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A PILOT STUDY ON THE PREVALENCE AND ASSOCIATION OF GPRC6A SINGLE NUCLEOTIDE POLYMORPHISMS IN SOUTH INDIAN PATIENTS WITH METABOLIC SYNDROME, METABOLIC SYNDROME AND INFERTILITY

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ABSTRACT

Aims: The G protein coupled receptor, class C, GPRC6A is identified to primarily modulate metabolic functions and reproductive health in males. The single nucleotide polymorphisms (SNPs) of GPRC6A rs2274911 and rs143913245 (F464Y) are reported to be associated with metabolic syndrome, infertility. However, there exists a lacuna in the knowledge regarding the incidence, prevalence of these SNPs in the Indian/south Indian population and hence the present study focuses to determine their influence in the metabolic syndrome (MTS), MTS and infertility patients. **Materials and Methods:** Genomic DNA from peripheral blood samples of healthy female/male controls, diabetic/obese male controls and patients with MTS, MTS and infertility were isolated and GPRC6A gene regions corresponding to exon 2, exon 4 were PCR amplified and sequenced. **Results**: Genotyping results revealed that rs2274911, rs6917467 was incident in the assessed population and that the percentage of rs2274911 wild type carriers (GG) significantly differed between the non-diabetic and diabetic groups (GG, $p<0.01$). Further, on comparison with the non-diabetic patients with MTS, the diabetic patients with MTS, diabetic patients with MTS and infertility ($p<0.05$; $p<0.001$ respectively) tended to present a significantly higher percentage of the rs2274911 heterozygous GA alleles. **Conclusion**: The GPRC6A rs2274911 is of frequent incidence in patients exhibiting metabolic abnormalities, infertility. Screening for GPRC6A in diabetic/ MTS, infertility patients will aid better therapeutic regimens and management of these disorders.

KEYWORDS: GPRC6A, SNP, F464Y, MTS (Metabolic Syndrome), Ocn (osteocalcin).

INTRODUCTION

The GPRC6A (G protein-coupled receptor family C, group 6, member A) located on chromosome 6q22.1 comprises of 6 exons, and is recognized as a critical candidate in metabolic regulation, sexual reproduction, hypothalamic-pituitary function and bone formation owing to its multi-ligand binding ability. Further, as a receptor for osteocalcin (Ocn), GPRC6A is also increasingly recognized for its role in glucose metabolism.[1] As such, under-carboxylated osteocalcin released into the circulation from the bone is evidenced to bind to the Ocn-sensing receptors in GPRC6A and regulate glucose metabolism in pancreatic β-cells and promotes insulin sensitivity in a wide variety of tissues such as skeletal muscle, liver and adipocytes.[2,3] On another hand GPRC6A is also evidenced to vitally participate in testosterone production via Ocn mediated functions, non-genomic membrane effects and negative regulation of the aromatization of androgens to estrogens.[4] The ability of GPRC6A to bind to cations such as calcium, zinc is further contributive to its participation in insulin regulation and testosterone production.[5]

Studies examining the functional impact of SNPs in the coding regions of GPRC6A in an Italian population brought to light that the rs2274911 is associated with insulin resistance, obesity, metabolic syndrome, testis failure and infertility. Similarly, the rs143913245 (F464Y) has been observed to be significantly associated with infertility.[6,7,8] Genome wide association studies have also widely implicated the rs2274911 SNP in prostate cancer. Based on such interesting research evidences the present study focused in examining the incidence, association of the widely reported GPRC6A polymorphisms rs2274911 and rs143913245 in MTS, infertility patients with MTS, of south Tamil Nadu, India.

MATERIALS AND METHODS

The present pilot study to determine the incidence and prevalence of the GPRC6A SNPs rs2274911 and rs143913245 were initiated in Alpha Hospital and Research Centre, Alpha health Foundation after obtaining consent from the Institutional Ethical Committee (IEC) and the informed consent from the participating patients. 5 healthy controls (1 female and 4 males) and 11 patients (all males) falling in the age group of 18-48 years, with MTS, and MTS with infertility, were included in the study based on the NCEP ATP III definition. Genomic DNA was isolated from peripheral blood samples of the controls, patients using a Qiagen Blood mini kit (C.No:51104). Assessment of clinical and anthropometric parameters in patients with MTS and infertility ascertained metabolic abnormalities such as dyslipidemia, high BMI, diabetes/hypertension. Patients included in the MTS + infertility group had a history of infertility and were ascertained to exhibit a continued trend for metabolic abnormalities by means of biochemical, clinical evaluation.

The region of interest in GPRC6A encompassing the exon 2 (400bp) that would enable the detection of rs2274911 (NC_000006.12; Pro91Ser) was amplified using the forward primer, 5' AATGAGATACAGCCATGTCCA 3' and reverse primer 5' GCAATGTTTGGAGGTAGCAC 3'. The exon 4 (284bp) region of the GPRC6A gene containing the allele for the F464Y SNP rs143913245 (NC_000006.12; p.Phe464Tyr) was amplified using the forward primer 5'TTGCAACATTTATATTAAGTGCTTATC3'and the reverse primer 5' CAAAACGGCCTACAACAAGG 3'.[8] The PCR conditions included hot start at 94 ºC for 10 minutes, amplified for 35 cycles of denaturation at 94ºC for 1 minute, annealing at 58ºC for 1 minute, elongation at 72 ºC degrees for 1 minute and final extension for at 72 ºC for 10 minutes. PCR products were gel extracted and the concentration, purity of the PCR products were evaluated from A260/A280 ratio using a Nano drop (Thermo Scientific, India). The products were sequenced using direct sequencing strategies (Scigenome/Agrigenome, Kerala) and sequence data were aligned and analyzed using Serial Cloner (2.6.1), NCBI BLAST searches.

Statistical Analysis

Data are presented as mean and SEM. Statistical analysis of the obtained data was assessed using GraphPad Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA and IBM SPSS version 20.0 for Windows SPSS Inc., Chicago, IL, USA.

RESULTS AND DISCUSSION

Cumulative evidences bring to light that GPRC6A is a pivotal regulator of complicated endocrine networks, energy metabolism, and is a versatile candidate that could impact critical signaling mechanisms owing to its ability to bind to osteocalcin, testosterone, basic amino acids and other functionally important cations. [9] SNPs in the functionally active region of GPRC6A have further been linked to defects in metabolic regulation, fertility. [10] However, till date, the incidence, influence of the GPRC6A SNPs rs2274911 and rs143913245 and their prevalence in infertile patients with metabolic syndrome had not been investigated in the Indian, south Indian population.

In order to identify the incidence/prevalence of the SNPs in the control, MTS, MTS with infertility patients, the present study strategized to PCR amplify and direct sequence a 400bp exon 2 region that encompasses the rs2274911 SNP and a 284bp exon 4 region that encompasses the rs143913245 (Fig.1-A,B and Fig.2-A,B,C).

As indicated in the images, genotyping of the 400bp product pertaining to rs2274911 revealed the incidence of the SNP in the assessed population. Genotypes corresponding to the wild type (GG), heterozygous (GA), homozygous (AA) allelic distribution were observed and the representative images for each genotype are presented in Fig.2-A, B, C. Genotyping for the

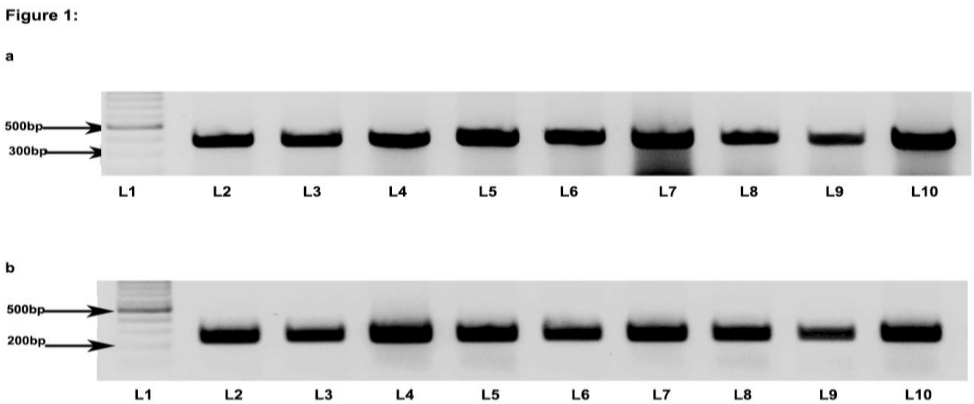


Figure 1: PCR amplification of exon 2, exon 4 regions of GPRC6A. (a) Representative image for the 400bp PCR product that encompasses the exon 2 region that would aid in the determination of the rs2274911 SNP. (b) Representative image of a PCR amplified 284bp product that encompasses the exon 4 region that would aid in the determination of the SNP rs143913245 (F464Y).

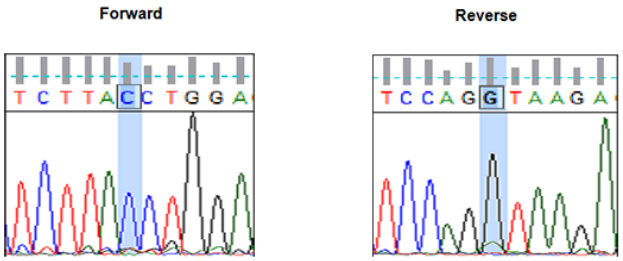
GPRC6A rs143913245 (F464Y) SNP in the exon 4 region (284bp product) revealed the absence of the SNP across the study groups (Fig.3A).

Figure 2 :

a

Alignment of Sequence_1: [BGrs22 400bp Fwd.txt.xdna] with Sequence_2: [BGrs22 400bp Rev.txt.xdna]

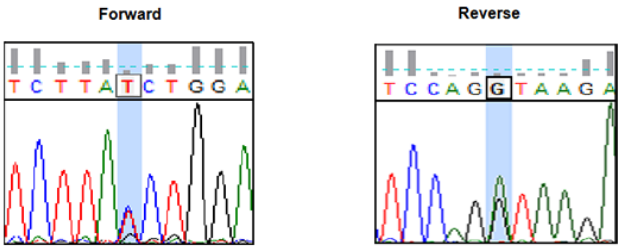
```
5′-GATGATCAACAATTCAACACTCTTAcCTGGAGTCAAACTGGGGTATGAAATCTATGACAC-3′
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3′-CTACTAGTTGTTAAGTTGTGAGAATgGACCTCAGTTTGACCCCATACTTTAGATACTGTG-5′
```



b

Alignment of Sequence_1: [ADK rs22 F.xdna] with Sequence_2: [ADK rs22 R.xdna]

```
5′-GATGATCAACAATTCAACACTCTTAtCTGGAGTCAAACTGGGGTATGAAATCTATGACAC-3′
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3′-CTACTAGTTGTTAAGTTGTGAGAATgGACCTCAGTTTGACCCCATACTTTAGATACTGTG-5′
```



c

Alignment of Sequence_1: [YA rs22 F.xdna] with Sequence_2: [YA rs22 R.xdna]

```
5′-GAGATGATCAACAATTCAACACTCTTAtCTGGAGTCAAACTGGGGTATGAAATCTATGAC-3′
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3′-CTCTACTAGTTGTTAAGTTGTGAGAATaGACCTCAGTTTGACCCCATACTTTAGATACTG-5′
```

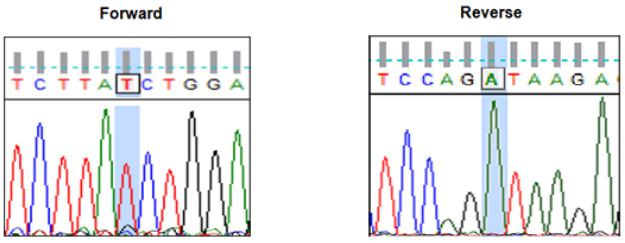


Figure 2: Determination of GPRC6A rs2274911 SNP by direct sequencing. All images presented are representative of triplicate data sets analyzed. (a) The forward and reverse sequences were aligned and the wild type alleles are presented for a rs2274911 negative patient (b) The forward and reverse sequences of a heterozygous positive patient were aligned and the T, G alleles are shown in the aligned sequence (c) The forward and reverse sequences of a homozygous positive patient were aligned and the T, A alleles are shown in the aligned sequence.

However, sequencing data analysis in the exon 4 revealed the presence of the reported SNP rs6917467 (NC_000006.12; p.His467His) that causes a synonymous codon change across the study groups (Fig.3B).

Figure 3:

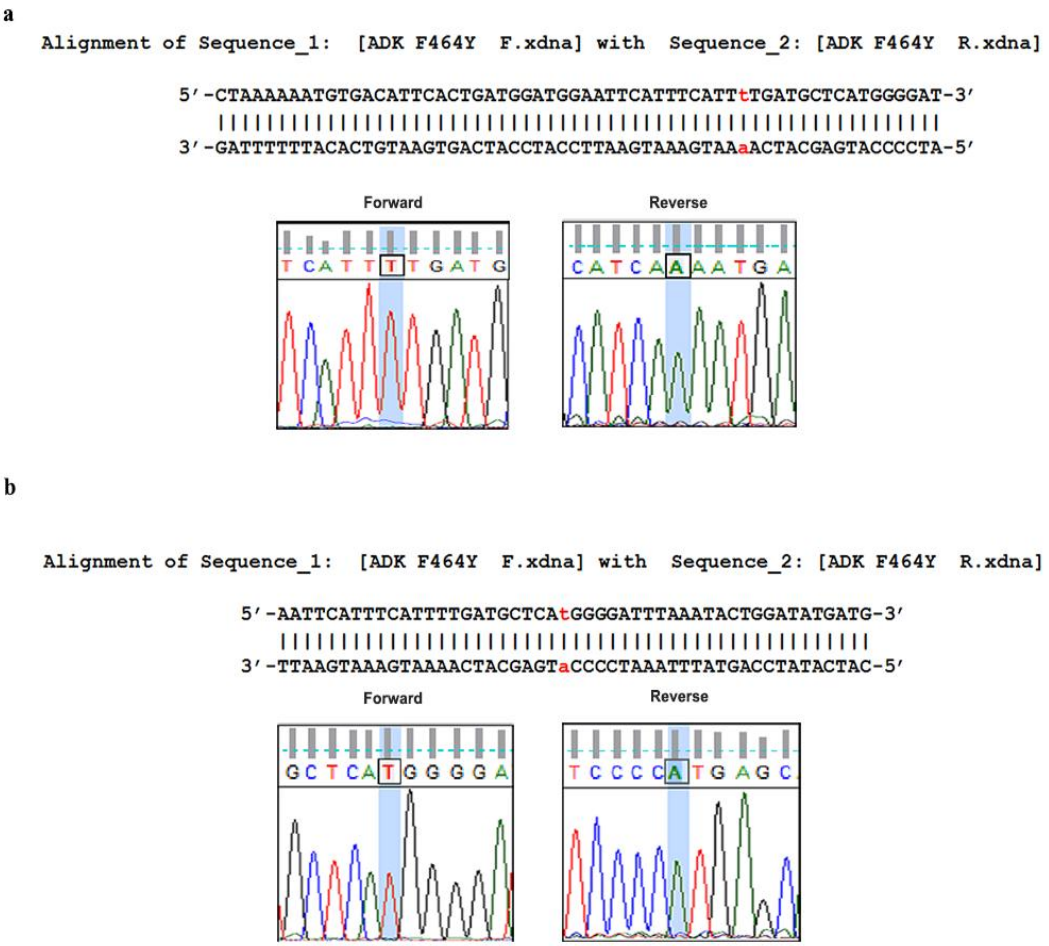


Figure 3: Determination of the GPRC6A rs143913245 (F464Y) SNP by direct sequencing. (a) Representative Images of the direct sequencing results for the PCR amplified 284bp product of rs143913245 negative controls, patients. The forward and reverse sequences were aligned and the wild type alleles T, A are shown in the aligned sequence. (b) Representative Images for the direct sequencing results for the PCR amplified 284bp product of GPRC6A of the reported SNP rs6917467. The forward and reverse sequences were aligned and the T, A alleles are shown in the aligned sequence.

Initial functional studies on GPRC6A exon 2 knock-out mice indicated phenotypes pertaining to metabolic and testicular abnormalities.[11] Subsequent population based large cohort studies from Italy bring forward that the GPRC6A SNPs rs2274911, F464Y are widely associated with metabolic abnormalities[6] and infertility, cryptorchidism.[12,13] In conjunction with such studies the present study results indicate that rs2274911 is incident and prevalent in patients with metabolic syndrome, infertility. Further, as represented in Fig.4A, the SNP rs2274911 was observed to be non-rare and widely incident in a heterozygous condition (GA) in 37% and in homozygous condition (AA) in 33% of the assessed population. However the wild type GG allele expression was incident only in 29% of the study population suggesting that obese/diabetic subjects who do not exhibit other metabolic abnormalities may also widely harbor the SNP.

Figure 4:

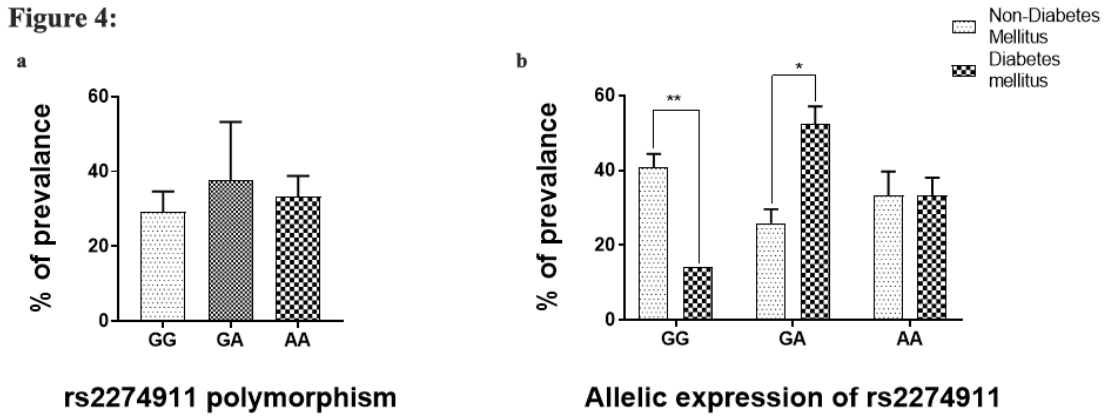


Figure 4: Incidence, prevalence of the GPRC6A rs2274911 polymorphism. (a) The percentage of allelic expression of the rs2274911 SNP was assessed using PCR, PCR product sequencing (b) The percentage of allelic expression of rs2274911 SNP was assessed in the non-diabetic, diabetic groups using PCR, PCR product sequencing. All values are mean ± SEM. Statistical analysis was carried out using a student's T test, chi-square/ logistic regression analysis as and when applicable. Significant p values are indicated as * p<0.05, ** p<0.01.

On similar grounds, the clinical implication of GPRC6A in male reproductive health (GPRC6A-osteocalcin, GPRC6A-testosterone) was brought to light in patients carrying the F464Y (rs143913245) missense variations. The clinical features of the patients carrying the rs143913245 variation involved abnormal male sex hormones, sub fertility and altered sperm parameters. Mechanistic studies further revealed that the functional capacity of GPRC6A leading the observed aberrations in reproductive health could be due to the retention of the ligand intracellularly, lack of surface expression of the ligand and reduced phosphorylation of ERK [8]. As pointed out by these early investigators rs143913245 is rarely incident and our negative results in the screening for the SNP may also likely reflect the rarity of the SNP. Further large scale studies may be required to ascertain the association of the SNP with regional, ethnic gene pool differences.

As the study aimed to assess the prevalence and allelic distribution of rs2274911 in the MTS, MTS patients with infertility, detailed analysis of the allelic variations in the study group patients based on BMI, lipid profile and the diabetic status was carried out. Significant differences in the allelic distribution for rs2274911 were observed between the diabetic and non-diabetic patients of the present study. In an overall, while 40% of the non-diabetic subjects (Fig.4b, Table.1) carried the wild type allele and were negative for rs2274911, only 14% of the diabetic patients were negative for rs2274911 (p=0.007).

Table 1: Allelic distribution of rs2279411 SNP in MTS, MTS with infertility patients. Significant differences were observed in the distribution of alleles between the study groups. Diabetic patients with MTS, MTS and infertility, were observed to significantly present the heterozygous GA alleles. Values are mean ± SEM. Significant p values are indicated as * p<0.05, ** p<0.01.

Study Groups	Allelic expression in %		
	GG	GA	AA
Non-Diabetic patients with Metabolic Syndrome	100	0	0
Diabetic patients with Metabolic Syndrome	16.6 ± 6.8	50 ± 11.7*	33.3 ± 6.8
Non-Diabetic patients with Metabolic syndrome and infertility	11.11 ± 9.0	22.2 ± 9.0	66.67
Diabetic patients with Metabolic syndrome and infertility	16.6 ± 13.6	83.3 ± 13.6***	0

It can be further observed that about 52% of the diabetic subjects were heterozygous positive for rs2274911 (p<0.05). Detailed analysis, as indicated in table 1 reveals that across the study groups controls/patients with diabetes had a significant difference in the distribution of the GG, GA alleles of the rs2274911 SNP. The distribution of the heterozygous GA among the MTS (50%, p< 0.05), MTS with infertility patients (83%, p< 0.001) is observed to be significantly higher in diabetic patients than in non-diabetic patients with MTS, MTS and infertility. Supportively, earlier cohort studies in the Italian normal, obese population have indicated that rs2274911 is associated with an increase in fasting glucose, triglycerides levels.[6] While earlier reports have clearly demonstrated the association of the homozygous AA alleles of the rs2274911 in glucose metabolic aberrations, infertility,[8] our present data indicate significant association with the heterozygous condition. Agreeably, an assessment with an increased sample size may shift the observed trend in favor of the homozygous allelic distribution.

Put together, for a non-rare and well examined SNP in metabolic abnormalities, male infertility, the present

rs2274911 study results fall in-line with earlier studies that demonstrate allelic variations in patients exhibiting an aberrant metabolic, reproductive phenotype. Additively, the present study results strongly inspire for further large scale cohort studies in the Indian population as this would promote better clinical outputs in MTS, MTS and infertility patients.

CONCLUSION

The GPRC6A SNP rs2274911 is significantly dispersed in the south Indian regional population and is prevalently incident in patients with metabolic abnormalities, infertility. Further large cohort studies are required to understand and promote therapeutic values in patients with MTS, infertility.

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