ORIGINAL ARTICLE

The significance and occurrence of TNF receptor polymorphisms in the Saudi population

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Faris Q. Alenzi *

College of Appl. Med. Sci, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia

Abstract *Background and objective:* On the basis that the inflammatory effects of TNF (tumour necrosis factor) are predominantly mediated through interaction with the TNF receptor-1 (TNFRSF1A), the current study was designed to establish the prevalence of the mutations, R92Q and P46L TNFRSF1A polymorphisms both in the general healthy Saudi population, and in Saudi patients carrying inflammatory diseases such as atherosclerosis or rheumatoid arthritis. We felt it important to report the frequency of the mutations, R92Q and P46L TNFRSF1A polymorphisms in healthy Saudi individuals, and those with inflammatory conditions, as well as to describe the pattern of immunological factors in individuals expressing R92Q or P46L TNFRSF1A. *Patients and methods:* We collected in PAX gene blood RNA tubes (for RT-PCR and sequencing) 500 blood samples from normal healthy individuals from the West and Center of Saudi Arabia, as well as 100 from patients with atherosclerosis, and 100 patients diagnosed with rheumatoid arthritis. All were screened for the levels of soluble TNF, C-reactive protein (CRP), interleukin6 (IL-6) and sTNFR1. In addition, they were screened for R92Q and P46L TNFRSF1A by RT-PCR. Moreover, phenotype and expression of peripheral blood mononuclear cells (PBMCs) was performed by flow cytometry (FACS). *Results:* Across 500 normal individuals, 8 (1.6%) expressed both R92Q and P46L mutations. By contrast, of the 100 patients in our study with atherosclerosis, 34% expressed both the R92Q and P46L mutations, whilst 42% of patients with rheumatoid arthritis expressed both mutations R92Q and P46L. No significant differences were observed between cell markers of normal individuals (CD3, 4, 8, 16, 56, 19, 25, ICAM-1, VLA-4 & L-selectin) and patients with atherosclerosis. There were significantly high values of cell markers in patients with rheumatoid arthritis compared with normal individuals both in terms of percentage and absolute counts ($p < 0.05$). Soluble IL-6 and sTNFR1 showed significant decreases in atherosclerosis and rheumatoid arthritis when compared with controls ($p < 0.05$). In addition, CRP and sTNF showed significant increases in the atherosclerosis and rheumatoid arthritis groups when compared to controls ($p < 0.05$). *Conclusion:* Our findings reasonably anticipate the presence of TRAPS disease (low pen-

* Address: College of Applied Medical Sciences, Prince Sattam Bin Abdulaziz University (PSAU), Al-Kharj, Saudi Arabia.
  E-mail address: fqalenzi@ksu.edu.sa

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1. Introduction

On the basis that the inflammatory effects of TNF (tumour necrosis factor) are predominantly mediated through interaction with the TNF receptor-1 (TNFRSF1A) (Paleolog et al., 1994), the current study was designed to establish the prevalence of the mutations, R92Q and P46L TNFRSF1A polymorphisms both in the general healthy Saudi population, and in Saudi patients carrying inflammatory diseases such as atherosclerosis or rheumatoid arthritis.

The relationship between TNFRSF1A and a variety of diseases is interesting and incompletely understood. For example, mutations in the ecto-domain of TNFRSF1A cause an autosomal dominant auto-inflammatory condition called TNF receptor associated periodic syndrome (TRAPS; MIM 142680) (McDermott et al., 1999), and which is typified by periodic fevers and a variety of tissue manifestations including abdominal pains, myalgia and erythematous skin rashes. Up to 15% of patients can get the life-threatening complication of amyloidosis. There are more than 50 variants of TNFRSF1A associated with TRAPS most of which are high penetrance mutations only seen in TRAPS patients and never in the general population. There are, however, two TNFRSF1A variants that do occur (rarely and asymptomatically) in the general population, although they were initially identified in TRAPS patients. As such, they represent low penetrance allelic polymorphisms. These polymorphic forms have the substitutions R92Q (glutamine replacing arginine at position 92) and P46L (leucine replacing proline at position 46). It is known that the R92Q and P46L forms of TNFRSF1A have an occurrence of approximately 1–2% (Aksentijevich et al., 2001; Ravet et al., 2006), although the P46L variant has been identified in almost 10% in a sub-Saharan population (Tchernitchko et al., 2005), and in almost 3% of Arabs living in North Africa (Dode et al., 2002).

Clinically, TRAPS associated with the high penetrance TNFRSF1A mutations represent more serious challenges to treat than in patients where TRAPS is associated with R92Q and P46L TNFRSF1A (Ravet et al., 2006). Also, the R92Q TNFRSF1A polymorphism appears to be both a susceptibility factor, and a disease-modifying factor, which impacts more common, inflammatory diseases. For example, it has been demonstrated that R92Q allele occurs in approximately 5% of patients with rheumatoid arthritis (Aksentijevich et al., 2001), and as many as 4% of patients with atherosclerosis (Poirier et al., 2004). The R92Q TNFRSF1A polymorphism has been linked to extra-cranial deep vein thrombosis in patients with Behcet's disease (Amoura et al., 2005), and also to manifestation of amyloidosis in juvenile idiopathic arthritis (Aganna et al., 2004).

Mechanistically, immunohistochemistry has shown that inflammatory tissue changes in TRAPS patient studies relate to an involvement of monocytes and macrophages in the inflammatory process. This was further supported by the observation that the myalgia suffered by individuals with TRAPS is due to a monocytic fasciitis and not myositis.

We therefore felt it important to report the frequency of the mutations, R92Q and P46L TNFRSF1A polymorphisms in healthy Saudi individuals, and those with inflammatory conditions, as well as to describe the pattern of immunological factors in individuals expressing R92Q or P46L TNFRSF1A.

2. Patients and methods

We collected in PAX gene blood RNA tubes (for RT-PCR and sequencing) 500 blood samples from normal healthy individuals from the West and Center of Saudi Arabia, as well as 100 from patients with atherosclerosis, and 100 patients diagnosed with rheumatoid arthritis.

The 100 Saudi patients diagnosed with rheumatoid arthritis were unrelated to each other. There were 28 males and 72 females; their age range was between 20 and 71 years. The 100 Saudi patients with atherosclerosis were also unrelated to each other, and comprised of 34 males and 66 females; their age range was between 26 and 65 years. The control group consisted of 500 unrelated, healthy blood donors, and comprised of 300 males and 200 females; their age range was between 25 and 65 years. Patients with other inflammatory/autoimmune diseases were not included in the study. Ethical approval for this study was obtained from research and ethics committee of PSMMC. Informed written consent was obtained from each subject before their recruitment onto this study.

Blood was collected in plain tubes for ELISA to detect soluble-TNFR1, IL-6, TNF and CRP. Three 3ml of blood was collected in tubes containing EDTA as anticoagulant for performing different CD analyses.

2.1. Measurement of soluble TNF-R1, CRP and cytokines (IL-6 and TNF)

This was achieved with a BioSource ELISA kit (BioSource Europe S.A). Samples of serum were diluted 1/100 in diluent. Then, 50 µl (of each standard, control and samples) were added to coated wells and incubated at room temperature with shaking, and then washed. Next, 200 µl of anti s-TNF-R1, TNF, IL-6 or CRP conjugate solution were added to all wells and the plate was incubated at room temperature on a horizontal shaker set at 7000 rpm. Following this, the plate was washed three times by aspirate and 400 µl of wash buffer was added to each well. Finally, 50 µl of chromogenic solution was added to all wells after which plates was incubated at room temperature in the dark for 30 min, after which 200 µl of stop solution was added to each well. Plates were read by an ELISA machine (Molecular Device, UK) and observing the absorbance of the plate at 450 nm within 30 min.

2.2. PAX gene to extract mRNA

PAX gene Blood RNA kits were used for purification of total RNA from 2.5 ml whole blood collected in a PAX gene Blood RNA Tube (BRT) for RT-PCR.

2.3. Procedure for isolation of mRNA from the samples to study the mutations in sTNF receptor 1

The BRT was centrifuged to pellet nucleic acids which were then washed and resuspended. Pellets were incubated in optimized buffers together with proteinase K (PK) to allow protein digestion. An additional centrifugation through the PAX gene shedder spin column (PSC) was carried out to homogenize the cell lysate and to remove residual cell debris. The supernatant of the flow-through fraction was transferred to a fresh microcentrifuge tube and ethanol was added to adjust binding conditions. The lysate was applied to a PAX gene RNA spin column (PRC) and, during a brief centrifugation, RNA was selectively bound to the PAX gene silica-gel membrane whilst contaminants passed through. Remaining contaminants were removed in several efficient wash steps. Between the first and the second wash steps, the membrane was treated with DNase I (RNFD) to remove any trace amount of bound DNA. After the washes, RNA was eluted in buffer and heat-denatured.

2.4. One step RT-PCR (QIAGEN)

This amplifies the DNA, which was formed from mRNA by reverse transcriptase. Two primers were designed for each mutation using oligonucleotide primer pair's specific for exons 3–4 of *TNFRSF1A*. Specifically because the aim was to focus on two mutations, R92Q and P46L, two primer pairs were designed to detect the R92Q in exon 4, whilst 3 primer pairs were designed to detect the P46L in exon 3 (Table 1). Two PCR runs were carried out for each sample (template).

One microlitre of enzyme mix (reverse transcriptase and Taq DNA polymerase), 1 μl of buffer, 1 μl of dNTP mix and 1 μl of samples (mRNA template from PAX gene tube) were added into 46 μl of RNase free water. This mix was added together in one microfuge tube (master mix). 50 μl from master mix of each sample were added to the PCR tube. The samples were transferred to PCR machine (Techne, UK) that had been preheated to 75 °C, and run on a PCR reaction programme (94 °C, for 50 s, 50 °C for 1 min, 72 °C for 1 min) × 35, and 72 °C for 5 min. One aliquot, used as a negative control, was left with no template. Five microlitres of PCR products were used for loading and gel electrophoresis.

2.5. Gel electrophoresis and transillumination

Gel electrophoresis was performed to confirm the appearance of the bands of the PCR products at 50 base pair (bp) length after the PCR reaction. Five microlitres of PCR product were added to 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficol in water). The agarose gel was prepared (2% agarose in TBE buffer with ethidium bromide 0.2 μl/ml). The TBE buffer was prepared (90 mM Tris, 90 mM borate and 2 mM EDTA pH 8.3) and the gel was placed in the gel electrophoresis tank, immersed with TBE buffer, and then loaded with the samples. The loaded gel was run at 80V for 45 min. The loaded gel was observed under UV-trans-illumination and photographed.

2.6. Sequencing – pre-sequencing enzyme treatment

In order to prepare the samples for the sequencing reaction, 0.5 μl of ExonucleaseI (10 U/μl) and 1 μl of SAP (shrimp alkaline phosphatase) (1 U/μl) were added to 5 μl of the PCR product. This mix was run on the enzyme treatment programme (37 °C for 15 min, 80 °C for 15 min) in a Techne PCR machine.

2.7. Sequencing reaction

One microlitre of enzyme treated PCR product was added to 4 μl of Big Dye terminator, 0.5 μl of primer and 5 μl of sterile de-ionized water. This mix was run on a sequencing reaction programme (96 °C for 30 s, 50 °C for 15 s, 60 °C for 4 min) × 25, and then 28 °C for 1 min.

2.8. Pre-sequencing cleaning

Ten microlitres of sequencing reaction product were purified by adding 124 μl of sequence cleaning mixture (ethanol:water:3 M sodium acetate pH 4.6–5.2 in a dilution 25:5:1). The samples were centrifuged at 4 °C and 20,000g for 15 min. The supernatant was removed and the pellet cleaned by 300 μl of 70% ethanol. The samples were centrifuged. The supernatant was removed and the pellet cleaned by ethanol and re-centrifuged. The supernatant was removed and the samples dried for the sequencing cycle. The cleaned samples

Primers	Sequence (in the 5′ to 3′ direction)
TNFRSF1A-R92Q-1-F	GCT GCT CCA AAT GCC GAA AG
TNFRSF1A-R92Q-1-R	GGT TTT CAC TCC AAT AAT GCC G
TNFRSF1A-R92Q-2-F	GAT GAC ATC CAC CAG AAG CGC ATG G
TNFRSF1A-R92Q-2-R	CCA TGC GCT TCT GGT GGA TGT CAT C
TNFRSF1A-P46L-1-F	CTT GTG TTC TCA CCC GCA G
TNFRSF1A-P46L-1-R	CTT TCG GCA TTT GGA GCA GC
TNFRSF1A-P46L-2-F	GGG CTC CTT CCT TGT GTT CT
TNFRSF1A-P46L-2-R	CTG ACT CTC CTG CCT GTG C
TNFRSF1A-P46L-3-F	TGT GTT CTC ACC CGC AGC CTA AC
TNFRSF1A-P46L-3-R	CCT GTG CAC ACT CAC CCT TTC

Table 1 Sequences of the primers.

were processed through the sequencing cycle by mixing them with POP7 polymer (ABI) and injecting them into sequencer machine (Applied Biosystem ABI, with 4 channels).

2.9. Flow cytometry analysis

Using a BD FACS Calibur by a direct immunofluorescence technique, flow cytometry analysis was used to assess the following cell surface markers; CD3, CD4, CD8, CD25 (Reg T), CD56 (NK), CD69 (gamma/delta T), CD14, CD16, CD19, ICAM-1, VLA-4, and L-selectin. Analyses were performed for all samples. This analysis was conducted on whole blood, and the lysis of erythrocytes was achieved using FACS lysing solution (10×) (BD catalogue No. 356457). The values were expressed as percentage and their absolute counts were calculated. All CDs were run along with negative isotypic controls (IgG1/IgG2).

2.10. Statistical analysis

Statistical analysis was carried out using SPSS version 16. Quantitative data were described in the form of mean ± SD, median and range. Comparisons between groups were performed using an independent t test, the Mann–Whitney U test was used for data that were not normally distributed. A $p > 0.05$ was considered not to be statistically significant whilst a $p < 0.05$ was considered significant. A statistical analysis of the sequencer and flow cytometry machines were carried out by their own software.

3. Results

3.1. RT-PCR

In normal individuals, 8 of 500 samples (1.6%) expressed both R92Q and P46L mutations. In contrast, of the 100 patients with atherosclerosis, 34% expressed both the R92Q and P46L mutations. In addition, 42% of the 100 patients with rheumatoid arthritis expressed both mutations (Fig. 1).
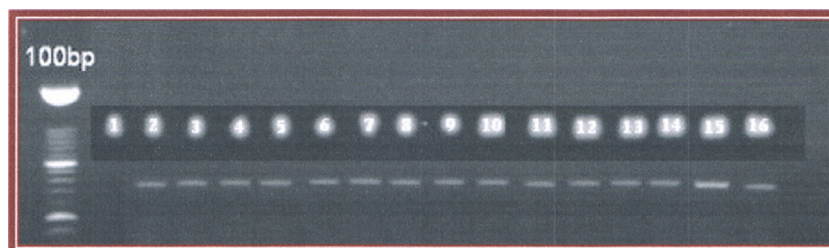
3.2. Levels of IL-6, sTNFR1, TNF and CRP in different groups studied

A significant increase in IL-6 was noted in the group of patients with rheumatoid arthritis when compared to the controls $p < 0.001$. Conversely, IL-6 in patients with atherosclerosis showed no significant difference. STNFR1 showed significant decreases in both atherosclerosis and rheumatoid arthritis groups when compared to controls ($p < 0.05$). CRP showed significant increases in both RA & AS groups when compared to the control group ($p < 0.05$). sTNF showed highly significant increases in both atherosclerosis and rheumatoid arthritis groups when compared to controls ($p < 0.001$). IL-6 and sTNFR1 in both atherosclerosis and rheumatoid arthritis groups had mutations and showed significant decreases when compared to controls ($p < 0.05$). 3/8 of those with mutations showed low values of sTNFRSF1A. The CRP and TNF in both atherosclerosis and rheumatoid arthritis groups showed significant increases when compared to controls ($p < 0.05$). Also, 2 healthy individuals with mutations showed high values of CRP (data not shown).

3.3. Cell surface marker analysis by flow cytometry

We found significantly higher levels of cell markers in patients with rheumatoid arthritis compared with normal individuals both in terms of percentage and absolute values ($p < 0.05$) (Table 2) (Fig. 2). However, no significant differences between cell markers of normal individuals and patients with atherosclerosis were noted (Table 3).

4. Discussion

The two TNFRSF1A variants, R92Q and P46L, were initially identified in TRAPS patients. These are variants which occur only in a small percentage of the general population, and who have no symptoms of TRAPS, and can thus be regarded as low penetrance allelic polymorphisms. In the current study, we found that 8 out of 500 normal individuals (1.6%)

Figure 1 RT-PCR of samples of normal individuals and of patients with atherosclerosis and with rheumatoid arthritis (sample 1 is a blank).

Table 2 Laboratory parameters in different groups studied.

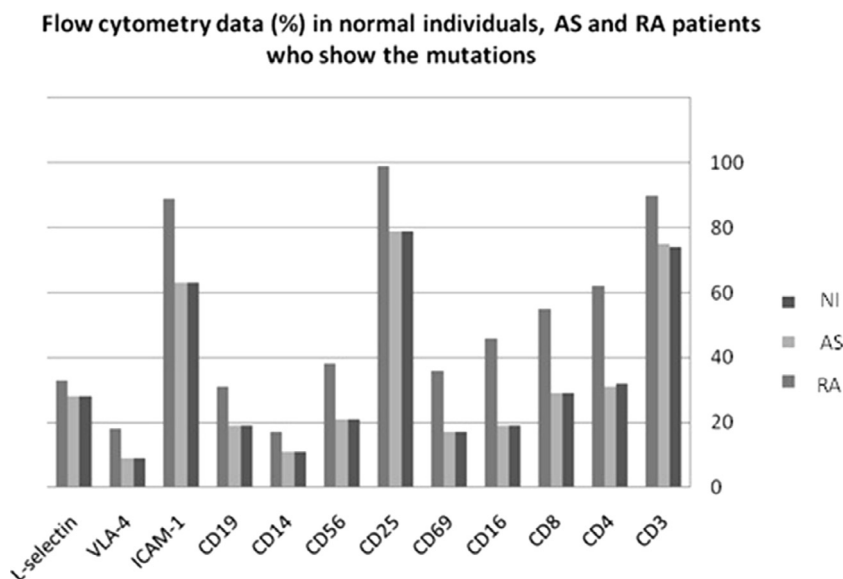| Marker	Control group median (range)	Rheumatoid arthritis median (range)	Atherosclerosis median (range)
IL-6 pg/ml	5 (0.5–16.6)	34.2 (3.5–233) $p < 0.005$	4.5 (0.5–16) $p > 0.05$
sTNFR1 ng/ml	0.4 (0.8–1.9)	0.1 (0–0.3) $p < 0.05$	0.1 (0–0.2) $p < 0.05$
CRP mg/dl	2 (1–4)	11.1 (2–37) $p < 0.05$	10 (3–35) $p < 0.05$
sTNF pg/ml	3.2 (0–7.3)	10 (6–45) $p < 0.001$	5 (2–9) $p < 0.001$

Figure 2 Probability data of cell markers in normal individuals and patients with atherosclerosis and rheumatoid arthritis who express mutations in TNFR1 (R92Q and P46L).

expressed both the mutations R92Q and P46L. In contrast, amongst patients 34% of patients with atherosclerosis and 42% of patients with rheumatoid arthritis expressed both these mutations. These findings lead us to conclude that the presence of TRAPS disease is likely to exist amongst the Saudi population.

In this study, TNFRSF1A was significantly decreased in patients with atherosclerosis and rheumatoid arthritis who had mutations (as compared to healthy individuals). In addition, 3/8 of normal individuals with mutations showed low values of sTNFRSF1A. It has been suggested that low levels of sTNFRSF1A may be the result of defective cleavage of the mutant receptor from the surface of activated cells (McDermott et al., 1999; Galon et al., 2000). Accordingly, it was suggested that the abnormally low levels of sTNFRSF1A circulating in patients with TRAPS may be insufficient to neutralize circulating TNF effectively, and which may therefore result in an exaggerated inflammatory response when the blood level of TNF becomes elevated.

When stimulated with TNF, cells from TRAPS patients actually tend to produce less IL-6 than those of normal individuals, a finding consistent with our own observations because the patients with mutations (atherosclerosis and rheumatoid arthritis) in our study showed significant decreases of IL-6 when compared to the control group (Aganna et al., 2003). However, Nowlan et al. (2006), reported IL-6 and IL-8 levels to be significantly elevated in C33Y TRAPS patients and more elevated in patients with rheumatoid arthritis. Significantly high levels of CRP have been found in patients with mutations who have atherosclerosis and rheumatoid arthritis, as well as in 2 healthy individuals with mutations. These findings are in line with previous work which reported that plasma laboratory findings in TRAPS were associated with typical inflammatory episodes (Aganna et al., 2003).

We found no significant difference between cell markers of normal individuals and patients with atherosclerosis (Table 2) (Fig. 2). In contrast, we noted significantly high values of cell

Table 3 Descriptive data of cell markers in normal individuals and patients (atherosclerosis and rheumatoid arthritis) who were positive to mutations (R92Q and P46L).

Cell markers (% and absolute)	Mean ± SD of normal individuals	Mean ± SD of AS	Mean ± SD of RA
CD3%	73.6 ± 11.5	74.5 ± 15.4	90 ± 10[**]
CD3 abs.	2371.8 ± 768	2330 ± 690	3600 ± 846[**]
CD4%	31.5 ± 10	31 ± 13	62 ± 18[**]
CD4 abs.	998 ± 402	1010 ± 554	2080 ± 654[**]
CD8%	28.6 ± 10.4	28.5 ± 9.8	55 ± 15.5[**]
CD8 abs.	909 ± 412	930 ± 510	1725 ± 496[**]
CD4/8 Ratio	1.7 ± 0.5	1 ± 0.4	2.5 ± 0.9[**]
CD16%	18.9 ± 7.9	17 ± 7.1	46 ± 13.3[**]
CD16 abs.	609.8 ± 326	520.6 ± 243	1200 ± 467[**]
CD69%	16.5 ± 7.5	15 ± 6.8	36 ± 12.3[**]
CD69 abs.	541.0 ± 292	455 ± 280	1320 ± 410[**]
CD25%	79.1 ± 13.4	75.3 ± 12.2	99 ± 1[**]
CD25 abs	2582 ± 880	2875 ± 790	3488 ± 1119[**]
CD56%	20.6 ± 10.3	23.1 ± 11.1	38.2 ± 14.4[**]
CD56 abs	743 ± 310	718 ± 348	1149 ± 401[**]
CD14%	11.3 ± 4.3	13.3 ± 5.1	16.5 ± 5.2[**]
CD14 abs	414.6 ± 215	312.6 ± 186	690 ± 254[**]
CD19%	18.6 ± 8	22.1 ± 7.8	31.2 ± 11.6[**]
CD19 abs	655.3 ± 302	601 ± 280	987 ± 298[**]
ICAM-1%	63.4 ± 9.6	62.3 ± 8.8	88.9 ± 10.1[**]
ICAM-1 abs	1987 ± 434	2258 ± 486	2687 ± 523[**]
VLA-4%	9.1 ± 2.2	7.9 ± 1.8	18.2 ± 3.9[**]
VLA-4 abs	313 ± 41	352 ± 48	475 ± 62[**]
L-selectin%	28 ± 6.7	27 ± 6.8	33 ± 7.9[**]
L-selectin abs	1267 ± 261	1251 ± 281	1612 ± 301[**]

[**] $p < 0.05$.

markers in patients with rheumatoid arthritis compared to normal individuals, and we found that these data correlated with the presence of acute or chronic inflammation in patients

with rheumatoid arthritis, where elevated migration of inflammatory cells would be expected.

Our results, especially when taken together with other research that has supported the association between TNF polymorphism and rheumatoid arthritis (Al-Mohaya et al., 2015; AlOkaily et al., 2015; Nasr et al., 2014; Alkhuriji et al., 2013; Al-Harthi et al., 2013 Jul 8; Al-Rayes et al., 2011), support the notion that proinflammatory cytokines and markers are potent inducers of inflammation, and further, that elevated levels of these cytokines, being frequently associated with the activation of macrophages, thereby influence the severity of inflammatory responses. We suggest that these results might well have prognostic utility for future clinical studies, and that TNFR polymorphism may serve as a guideline in determining the clinical response to anti-TNF-α therapy.

In conclusion, these data may reasonably anticipate the presence of TRAPS disease (low penetrance mutations) amongst the Saudi population although further studies are needed to confirm these results.

Acknowledgement

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