
GG *vs* GA+AA	71 (71%)	62 (62%)	1.50 (0.83–2.71)	0.17
G	168 (0.84)	158 (0.79)	Position	Position
A	32 (0.16)	42 (0.21)	0.71 (0.43–1.19)	0.19

used for comparing the observed and anticipated genotype frequencies using control subjects. The odds ratios, upper, and lower ranges of 95% confidence intervals (95% CI) for C481T and G857A polymorphisms in the NAT2 gene were used in the genotype differences between AML cases and healthy control subjects. The $P<0.05$ were considered statistically significant (Khan *et al.*, 2019).

RESULTS

Clinical details for AML cases and controls

Table 2 documents the clinical characteristics between AML and non-AML (controls) involved in this study. In this case-control study, 100 AML cases and 100 healthy controls were selected within the Saudi population. 38.9±15.1 and 39.9±12.06 was the known mean age for AML cases and control subjects documented with non-signifcant association ($P=0.60$). Between 19–82 is the minimum and maximum age for AML cases and 18–63 is the minimum-maximum age for the control subjects documented in this study. The male and female patients in the AML cases were 61% and 39%, respectively, whereas the male and female participants in the controls were 54% and 46%.

HWE analysis

HWE analysis was performed in both the C481T and G857A polymorphisms and HWE was not in accordance with the control subjects for C481T; VAF=0.37; $\chi^2=10.98$ and $P=0.0009$ and for G857A is in accordance with the control subjects VAF=0.21; $\chi^2=0.60$ and $P=0.80$.

Genotype analysis for C481T and G857A polymorphisms

The genotype frequencies between CC, CT and TT were found to be 51%, 30% and 19% in the AML cases, whereas in the control subjects 48%, 31% and 21% were documented as CC, CT and TT genotypes. 66% of the C allele and 34% of the T alleles have been documented in AML cases and in the control subjects, 0.365% of the T allele and 0.635% of the C allele were confirmed. The genotype [CT *vs* CC; OR-0.91; (95% CIs: 0.48–1.72) and $P=0.77$ and TT *vs* CC; OR-0.85; (95% CIs: 0.40–1.77) and $P=0.66$] and dominant model also showed the non-signifcant association (CC *vs* CT+TT; OR-1.12; (95% CIs: 0.64–1.96); $P=0.67$ allele frequencies [T *vs* C; OR-0.89; (95% CIs: 0.59–1.35) and $P=0.60$] was also confirmed and non-significantly associated. Table 3 confirms the genotype distribution as well as allele frequencies in AML cases and control subjects. The GG, GA, and AA genotype frequencies of G857A polymorphism were 71%, 26%, and 3% in AML cases, respectively, and 62%, 34%, and 4% in control genotypes. The allelic discrimination between G and A alleles in AML cases was found to be 84% and 16%, respectively, whereas in control subjects it was 79% and 21%. The genotype [GA *vs* GG; OR-0.66; (95% CIs: 0.36–1.23) and $P=0.19$ and AA *vs* GG; OR-0.65; (95% CIs: 0.14-3.04) and $P=0.58$] and dominant model also showed the non-signiifcant association (GG *vs* GA+AA; OR-1.50; (95% CIs: 0.83–2.71); $P=0.17$ allele frequencies [A *vs* G; OR-0.71; (95% CIs: 0.43–1.19) and $P=0.19$] was also confirmed and non-significantly associated.

DISCUSSION

In this case-control study, C481T and G857A polymorphisms in the *NAT2* gene were studied in the Saudi patients diagnosed with AML and the study results confirmed the negative association with any of the allele or genotype frequencies. AML is one of a particular type of cancer that modifies the drug-metabolization enzymes and mainly because of genetic heterogeneity of the various human populations in the fields of drug metabolism and disease sensitivity, NAT2 is a key subject in pharmacogenetic study. Seven SNPs of the *NAT2* gene were studied in human diseases at 191, 282, 590, 857, 481, 803 and 314 positions, and many more SNPs have been reported in worldwide studies (Adole *et al.*, 2016). The NAT2 isoform is encoded by the *NAT2* gene, which is situated on chromosome 8 (8p22), and this gene is positioned combined with the *NAT1* gene and the pseudogene *NATP*. the observed variety of the described alleles derives from the combination of single-base mutations selected from among the several bases. Enzyme activity, affinity for substrate, and stability of resultant protein can all be affected by existing SNPs in

the coding region of the gene. The C481T (rs1799929), G590A (rs1799930), G857A (rs1799931), and G191A (rs1801279) have been useful in the context of NAT2 as an acetylator phenotype to detect them (Santos *et al*., 2016). C481T polymorphism is a synonymous SNP frequently associated with T341C, which occurs in clusters NAT2*5, as A, B, F, G, H, I, L, and M in the variant alleles 6E, 11A&B, 12 and 14 C. C481T (NAT2*11A) is exceedingly rare, and has not been found in over 2600 individuals of European descent (Garcia-Martin, 2008). G857A substitution results in a change in the amino acid glutamate in the protein (G286E). This mutation alters the active site of the enzyme, lowering its selectivity and capacity to operate as a catalyst. The polymorphism in the *NAT2* gene identified in the G857A variant reduces aromatic and heterocyclic amines. Only 11 NAT2 alleles have the mutation G857A, and two of these are the result of a slow acetylator phenotype, while the others have not yet been identified (Rajasekaran *et al*., 2011). In this study, only C481T and G857A polymorphisms have been studied in the Saudi patients confirmed with the AML disease. The current study results with G857A polymorphism were associated with a previous study documented in the Jordanian population (Jarrar *et al*., 2010).

A function for *NAT2* gene polymorphism in several cancer types has been proposed, and AML has been documented in multiple studies in the global population (Gra *et al*., 2008; Majumdar *et al*., 2008; Zanrosso *et al*., 2012; Zou *et al*., 2017). Limited studies have been recorded in the Saudi population with the *NAT2* gene in different diseases. C481T and G857A polymorphisms have been linked to T2DM in the Saudi Arabian population, and the study results validated the favorable connection with G857A polymorphism. However, none of the SNPs was shown to be positively associated in this study, which may be due to the function of the specific disease (Al-Shaqha *et al*., 2015). A previous study in the Arab control population in Saudi Arabia with both the C481T and G857A polymorphisms in the *NAT2* gene was performed (Bu *et al*., 2004). A similar study on the *NAT2* gene was conducted in the Saudi Arabian population in Al-Ahsa, where it was discovered that persons with the *NAT2* gene had an elevated risk of slow acetylators, potentially impacting the efficacy and vulnerability to numerous diseases (Zahra *et al*., 2020). A similar pattern of this study was replicated in the Jordanian population (Jarrar *et al*., 2018). The frequency of *NAT2* gene polymorphism in Saudi Arabia, Oman, and Emirati populations varies(Al-Ahmad *et al*., 2017; Tanira *et al*., 2003; Zahra *et al*., 2020). The *NAT2* gene was screened in the Egyptian population in children diagnosed with lymphoblastic leukemia, and the study results indicated that the *NAT2* gene is slowly related with the acetylator phenotype with ALL risk in pediatric children (Kamel *et al*., 2015). In the NCBI dbSNP *NAT2* C481T and G857A database, the worldwide Minor All Frequency was T=0.27 and A=0.08. G857A is a rather infrequent polymorphism, according to these frequencies (Fayez *et al*., 2018). The proportion of mutant alleles was validated in this study as 0.34 in T-allele and 0.16 in A-allele of both described polymorphisms in the *NAT2* gene.

A meta-analysis of *NAT2* gene variants in isoniazid-induced hepatotoxicity (IIH) found that these genetic variants had a substantial impact on IIH. The *NAT2* genotyping test can help with a better knowledge of drug-enzyme metabolism as well as an earlier prediction of IIH (Khan *et al*., 2019). A documented meta-analysis found rs1799931 to be a protective factor against cancer development (Tian *et al*., 2014), which was consistent with the current report's results as well as those of others (Zou *et al*., 2017). A meta-analysis study on acute leukemia with the NAT2 gene was conducted (Zhu *et al*., 2019). This study has added strengths and limitations and one of the strengths of this study was opting for a minimum of 100 Saudi patients diagnosed with AML cases and 100 healthy Saudi controls involved in this study. Opting for only 2 SNPs involved in this study is one of the major limitations of this study. Missing of anthropometrical and clinical data was another limitation of this study.

CONCLUSION

In conclusion, the current study findings revealed a negative correlation as well as a protective factor for AML with the C481T and G857A polymorphisms in the *NAT2* gene. The current study findings verified the comparable correlation found in Chinese studies.

Declarations

Conflict of Interest: I don't have any conflict of Interest towards this manuscript.

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