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A PCR-RFLP method for detection of the *LNPEP* encoding human insulin-regulated aminopeptidase (IRAP) rs4869317 polymorphism

María Jesús Ramírez-Expósito[1], José Manuel Martínez-Martos[1], Teresa Palomeque[2] & Pedro Lorite[2]

[1]Experimental & Clinical Physiopathology Research Group, Department of Health Sciences, University of Jaén, 23071 Jaén & [2]Unit of Genetics, Department of Experimental Biology, University of Jaén, 23071, Jaén, Spain

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Background & objectives: **Insulin regulated aminopeptidase (IRAP) has been related to certain pathologies such as breast cancer, Alzheimer´s disease and septic shock. IRAP is encoded by the leucyl/cystinyl aminopeptidase (*LNPEP*) gene. The genetic variation in the *LNPEP* gene has been analyzed in relation with the mortality and vasopressin clearance in septic shock. The *LNPEP* rs4869317 SNP (single nucleotide polymorphism) was the most significantly associated SNP with vasopressinase activity, being TT genotype associated with increased mortality. The objective of the present study was to develop a simple method to allow a quick and affordable genotyping for the rs4869317 SNP of *LNPEP* gene.**

Methods: **Blood DNA samples were obtained from randomly selected healthy volunteers (n=28). A pair of primers was designed to amplify an 834 bp region of the *LNPEP* gene containing the rs4869317 SNP. The two alleles (T or A) were detected by digestion of the PCR products with the *Pac*I restriction endonuclease. This enzyme only cuts the PCR products when the adenine is present in the SNP.**

Results: **All individuals showed RFPL (restriction fragment length polymorphism) fragments for the expected genotypes (TT, TA or AA). The methodology was validated by sequencing of the amplified DNAs from several 'T/T' and 'A/A' homozygotes and 'T/A' heterozygotes. The results from both methods showed agreement.**

Interpretation & conclusions: **The PCR-RFLP is a simple and reliable method that allows a quick genotyping for the rs4869317 SNP of *LNPEP* gene. The study of this polymorphism could be useful in future investigations to analyze the role of genetic variants of IRAP in several physiological/pathological conditions.**

Key words Human - insulin-regulated aminopeptidase - IRAP - *LNPEP* - PCR-RFLP - single nucleotide polymorphism

Insulin-regulated aminopeptidase (IRAP) is an enzyme with a broad tissue distribution[1,2]. The physiological role of IRAP is not well understood, but it has been described as the protein that accompanies the glucose transporter GLUT4 to the plasma membrane following insulin stimulation to facilitate glucose uptake[3,4] and also as the angiotensin IV (AngIV) receptor subtype AT_4 in the renin-angiotensin system (RAS)[5,6], which has been also identified as the hepatocyte growth factor (HGF)/c-Met receptor[7].

Also, IRAP has been involved in the generation of antigenic peptides for cross-presentation in endosomal compartments[8-10] in major histocompatibility class (MHC) class I antigen processing. However, the best known is the IRAP role as regulator of oxytocin and vasopressin levels and as AT_4/HGF/c-Met receptor for AngIV. IRAP has been confirmed as a regulator of oxytocin functions in the prostate[11]. It has been also involved in pathologies such as breast cancer and Alzheimer´s disease[12-14].

Regarding its molecular characteristic in humans, the IRAP enzyme is encoded by the *LNPEP* gene[1]. The *LNPEP* gene consists of 18 exons (17 introns) on chromosome 5q15 covering 94 kb of genomic DNA and has two major transcript variants[15]. The *LNPEP* rs4869317 SNP (single nucleotide polymorphism) was found to be the most significantly associated SNP with the vasopressinase activity[15]. The rs4869317 SNP is located in the intron 1 of the transcript variant 1 (GenBank Acc. No. NM_005575.2, *http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4869317)*. This SNP is located at position 4605876 in the *Homo sapiens* chromosome 5 genomic contig GRCh37.p10 (Genbank Acc. No. NT_034772.6) and it is generated by a T/A transversion. In patients with septic shock the T/T genotype was associated with increased 28-day mortality compared with patients who had the AT or AA genotype probably due to altered clearance of vasopressin[15].

Due to the putative importance of *LNPEP* gene variants in the different physiological/pathological conditions in which IRAP was involved, we undertook this study to develop a simple method for quick genotyping of rs4869317 SNP that could be useful in future investigations to analyze the role of the *LNPEP* gene polymorphism in other human pathologies.

Material & Methods

This study was conducted in the department of Health Sciences and Experimental Biology, University of Jaén, Jaén, Spain during period of study. Blood samples were obtained by venipuncture in vacutainer tubes with heparin as additive, from 28 randomly selected healthy volunteers, graduate students and personal staff of the University. Genomic DNA was extracted from 200 µl aliquots of blood using NucleoSpin® Blood (MACHEREY-NAGEL, Germany) according to the manufacturer's procedure. The study was conducted during 2013 and approved by the university bioethics committee, and all volunteers signed an informed consent form.

Genotyping for this SNP was performed by a direct PCR-based RFLP (restriction fragment length polymorphism) method[16]. The presence of adenine in the SNP position resulting in the presence of a target for the *Pac*I restriction endonuclease (TTAATTAA) that is absent in the 'T' allele (TTTATTAA). An 834 bp length fragment containing the SNP was amplified by PCR. For this purpose we designed two primers around the SNP, LNPEP-F: 5´- AGTAGATGTGGTTACTTGGG and LNPEP-R2: 5´ - AGGACATGTAGGGCAATATC (Fig. a). PCRs were carried out using the following cycling profile: initial denaturation at 94°C (4 min), 40 cycles at 94°C (30 sec), 58.5°C (1 min), 72°C (1 min), with a final elongation step of 72°C for 10 min. Reactions were set up in a 25 µl mixture containing 25-50 ng of genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each primer and 1 U of BIOTAQ™ DNA Polymerase (BIOLINE, UK). PCR products were analyzed by electrophoresis in 2 per cent agarose gels stained with ethidium bromide; 5 µl of the PCR reaction mixture was used to test the DNA amplification. The remaining 20 µl was directly digested by addition of 5 units of *Pac*I (New England Biolabs, USA) in a 40 µl of reaction mix with the 1X CutSmart™ Buffer (supplied with the restriction enzyme). This process did not require purification of PCR products. Digested DNA was analyzed by electrophoresis in 2 per cent agarose gels stained with ethidium bromide. As internal control in the PCR products digestion a plasmid carrying only a target for *Pac*I was used. Only digestions with totally linearized plasmid were considered.

Results & Discussion

PCR amplifications resulted in a clear and unique amplicon with the predicted size from the designed primers (834 bp). After incubation with *Pac*I of a 'T/T' homozygote, the amplified bands were not affected since the amplicon lacked *Pac*I targets (Fig. b, lanes 4,5). By the other way in 'A/A' homozygote the amplified 834-bp fragment was cleaved into two fragments of 331 and 503 bp after digestion with *Pac*I (Fig. b, lane 7). Three bands of 834, 503 and 331 bp in length were visible after electrophoresis when the heterozygote 'T/A' DNA was amplified and digested with *Pac*I (Fig. b, lanes 3, 6, 8).

To test the validity of the PCR-RFLP method developed in this study, amplified DNAs from several 'T/T' and 'A/A' homozygotes and from several 'T/A'

heterozygotes were sequenced using the LNPEP-F primer. Sequencing of the *LNPEP* products confirmed the genotype estimated by PCR-RFLP methods (Fig. c). However, in some of the sequenced individuals the existence of other polymorphisms by insertion/deletion makes it impossible to determine the sequence around the SNP since overlapping sequencing fluorograms are generated. In these cases it was necessary to use the LNPEP-R2 primer for sequencing the amplified fragment in the other sense to confirm the genotype. Among the 28 individuals, there were 16 'T/A', 10 'T/T' and two 'A/A', which gave allelic frequencies of 0.64 for 'T' and 0.36 for 'A'.

At present, there are numerous technologies available for SNP genotyping[17]. Its disadvantages are associated with genotyping methods, which include significant investments of expensive probes and/or high cost equipment, that are usually prohibitively expensive for the majority of those affected, particularly in the third world countries and the developing countries. In comparison to alternative methods the number of samples that can be analyzed simultaneously by PCR-RFLP is low. However, its advantage is that it does not require large infrastructure and can be carried out in laboratories with basic equipment, so must be sufficiently accessible to be introduced in the most common clinical laboratories.

An other simple method to analyze a SNP, also based in conventional PCR, is the AS-PCR (allele-specific PCR), that requires similar equipment and cost as the PCR-RFLP[18]. Each SNP is detected using an allele-specific PCR primer with the terminal nucleotide of the primer corresponding with the specific SNP site. The AS-PCR is based on the inefficient extension of the polymerase when the 3´ end is not perfectly complemented to the template. However, this technique has the problem of potential errors associated with non-specific primer binding, especially in the detection of 'AT' heterozygous.

Our method also implies the use of an internal control in the PCR products digestion. We have used a plasmid carrying only a target for *Pac*I, but any other DNA having a single target for this enzyme could be

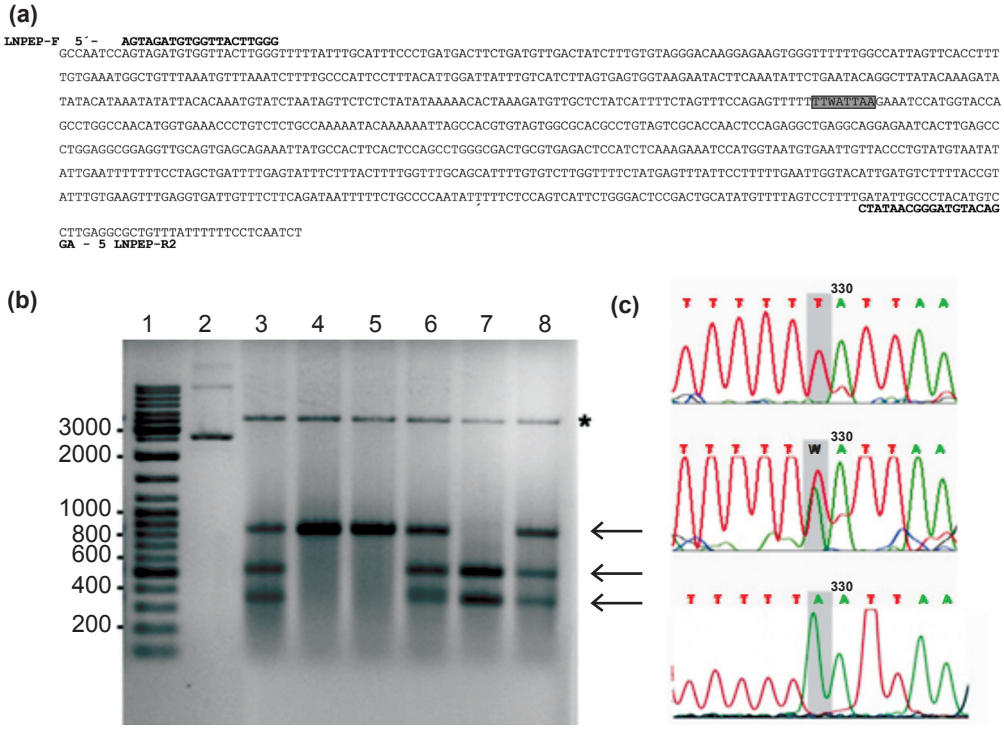

Figure. (a) DNA sequence of the amplified *LNPEP* gene fragment. The sequences of the primers (LNPEP-F and LNPEP-R2) are shown as well the target site for the *Pac*I endonuclease in a grey box (W=A or T). **(b)** Electrophoresis of the *Pac*I digested PCR products of the *LNPE* gene on a 2 per cent agarose gel, showing the different banding patterns obtained for each genotype. Lane 1 = 100 bp DNA size marker. Lane 2= undigested plasmid used as internal control of the *Pac*I digestion.[T/T] homozygote (lanes 4, 5), [A/A] homozygote (lane 7), [T/A] heterozygote (lanes 3, 6, 8). Asterisk marks linearized form of the plasmid used as internal control. **(c)** Sequencing fluorograms of the *LNPEP* products showing the region around the single nucleotide polymorphism in [T/T], [T/A] and [A/A] individuals, respectively.

used. The use of an internal control avoids genotyping mistakes by partial digestion of the PCR products by the restriction endonuclease. Another advantage is that the PCR buffer does not seem to interfere with the activity of the *Pac*I endonuclease. This means that previous purification of the PCR product is not necessary to carry out the RFLP, making the process quicker and cheaper. Genotyping by PCR-RFLP can be used to analyze other SNPs. The only limitation is that the SNP is part of the target for a restriction endonuclease so that the endonuclease cuts in one allele but not in the other.

In conclusion, our findings showed a simple and reliable method to analyze a SNP of the *LNPEP* gene encoding for IRAP protein. This will allow a rapid analysis of gene variants in the different physiological/ pathological conditions in which IRAP is involved.

Conflicts of Interest: None.

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Reprint requests: Dr Pedro Lorite, Unit of Genetics. Department of Experimental Biology.
University of Jaén. 23071, Jaén. Spain
e-mail: plorite@ujaen.es