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IN SILICO DESIGN AND VALIDATION OF A NOVEL PCR-RFLP ASSAY FOR GPRC6A RS2247911 POLYMORPHISM INVOLVED IN MALE INFERTILITY

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Male infertility is a prevalent disorder affecting approximately 7% of the male population, at least 2,000 genes are involved in spermatogenesis, availability of genetic investigations is a challenge in developing countries, GPRC6A, G-protein coupled receptor, activated by Osteocalcin (OCN)an osteoblast secreted hormone, is a master regulator of a complex metabolic and reproductive networks. rs2247911 in GPRC6A plays a role in male fertility and glucose metabolism, Although DNA sequencing is regarded the golden standard tool for genotyping, it is less affordable in developing world laboratories, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay is an inexpensive and highly valuable tool for genotyping, this study aimed to develop (PCR-RFLP) method for assessment of rs2247911 polymorphism.10% of male subjects genotyped previously by sequencing were genotyped the by PCR-RFLP method based on Geneious® bioinformatics tool suggested restriction enzyme (ScrFI), PCR-RFLP 100% correctly identified genotypes. we present for the first time an accurate and inexpensive method for genotyping rs2247911.

***Keywords**: GPRC6A, PCR-RFLP, Sequencing, Osteocalcin, testis failure.*

INTRODUCTION

Male Infertility multifactorial pathological condition affecting approximately 7% of the male population, at least 2,000 genes are involved in spermatogenesis, about 20% of cases are ascribed to genetic factors, such as monogenic and chromosomal defects[1]. The GPCR, class C, group 6, subtype A (GPRC6A), is a member of G-protein coupled receptors, first deorphanized in 2004 using the protein sequences of class C GPCRs as a query. The GPRC6A gene is located on chromosome 6q25, composed of 6 exons and 7 introns[٢], the most abundant variant of GPRC6A corresponds to a 926 amino-acids protein. It is characterized by an uncommon long amino-terminal extracellular domain, a 7-transmembrane (7TM) domain, with three extra- and intracellular loops, and an intracellular C-terminal. The mature receptor is a homodimer of two proteins interacting through a large hydrophobic interface[٢]. GPRC6A transcripts have been detected in several tissues, including brain, lung, liver, heart, kidney, skeletal muscle, pancreas, placenta, spleen, ovary, testis, leukocytes, monocytes and adipocytes[4&5].

The recognized ligands of GPRC6A, validated through genetic approaches in animal models, are the bone protein Osteocalcin in its undercarboxylated form (ucOCN) and Testosterone(T), but other possible ligands have been discovered in vitro[6&7].it has been reported that the deletion of GPRC6A gene in male mice results in feminization, including reduced circulating T levels and testis weight[8]. Conditional deletion of GPRC6A gene in

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Leydig cells associated with a conserved ucOCN-related hypogonadism and decreased sperm counts[9]. The similar phenotypes were displayed by *GPRC6A*$^{-/-}$ and *OC*$^{-/-}$ mice and the additive phenotypic effects in compound *GPRC6A*$^{+/-}$*/Ocn*$^{+/-}$ mice, support the presence an endocrine network in mice[10].

These observations were later supported by the findings of Oury et.al, where the involvement of GPRC6A in human male reproduction was addressed by the identification of two individuals harboring a rare heterozygous missense variant in the extracellular domain of GPRC6A (F464Y). This variant prevented the receptor from localizing to the cell membrane and blunted its signaling pathway resulting in hypogonadism and infertility[11].

DNA-based analyses for Genotyping of rs2247911 was investigated via direct sequencing which is considered the gold standard for the detection of genetic variations. However, this technology is labor intensive, requiring dedicated instruments and personnel, unavailable in all fertility centers. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a technique used to distinguish homologous DNA sequences detected via fragment length difference after DNA digestion by specific endonucleases[12]. The lack of facilities for DNA sequencing in developing countries led our group to set up a restriction enzyme-based method that can recognize the genotype as alternative method of sequencing to investigate GPRC6A rs2247911 by validation of bioinformatic based suggestion.

Subjects and methods

The current comparative study was approved by the Ethics Committee of Damascus University, (N.18FP) and included randomly chosen 10% samples out of 190 male subjects genotyped by sequencing technique in a previous study of our group[13], attending the Orient Hospital for Assisted Reproduction Treatment for fertility problems were recruited and provided an informed consent. Participants provided 5ml venous blood by venipuncture, using EDTA coated tubes for whole blood collection and stored at -80°C until use.

Genetic screening for GPRC6A rs2274911 polymorphism via Sequencing

Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer's protocol. The oligonucleotide primers used for the analysis of the polymorphism rs2274911 (c.117130704; G→A; Pro91Ser) were adapted from previous studies (forward 5'-AATGAGATACAGCCATGTCCA-3' reverse5'-GCAATGTTTGGAGGTAGCAC3'[14,15].
Amplification was performed in a 25 µL reaction volume containing 100 ng genomic DNA, 1.5 U AmpliTaq Gold (Applied Biosystems), 2.5X PCR Gold buffer (Applied Biosystems), DMSO 100%, 2 µM of each dNTP (Amersham Biosciences) and a mix of forward and reverse primers (1 µmol/L each). Samples were denatured at 94°C for 10', amplified for 35 cycles of 94°C for 60", 58°C for 60", 72°C for 60", and then extended at 72°C for 10', using thermocycler GeneAMP PCR System 2700 (Applied Biosystems). PCR products were then purified and analyzed by direct sequencing (ABI 3130xl Genetic Analyzer, Thermofisher). Sequence analysis was performed using the online free gap4 software of the Staden Package.

Genetic screening for GPRC6A rs2274911 polymorphism via PCR-RFLP

Restriction endonuclease used to investigate the genotype was ScrFI (New England Biolabs, Inc.) (NEB)upon suggestion of Geneious® software for Bioinformatics, 10µL products of PCR amplicon was digested with 2 µL of restriction endonucleases ScrFI 5000 units, 2 µL buffer and 6 µl nuclease free water, for 4 hours digestion time at 37 °C, for fragments documentation samples were electrophoresed on a 2.5% agarose gel in 1×TAE buffer, stained with ethidium bromide, and visualized under UV light, a DNA ladder 100bp Thermo scientific™ was used as a marker.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 (SPSS Inc., Chicago, IL). Data are expressed as frequencies and percentage for genotype, χ^2 test Goodness of fit was performed

to examine differences in frequencies of genotypes between experimented method (RFLP) and reference method(sequencing).

RESULTS AND DISCUSSION

Results

Polymerase Chain Reaction PCR yielded an amplicon of 400bp, of rs2247911 (c.117130704 G> A, Pro91Ser). genotypes were GG, AA homozygotes and AG heterozygote detected by sequencing technique, chromatogram is illustrated in (figure1), Digestion by ScrFI in silico yielded two fragment (59,341 bp) when the nucleotide was A and three fragments (59,196,145 bp) when the nucleotide was G (figure 2),when implemented in vitro, gel electrophoresis displayed an identical fragment length to the ones proposed in silico (figure 3), sequencing based frequencies of genotype for chosen samples was 47.3%,31.5%,21% for AA, AG, GG respectively, when examined by χ^2 goodness of fit test to compare genotype frequencies obtained by sequencing as a reference method and RFLP as an experimental method, complete concordance was shown between the two methods P= 1, (table 1).

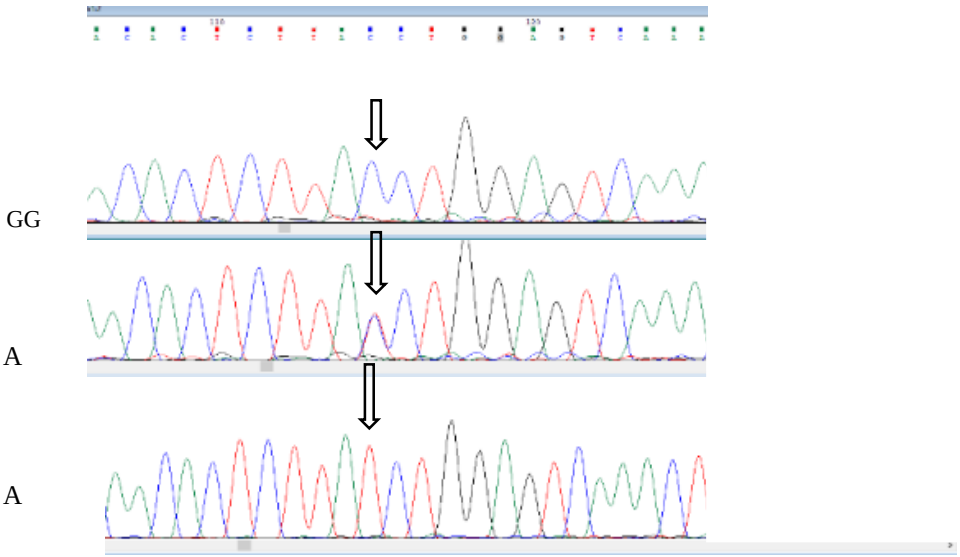

Fig. 1: Sequencing chromatogram of rs2247911, arrows show the polymorphic site in GG, AG, AA genotypes .

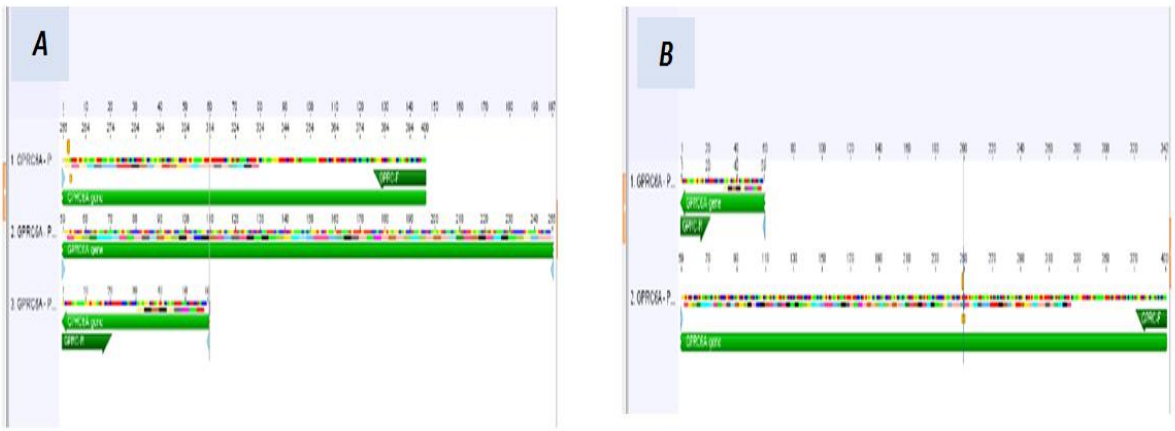

Fig.2: In silico fragments produced by ScrFI digestion, ***A*** three fragments when the nucleotide is G, ***B*** Two fragments when the nucleotide is A.

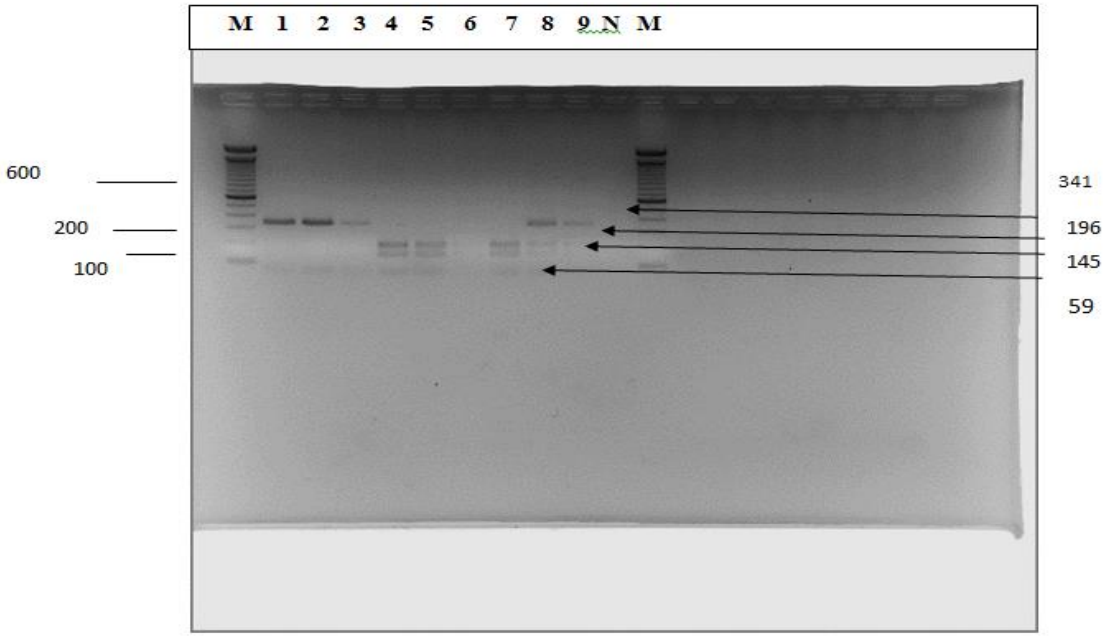

Fig. 3: Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) electrophoresis results of GPRC6A rs2247911 polymorphism, M marker 100bp, N negative, lane 1-3 are AA genotype, lane 4-7 are GG genotype, lane 8-9 are AG genotype, 2.5% agarose was used.

Table 1: Frequencies of genotype detected by the two methods 19 samples.

Genotype (Sequencing)	Frequency	Genotype (PCR-RFLP)	Frequency
AA	9/19(47.3%)	AA	9/19(47.3%)
AG	6/19(31.5%)	AG	6/19(31.5%)
GG	4/19(21 %)	GG	4/19(21%)
P=1 goodness of fit is 100%, data are presented as n/N%			

Discussion

Infertility in developing countries was estimated to affect 3.5–16.7% of population[16], reaching 30% in some developing regions[17], male infertility, contributes to more than half of all cases of childlessness, in the Middle East region 60–70% of couples male factor is included as one of multiple factors of infertility[18], this is particularly problematic in this area where fatherhood is equivalent to manhood[19]. This may lead to stigma of the affected male resulting in higher rates of depression[20]. these countries encounter a deficiency in facilities for infertility diagnosis and treatment and lack of trained personnel, essential infrastructure and equipment[21],

Reproductive healthcare systems should include accessible infertility diagnosis and treatment and simplification of the diagnostic and therapeutic procedures would minimize the complication rate[22], The World Health Organization (WHO) has recommended that infertility to be considered as global health problem and has encouraged adaptation of assisted reproductive technologies (ARTs) for poor-resource countries; stating "research should be directed towards finding effective, low-cost solutions to infertility and this exploration should extend to ART"[23]. In this contingent situation, adapting very informative and non-expensive investigation methods without losing accuracy, can be a successful

approach. It is the specific case of genetic screening of *GPRC6A*, a receptor involved in male fertility as a component of a hormonal access of OCN-GPRC6A that induces testosterone biosynthesis promoting the survival and proliferation of the germ cells independently of LH mediated one[24,25]. Investigation of signaling pathways activated by the osteocalcin-GPRC6A had revealed the involvement of CREB mediated pathways stimulating steroidogenesis and T production[26], The involvement of the OC-GPRC6A in the regulation of testis function in humans was first proved by the description of two individuals harboring a rare heterozygous missense variant in the extracellular domain of GPRC6A (F464Y), that prevented the receptor from localizing to the cell membrane and blocked the signaling pathway and was associated with primary testis failure and feature of metabolic syndrome[11]. additional evidence was derived from a genome wide association study where, the coding SNP mostly associated with prostate cancer was the common NS variant rs2274911 (Pro91Ser) in the *GPRC6A* gene, with the minor allele G presenting a protective effect[27] this gene variant was then detected for its involvement of infertility and/or cryptorchidism along with F464Y mutation, by De Toni et al, the risk allele A of rs2247911 *GPRC6A* was associated with increased LH levels and almost doubled the risk of impairment of spermatogenesis. in vitro model showed that the A allele, either in homozygosity or heterozygosity, was associated with a malposition of the receptor on cell membrane and a reduction in downstream phosphorylation of ERK1/2[15], GPRC6A gene subsequently, proved role in metabolic derangements, the risk allele A was associated with increased fasting insulin and HOMA-IR, suggesting that the polymorphism pre-dispose individuals to the development of insulin resistance, regardless of metabolic phenotype[14]. Recently, the role of rs2247911in *GPRC6A* role in testis function was validated on other population, our group provided evidence that genotype of *GPRC6A* is an independent predictor of Testosterone, and the (OC-GPRC6A) axis flanks the classical hypothalamus/pituitary/gonadal in determining serum T levels and the spermatogenic pattern in cohort of Syrian infertile males with testicular failure[13].

In this methodological comparison study, the detection of rs2247911 polymorphism in GPRC6A via PCR–RFLP was implemented to be 100% in concordance with DNA sequencing. The straightforward application of this method has a dramatic effect on the reduction of turnaround time and costs for the identification of clinically important variants and can be applied in resource-limited settings that lack sophisticated equipment, this strategy is implemented as a principle in other polymorphisms and genes for providing a low cost methodology to diagnose critical diseases as *IDH1* Mutations in Gliomas[28]and *IFNL4* rs368234815 Polymorphism in Chronic Hepatitis C[29].

For the first time the RS2247911 genotype in *GPRC6A* is detected via RFLP-PCR an economic method which can fit labs with big number of teste and limited resources.

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تصميم دراسة مجراة حاسوبياً واختبار المصدوقية لمقايسة جديدة للتحري عن التعدد الشكلي rs2247911 في جين GPRC6A المتورط في العقم الذكري

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إن العقم لدى الذكور مرضٌ منتشرٌ وتمثّل التحريات الجينية عنه تحدياً بالنسبة للبلدان النامية، يعد المستقبل GPRC6A المقترن بالبروتين G المفعّل من قبل الأوستيوكالسين منظماً رئيسياً لشبكات استقلابية وتكاثرية، يلعب التعدد الشكلي rs٢٢٤٧٩١١ دوراً في الخصوبة الذكرية واستقلاب الغلوكوز. على الرغم من اعتبار سلسلة الدنا التقنية الذهبية القياسية للتنميط الجيني فإنها أقل توافراً في مختبرات العالم النامي أمّا طريقة(تعدد أشكال أطوال الشدف المقتطعة) فهي تقنية غير مكلفة ذات أهمية كبيرة لهذا فقد هدفت هذه الدراسة إلى تطوير طريقة تنميط جيني معتمدة على تقنية (تعدد أشكال أطوال الشدف المقتطعة) لتحرّي التعدد الشكلي rs٢٢٤٧٩١١ جرى تحرّي النمط الجيني بهذه الطريقة اعتماداً على إنظيم التقييد المقترح من قبل برنامج Geneious® للمعلوماتية الحيوية (ScrFI) وذلك ل١٠% من العينات لذكورٍ كانت قد خضعت مسبقاً للتنميط الجيني بطريقة السَلسَة وقد تمكنت بمطابقة نسبتها ١٠٠% من التعرف على النمط الجيني، نقدمّ هنا وللمرة الأولى طريقة دقيقة غير مكلفة للتنميط الجيني لـ rs٢٢٤٧٩١١ .