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Distribution of arylamine N-acetyltransferase 2 (NAT2) genotypes among Omanis

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توزيع الأنماط الجينية لخميرة ال NAT٢ بين العمانيين

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المستخلص: الهدف: تعيين الأنماط الجينية المتعددة لخميرة ال NAT٢ بين مجموعة مكونة من ١٢٧ عماني لا تربطهم صلة قرابة. **الطريقة:** تحديد تواجد أكثر الطفرات المعروفة لهذه الخميرة وهي G١٩١A, C٢٨٢T, C٣٤١T, C٤٨١T G٥٩٠A, A٨٠٣G, G٨٥٧A بواسطة طريقة التحليل المعروفة ب PCR-RFLP. **النتائج:** أظهرت النتائج أن أكثر الأنماط شيوعا هي الأنماط NAT*٤, NAT٢*٦A, NAT*٥B التي تواجدت بنسب ٠,٣٦٢, ٠,٢٤٨, ٠,١٨٩ على التوالي. وبلغت نسبة التكرار لمجموعة الأفراد الذين تتواجد فيهم الخميرة سريعة العمل ٠,٢٥ من مجموع المشاركين في الدراسة. **الخلاصة:** تم اكتشاف أليل جديد لم يسبق التعرف عليه، يحتوي على الطفرات T٣٤١C, C٢٨٢T, G٥٩٠A وتم تسميته ب NAT٢*٥J. وكانت الأنماط الجينية الأكثر شيوعا هي B/٥B, ٥B/٦A, ٤/٥B, ٤/٦A التي تواجدت بنسب ٠,١٦٥, ٠,١٥٧, ٠,١١٨, ٠,١١٠, ٠,٠٧٩.

ABSTRACT. ***Objective***: to determine the genotypes of arylamine *N*-acetyltransferase (NAT2) among 127 unrelated apparently healthy Omanis. ***Method:*** Identify the most common known polymorphisms of NAT*2 gene namely, $G^{191}A$, $C^{282}T$, $C^{341}T$, $C^{481}T$, $G^{590}A$, $A^{803}G$ and $G^{857}A$ using PCR-RFLP analysis. ***Results***: Eleven allele variants (3 alternative) and 30 different genotypes were determined. The commonest alleles were found to be NAT*5B, NAT2*6A and NAT*4 with corresponding frequencies of 0.362, 0.248 and 0.189 respectively. The overall frequency of rapid acetylator alleles was 0.25. ***Conclusion:*** A new allele variant containing $G^{590}A$, $C^{282}T$ and $T^{341}C$ polymorphisms was found in one subject (was named NAT2*5J). The commonest genotypes were found to be 5B/5B, 5B/6A, 4/5B, 4/6A with frequencies 0.165, 0.157, 0.118, 0.110 and 0.079 respectively.

Keywords: Omani, *N*-acetyltransferase (NAT2), genotyping

THERE IS AMPLE EVIDENCE TO SUPPORT THE concept that the inter-ethnic genetic variation may be accountable, at least in part, for the variability in drug response.[1,2] Genetic polymorphism that may influence a drug's response may exist in drug metabolizing enzyme genes[3,2] and/or drug receptor genes.[4,5]

Drug metabolizing enzyme (DME) polymorphisms play a significant role in determining the outcome of using drugs in therapy and may be a risk factor in some human diseases.[6,7,3] Genetic polymorphism of *N*-acetyltransferase (NAT) is among the most studied DME polymorphism. NAT has two isoenzymes (NAT1 and NAT2) that have been identified in humans and animals. Both genes are single, intronless protein coding exons with 870 bp open reading frames encoding 290 amino acids protein that exhibit polymorphism.[8]

In a recent review, Hein *et al,*[9] and Hein[10] presented the most significant information on the molecular genetics and cancer epidemiology of NAT. The authors also provided a list of reviews published in the past years, which dealt with this topic. Furthermore, they included an updated nomenclature for NAT1 and NAT2 based on a consensus system published by Vatsis *et al*[11] and the International NAT Nomenclature Committee guidelines.[12]

A few reports have been published on NAT genotyping of Arab populations. Those available are on Egyptians,[13] Emiratis,[14] Lebanese,[15] and Saudis,[16,17] all of which are suggestive of different patterns of polymorphism. No information is available on Omanis of Arab descent in this respect. In this work we present the first report on NAT2 genotyping of apparently healthy Omani adults.

MATERIALS AND METHODS

The Research and Ethics Committee of the College of Medicine, Sultan Qaboos University, approved this study. Blood from 127 apparently healthy unrelated

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Omani adults (males and females; age range 20–30 years) was used.

Polymerase Chain Reaction (PCR)

Amplifying 999 bp segment of NAT2 gene sequence*

Genomic DNA was obtained from the buffy coat of whole blood samples of subjects by phenol/chloroform extraction. Amplification was carried out by PCR using 0.2 μM of oligonucleotide primers, Nat-Hu 14 (forward primer; 5'-GAC ATT GAA GCA TAT TTT GAA AG-3') and Nat-Hu 16 (reverse primer; 5'-GAT GAA AGT ATT TGA TGT TTA GG-3') described by Hickman and Sim (1991) in a 100 μl mixture of 0.01 M Tris buffer (pH 8.3), MgCl2 2.5 M, dNTP 1.25 μM, Taq polymerase 1.25 U, and genomic DNA 1 μl. Reagents and mixture were kept on ice during preparation. Negative control (DNA not added) was used for quality control. Amplification conditions were denaturation 1 cycle at 95°C for 5 min, 35 cycles (94°C 30 sec, 56°C 1 min, 72°C 2 min), then 1 cycle 72°C 8 min.

Amplifying 360 bp and 683 segments from the 999 bp of NAT2 gene sequence*

Two other PCRs were performed to amplify 683 bp and 360 bp sequences from the 999 bp product of genomic DNA. Forward primers MS 341cC-M (5'-CAC CTT CTC CTG CAG GTG ACC CC-3') and MS 342RC (5'-CGA CAA TGT AAT TCC TGC CGT CC-3') were used respectively. Nat Hu 16 was the reverse primer in both cases. The NAT2 gene amplification product (999 bp) was diluted 1:100 and used as a template. PCR conditions were similar to the above. The 360 bp fragment was used for $T^{341}C$ polymorphism detection, whereas the 683 bp fragment was used to confirm genotyping as explained below. PCR was performed using Hybaid OmniGene or Perkin Elmer System 480.

Restriction Fragment Length Polymorphism (RFLP)

The presence of polymorphisms $G^{191}A$, $C^{282}T$, $C^{481}T$, $G^{590}A$, $A^{803}G$, $G^{857}A$ on NAT2 gene was determined by RFLP using the restriction endonucleases *Msp* I, *Fok* I, *Kpn* I, *Taq* I, *Dde* I *and Bam* HI respectively. The presence of polymorphism $T^{341}C$ was determined using *Nco* I enzyme as described by Simsek *et al*.[18] The method is based on mutagenesis-directed PCR to create an *Nco* I site for the wild-type allele.

Enzymatic digestion was carried out in a total volume of 15 μl using 10 μl of the NAT2 gene amplification product (999 bp), the appropriate restriction enzyme (10 U) and its buffer. Distilled water was added to bring the final volume to 15 μl. Digestion was carried overnight at 37°C for all enzymes but at 56°C for *Taq* I, under mineral oil. Digested DNA (10 μl) was separated by electrophoresis on 2% agarose or 10% polyacrylamide gel (90V cm^{-1} for 90 min or 180 min respectively) with DNA molecular size marker. Bands were visualised with ethidium bromide and UV transillumination.

DNA sequencing

An amplified 998 bp fragment of *NAT2* gene from a new variant (5J) was sequenced in both chains (forward and reverse) using the dideoxy termination method on an ABI-310 DNA sequencer. The sequencing primers utilized were Nat Hu 14 for the forward sequencing, and Nat Hu 16 for the reverse.

Nomenclature

Using RFLP analysis, the presence/absence of polymorphism(s) in each DNA sample was identified. Accordingly, the genotype of each subject was determined as per the NAT nomenclature scheme published by Hein *et al*.[9] and the website (http://www.louisville.edu/medschool/pharmacology/NAT.html).[19]

Allelic Linkage Analysis

In 41 DNA samples, the detected combination of polymorphisms matched more than one genotype. For these samples, polymorphism linkage analysis was performed to confirm their genotype using method detailed in Cascorbi's *et al*.[20] The details of polymorphisms, and alternative genotypes of these samples are listed in Table 1.

Reagents were purchased from GibcoBRL. *Taq* polymerase was from Pharmacia.

RESULTS

The summary of the RFLP data, which follows, is presented by the name of enzyme followed by the size of produced fragments (in parentheses), the type of the digested allele and lastly the size of the initial PCR segment size used for RFLP. *Msp* I (810 bp and 189 bp, wild allele, 999 bp segment), *Fok* I (269 bp and 91 bp, wild allele; 360 bp segment), *Kpn* I (521 bp and 478 bp, wild allele, 999 bp segment), *Taq* I (359, bp, 244 bp, 226 bp and 170 bp, wild allele, 999 bp segment), *Dde* I *(*345 bp, 278 bp, 203 bp, 150 bp and 23 bp, mutant allele, 999 bp segment*), Bam* HI (853 bp and 146 bp, wild allele, 999 bp segment) and *Nco* I (198 bp, 136 bp and 23 bp wild allele; 360 bp segment). Cutting sites other than polymorphism site were due to the presence of naturally existing non-polymorphic restriction sites. The latter was found in cases of *Taq* I, *Dde* I and *Nco* I enzymatic digestion.

Table 1. ***Combination of polymorphisms detected in 41 DNAs and their NAT2* genotypes or alternative genotype among 127 unrelated healthy Omani subjects.***

Group	Number of DNAs	Polymorphisms	Genotype	Alternative Genotype
1	14	341, 481, 803	NAT2*4 / NAT2*5B	NAT2*5A / NAT2*12A *or* NAT2*5D/NAT2*12C
2	14‡	282, 590	NAT2*4 / NAT2*6A	NAT2*6B / NAT2*13
3	4	282, 341, 481, 803	NAT2*5B / NAT2*13	NAT2*5A / NAT2*12B
4	3	341, 803	NAT2*4 / NAT2*5C	NAT2*5D / NAT2*12A
5	3	282, 857	NAT2*4 / NAT2*7B	NAT2*7A / NAT2*13
6	2	341 (m/m), 481, 803	NAT2*5A / NAT2*5C	NAT2*5B / NAT2*5D
7	1	341, 381, 803 (m/m)	NAT2*5B / NAT2*12A	NAT2*5C / NAT2*12C

Note: Linkage analysis was performed using Cascorbi *et al* 1995 method.

‡ Linkage analysis in these 14 samples could not be performed.

All enzymes except *Dde* I cut the wild allele. Hence, the presence of the 999 bp fragment (undigested initial PCR product) marked the presence of polymorphisms $G^{191}A$, $C^{481}T$ and $G^{857}A$. Similarly, a 360 bp fragment indicated the presence of $C^{282}T$ polymorphism. However, for polymorphisms $C^{341}T$, $G^{590}A$ and $A^{803}G$, the presence of a 221 bp, 396 bp and 203 bp fragment, respectively, was diagnostic.

A new allele variant, containing $C^{282}T$, $T^{341}C$, and $G^{590}A$, was characterized using both automated DNA sequencing and two different RFLP methods. Identical genotyping results were obtained for the $C^{282}T$ and $G^{590}A$ polymorphism by the sequencing and RFLP methods. However, the genotyping results at the $T^{341}C$ polymorphic site were different. Two independent RFLP methods showed presence of a heterozygosity (T/C) at the $T^{341}C$ site, while DNA sequencing detected no heterozygosity in this position (mainly T in the forward, and A in the reverse sequencing). The discrepancy about the $T^{341}C$ site is elaborated in the discussion section.

The distribution of seven investigated NAT2* gene polymorphisms is detailed in Table 2. None of the tested alleles was found to contain $G^{191}A$ polymorphism, indicating the absence of this polymorphism in our examined population. All polymorphism distribution was in Hardy-Weinberg equilibrium. Polymorphisms with the highest frequency (0.44) were $C^{341}T$ and $A^{803}G$ whereas the $G^{857}A$ polymorphism was least frequent (0.04) and was found in heterozygote but not in homozygote DNAs. Polymorphisms $C^{481}T$, $C^{282}T$, $G^{590}A$ were found in 39%, 33% and 27% of all alleles, respectively.

The allele identification as per Hein *et al*,[9] showed that the frequency of various alleles among our studied samples of Omanis is as represented in Table 3. Alleles *5B *6A and *4 (89, 63 and 45 alleles respectively) were repeated in more than 0.78 of the sample size. The least frequent allele was a previously non-reported variant. It contained polymorphisms $G^{590}A$, $C^{282}T$ and $C^{341}T$. Alleles 5D, 5F, 6C, 6D, 7A and 14 A were not detected.

Table 4 summarises the frequency of genotype distribution. The commonest genotypes were found to be 5B/5B, 5B/ 6A, and 4/6A (21, 20 and 15 individuals respectively) reflecting the abundance of their alleles in the studied population.

DISCUSSION

This is the first report on NAT2* genotyping of an Arab population in which all known seven polymorphisms of this gene have been fully and thoroughly tested. For example, the detection method for polymorphism $C^{481}T$, which was not previously detected in an Arab population, was based on two different complementing RFLP procedures.[21] Also, in a number of samples, direct automated DNA sequencing[18] confirmed the presence/absence of some polymorphisms. Furthermore, when combination of polymorphisms theoretically matched more than one genotype, further RFLP procedures were developed to identify the correct genotype. Hence, when Hardy-Weinberg equation was applied, the number of observed alleles (254) exactly matched the expected number in all polymorphisms except for polymorphisms $C^{282}T$ and $C^{481}T$ where the expected number was 253 and 255 respectively. In complementation to our experimental work, we used the latest published NAT2* nomenclature system for allele nomenclature as per the NAT website.[19]

The distribution of polymorphisms in our studied population appears to be similar to that in other ethnically

Table 2. *Distribution of 7 polymorphisms in the NAT2* gene among 127 unrelated healthy Omani subjects*

frequency	$G^{191}A$		$C^{282}T$		$C^{341}T$		$C^{481}T$		$G^{590}A$		$A^{803}G$		$G^{857}A$	
	0.00		0.33		0.44		0.39		0.27		0.44		0.04	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%
w/w	127	100	60	47	42	33	51	40	71	56	44	35	117	92
w/m	0	0	50	39	57	45	53	42	43	34	55	43	10	8
m/m	0	0	17	13	28	22	23	18	13	10	28	22	0	0

\# Number of polymorphism per DNA.

% Percentage (Total may not add up to 100 because figures were rounded to the nearest integer.)

* Percentage of mutant allele from total alleles, i.e., 254 alleles.

related Arab populations, especially in the neighbouring countries such as the United Arab Emirates.[14] However, no $G^{191}A$ polymorphism (the "African polymorphism") was found in our group, whereas two individuals having this polymorphism were identified in the UAE study. This may be explained by the fact that our Omani population was sampled from the "internal" Omani region and it was ensured by pedigree experts of the area to have not mixed with other ethnic groups.

In addition, we found an allele variant that was not reported in Hein *et al*.[9] It contained the following polymorphisms $C^{282}T$, $T^{341}C$, and $G^{590}A$. The suggested name of the new allele, NAT2*5J, has been accepted by the NAT2 Nomenclature Committee. The allele has been listed at the NAT web site, with a note "The SNPs on NAT2*5J were determined by RFLP methods which are indirect".[19] (This note will be removed once a direct approach is applied to identify the SNPs in the allele.)

All three polymorphic changes ($C^{282}T$, $T^{341}C$, and $G^{590}A$) in our new variant were characterized independently both by automated DNA sequencing and two different RFLP methods. DNA sequencing of a 999 bp fragment of the *NAT2* gene confirmed the genotyping results obtained by RFLP methods at the $C^{282}T$, and $G^{590}A$ sites. However, there was a discrepancy for the genotyping results at the $T^{341}C$ site, where two independent RFLP methods detected heterozygosity (T/C) at the 341 site while DNA sequencing showed a main peak signal for T and a small peak, less than 10% for C. Since the threshold value for heterozygote detection is usually set at 30% in sequencing, the presence of heterozygosity at the $T^{341}C$ site was missed by automated DNA sequencing. A similar conflicting result between DNA sequencing and RFLP methods has recently been described by Simsek *et al*[18,21] who showed that two complementary PCR-RFLP methods are better than automated DNA sequencing for the detection of heterozygotes in the NAT2 gene.

Almost all groups with published work on NAT2, subdivide NAT2* phenotypes to either "slow" or "rapid" acetylators. However, a third type of acetylators, namely "intermediate" is also mentioned in Hein[8] and Hein *et al*.[9] The genotype/phenotype relationship is thoroughly discussed by these authors. One added evidence to support

Table 3. *Frequency of various NAT2* gene allele variants among 127 unrelated healthy Omani subjects*

Allele variant	Predicted Phenotype	Number	Frequency
*4	R	45	0.177
*5A	S	8	0.031
*5B	S	89	0.350
*5C	S	9	0.035
*5E	S	1	0.004
*6A	S	63	0.248
*6B	S	4	0.016
*7B	S	10	0.039
*12A	R	6	0.024
*12B	R	4	0.016
*12C	R	3	0.012
*13	R	6	0.024
*5J?	S	1	0.004
total		254	1.000

* Total value adds up to higher than 1.000 due to rounding to the third decimal point.

Table 4. *Frequency of various NAT2* genotypes among 127 unrelated healthy Omani subjects*

Genotype	Predicted Phenotype	Number‡	Frequency
5B/5B	S	21	0.165
5B/6A	S	20	0.157
4/5B	I	15	0.118
4/6A	I	14	0.110
6A/6A	S	10	0.079
5A/12B	I	4	0.031
5B/7B	S	4	0.031
4/4	R	3	0.024
4/7B	I	3	0.024
5B/5C	S	3	0.024
5D/12A	I	3	0.024
6A/13	I	2	0.016
6A/6B	S	2	0.016
5B/12A	I	2	0.016
5B/5D	S	2	0.016
5C/6A	S	2	0.016
5C/5C	S	2	0.016
5C/12C	I	2	0.016
Total		127	1.000

‡NAT2* genotypes, 4/5A, 4/6B, 4/12A, 4/12C, 4/13, 5A/5A, 5C/6A, 5B/6B, 6B/6B, 5E/6A, 7B/13, 13/13, ?/6A were detected in only 1 subject each (frequency 0.008).

I = intermediate; R = rapid; S = slow.

their view can be lent from an experimental observation reported by Cascorbi *et al*.[6] These authors unequivocally showed that individuals carrying a homozygote rapid genotype present with statistically significant higher acetylation capacities than those who are heterozygotes, i.e., slow/rapid genotype.

The predicted phenotype in our study was deduced from genotypes as three sub-types of acetylators as adopted from Hein *et al*.[9] A slow acetylator was predicted if the genotype was comprised of 2 slow alleles; a rapid acetylator genotype would consist of 2 rapid alleles and an intermediate acetylator would be predicted if its genotype contained one slow and another rapid acetylator alleles. The alleles considered rapid were the wild (NAT2*4) ones and those containing polymorphisms $C^{282}T$ (NAT2*13) and $A^{803}G$ (NAT2*12). All other alleles were considered slow acetylators.[6,9]

Accordingly, the prevalence rate of genotypes (and hence the predicted phenotype) was 0.57, 0.38 and 0.05 for slow, intermediate and rapid acetylators. As mentioned earlier, most published data classify NAT2* acetylators into two (slow and rapid) subtypes. Having our population classified into three subtypes makes it difficult to compare our NAT2* genotype/phenotype prevalence with that of other studies.

CONCLUSION

Our data are in line with the published data from populations of similar ethnic background. However, we detected a new allele variant in one subject whose other allele was found to be NAT2*6A. The new allele, NAT2*5J, has been accepted by the NAT2 Nomenclature Committee, and listed at the NAT web site.[19]

In addition, from our experience, we suggest a re-visit of all published data on NAT2* genotyping and phenotyping in such a way that addresses two issues, first to name alleles according to the latest published nomenclature system[9] and second, to re-classify phenotypes as three, rather than two sub-types. This may help in making the published work on NAT2* correlating and more efficacious.

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