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Original Article

## Effect of PTPRδ rs17584499 C/T Polymorphism on Therapeutic Efficacy of Metformin in Bangladeshi Patients with Type 2 Diabetes


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**ABSTRACT:** Type 2 diabetes mellitus (T2DM) is a polygenic and complex disorder that is difficult to treat effectively in the long term without oral anti-diabetic agents. Genetic variants of these susceptible genes provided the new targets for prevention, diagnosis and treatment of T2DM. Metformin is the first-line agent for T2DM patients that reduces gluconeogenesis by increasing hepatic sensitivity to insulin. To investigate the effect of the rs17584499 C/T polymorphism on metformin therapeutic efficacy in BangladeshiT2DM patients, a fragment of 540 bp of protein tyrosine receptor type delta (PTPRδ) was analysed in 50 T2DM patients having only metformin and 25 healthy controls. In this study it was revealed that T2DM patients having only metformin to treat their disease for 3.62±2.97 years managed their glucose level and lipid profile to within the reference value. Two additional SNPs (rs1978741G/A and rs7865131C/G) within target SNP rs17584499 C/T were also identified. It was found that, the probability of the association of target rs17584499 C/T and newly identified rs1978741G/A with development of T2DM and their effect on metformin therapeutic effect on metformin in Bangladeshi T2DM patients is very low. But carriers of another newly identified rs7865131C/G: the G allele might be having higher susceptibility to T2DM as well as might have adverse effect on therapeutic effect on metformin in patients with T2DM.



**KEYWORDS:** Type 2 diabetes mellitus (T2DM), Metformin, Bangladesh, Polymorphism, Therapeutic effect.

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### INTRODUCTION

Diabetes mellitus is a leading cause of death and disability worldwide [1, 2]. In Bangladesh, a recent meta-analysis showed that the prevalence of diabetes among adults had increased substantially, from 4% in 1995 to 2000 and 5% in 2001 to 2005 to 9% in 2006 to 2010 [3]. According to the International Diabetes Federation, 2011 this prevalence will be 13% by 2030.

Type 2 diabetes (T2D) is a metabolic disorder that generally appears later in life but may occur in childhood and is characterized by the combination of insulin resistance and relative insulin secretion deficiency [4]. Type 2 diabetes is usually not diagnosed until health complications such as stroke, hypertension, amputation, nephropathy, neuropathy, retinopathy, cardiovascular, impotence, skin lesions have occurred

Type 2 diabetes mellitus (T2DM) is a progressive and complex disorder that is difficult to treat effectively in the long term. The majorities of patients are overweight or obese, and will be unable to maintain long-term glycogenic control without oral ant-diabetic agents [5]. The disease is considered to be a polygenic disorder in which genetic variants confers a partial and additive effect. Genetic discoveries have provided the new targets for prevention, diagnosis and treatment of T2DM [6, 7]. Till now, it was identified that more than 40 susceptibility genes associated with T2DM in different population, these discoveries may give some new clues to explore the pathogenesis and the new targets for treatment T2DM [8, 9].

Previously it was reported that protein tyrosine phosphates receptor type delta gene (PTPRδ), a novel susceptibility gene significantly associated with the development of T2DM in Taiwan population by GWAs analysis[10]. Protein tyrosine phosphates (PTPs) are key regulators of the insulin receptor signal transduction pathway [11, 12]. Therefore, PTPRδ gene may play an important role in glucose homeostasis and insulin action.

Metformin is the first-line agent for T2DM patients that reduced gluconeogenesis by increasing hepatic sensitivity to insulin and decreased the hepatic extraction of certain gluconeogenesis substrates (e.g. lactate) [13-15] identified that metformin significantly decreased the levels of PPG ($P < 0.05$) and CHO ($P < 0.05$) of patients with PTPRδ rs17584499 CT+TT genotypes compared of individuals with CC genotype. Metformin efficacy may be affected by gene polymorphisms.

However to date, there are no reports about the impacts of PTPRδ rs17584499C/T polymorphism on metformin

therapeutic efficacy in Bangladeshi Type 2 diabetic patient. In this study we want to explore the association of PTPRδ genetic polymorphism with the development of T2DM and assess the effects of PTPRδ rs17584499C/T polymorphism on metformin efficacy in Bangladeshi patients with T2DM.

## Methods and materials

### *Subjects*

A total of 50 unrelated T2DM patients aged ranging from 27 to 62 years and 25 healthy controls aged ranging from 23 to 50 years were selected to conduct this study. All patients were enrolled in BIRDEM General Hospital (Dhaka, Bangladesh). Written consents were obtained from all participants before the start of this study. Patients were diagnosed according to the diagnosis criteria of the World Health Organization made in 1997 by fasting plasma glucose (FPG≥7.0 mmol/l) and/or postprandial plasma glucose (PPG≥11.1 mmol/l). The criteria for controls were no past diagnostic history of T2DM. All of patients and healthy controls in this study were of Bangladeshi population. Approximate 3-5 ml of blood sample was collected in EDTA coated vacutainer from both patients and healthy controls and stored at -20$^{o}$C until analysis.

### *Clinical laboratory tests*

To test the basic clinical characteristics, blood samples were collected after an overnight fast and at 2 h after breakfast from the patient who on only metformin medication. To check their glucose level plasma concentrations of Fasting Plasma Glucose (FPG), Postprandial Plasma Glucose (PPG) and Glycated Hemoglobin (HbA1c) were determined. Cholesterol (CHO), Triglycerol (TG), Low-density Lipoprotein-cholesterol (LDL-c) and High-density Lipoprotein-cholesterol (HDL-c) were also determined to check their lipid profile. These clinical laboratory tests were done in BIRDEM General Hospital limited in Bangladesh. This study was done by collaboration between Center for Advanced Research in Sciences (CARS), Dhaka University and BIRDEM General Hospital limited in Bangladesh. Body Mass Index (BMI) of patients was calculated as weight (kg)/height ($cm^2$).

### *Molecular Analysis*

Genomic DNA was extracted from whole blood by a standard procedure of phenol/ chloroform/isoamyl alcohol extraction method [16]. The quality and quantity of extracted DNA was measured by NanoDrop 2000 spectrophotometry and visualized by 0.8% agarose gel electrophoresis in 1×TAE buffer. A fragment of 540bp from 8878815-8879354 bp in intron 11 of PTPRδ was amplified to identify the presence or absence the PTPRδ rs17584499 in Bangladeshi T2DM patient who on metformin medication by using Lab designed primer pairs. Amplifications were performed in a 20 ml volume containing one unit of AmpliTaq Gold (Promega, USA), 1× polymerase chain reaction (PCR) buffer, 1.87 mM $MgCl_2$, 200 µM deoxynucleotides triphosphates, 5 pmol each of forward and reverse primer and 70-80ng of genomic DNA. The PCR program consisted an initial denaturation at 95° C for 5 min, followed by 35 cycles of 1 min at 95° C, 45 second at 60° C, 1 min at 72° C and with a final elongation at 72 ° C for 7 min. PCR products were visually verified on 1% agarose gels and directly sequenced using a Big Dye Terminator cycle sequencing kit V3.1 (Applied Biosystems, Foster City, CA) and by ABI PRISM® 3130 Genetic analyzer (Center for Advanced Research in Sciences or CARS, Dhaka University, Bangladesh). Each sequence was reconfirmed by second time sequencing.

The chromatograms generated from the genetic analyzer along with the base sequences were analysed by Bio-edit Sequence Alignment editor (V 7.0)[17]. A fragment of PTPRδ gene sequences of patient's and healthy control's samples were compared with National Centre for Biotechnology Information (NCBI) RefSeq entry of PTPRδ (NC_000009.12|) using NCBI BLAST (bl2seq) tools[18]. NCBI>Variation>Tools>1000 Genomes Browserdatabase [19] was used to identify the presence of identified mutations in other populations. The Human Splicing Finder (HSF) matrices [20] were used to analyze the effect of PTPRδ rs17584499C/T, rs7865131C/G and rs1978741G/A on putative splicing regulatory sequences

### *Statistical analysis*

Data were expressed as mean (±SD) and number (percentage) as appropriate. The mean and standard deviation of each basic Clinicopathological parameter were calculated using Microsoft Excel 2010. The Genotype and allelic frequencies of polymorphisms between patients and healthy controls were compared using Pearson-$\chi 2$ test. The test for statistical significance were calculated by using GraphPad statistical software [21] and a P value less than 0.05 was considered to be significant.

## Result

T2DM patients having only on metformin for 3.62±2.97 years managed their glucose level and lipid profile to within reference value [22-24] (Table 1). Their CHO level (189.12±36.06) is within control's reference value and their fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) were very close to control's reference value (Table 1). Their HDL-c level (46±9.842mg/dl) were also in good condition that means >40 mg/dl. Other parameters such as PPG, TG and LDL-c were slightly higher than reference value (Table 1).

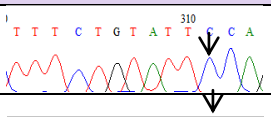
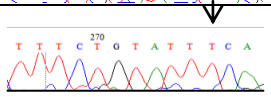
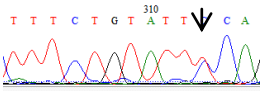
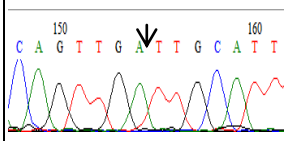
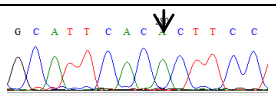
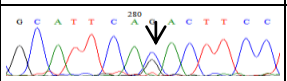
A fragment of 540bp of PTPRδ containing target SNP rs17584499 C/T was successfully amplified and sequenced from all patients and healthy controls. In this study, two additional SNP (rs7865131C/G and rs1978741G/A) was also identified in Bangladeshi people. These two SNP previously identified in China, Japan Europe and Sub-Saharan Africa but in Bangladesh this is first time reported. Target rs17584499 C/T polymorphism was observed mostly heterozygous genotype in both patients and healthy controls (Table2). On the other hand one additional SNP rs1978741G/A identified as only as homozygous genotype in all of patients and healthy controls whereas another additional SNP rs7865131C/G identified as heterozygous genotype in only patients but not in healthy control samples (Table2). Therefore this rs7865131C/G SNP might be having a significant role in the development of T2DM in Bangladeshi patients.

**Table 1:** Basic clinical characteristics of T2DM patients

| Parameters | T2DM patient (n=50) | Healthy control's reference value* |
|---|---|---|
| Duration of taking Metformin | 3.62±2.97 | - |
| Sex Male | 14/50 | - |
| Female | 36/50 | - |
| Age | 48.06±8.99 | - |
| BMI | 25.47±2.41 | - |
| FPG (mmol/l) | 6.37±1.16 | <6.1 |
| PPG (mmol/l) | 8.33±1.93 | <7.8 |
| HbA1c (%) | 6.88±0.56 | <6.5 |
| CHO (mg/dl) | 189.12±36.06 | <200 |
| TG (mg/dl) | 163.04±71.024 | <150 |
| HDL-c (mg/dl) | 46±9.842 | >40 |
| LDL-c (mg/dl) | 109.6±29.19 | 60-100 |

**Healthy control's reference values as [19-21]*

**Table 2:** Nucleotide polymorphisms at PTPRδ in Bangladeshi T2DM patients and healthy controls

| SNP | SNP location in genome | SNP status | Chromatogram | No of patients & control | Remarks |
|---|---|---|---|---|---|
| C/T | 8879118bp at chr-9 (NC_000009.12) *target SNP* | Normal |  | 34 patients 19 controls | rs17584499 |
| | | Mutant (Homo) |  | 1 patients | |
| | | Mutant (Hetero) |  | 15 patient 6 controls | |
| G/A | 8878961bp at chr-9 (NC_000009.12) *156bp upstream of target SNP* | Mutant (Homo) |  | 50 Patients 25 Controls | rs1978741 |
| C/G | 8879086bp at chr-9 (NC_000009.12) *32bp upstream of target SNP* | Normal |  | 46 Patients 25 Controls | rs7865131 |
| | | Mutant (Hetero) |  | 4 patients | |

*Homo=Homozygous, Hetero=Heterozygous*

The genotypes of PTPRδ rs17584499 C/T, rs7865131C/G and rs1978741G/A polymorphisms were determined in 50 T2DM patients and 25 healthy controls by the sequencing method. Genotype and allelic frequencies of these polymorphisms was consistent with Hardy-Weinberg equilibrium (P>0.05). T allele frequency of target rs17584499 C/T was almost equal between patients and healthy controls and is more frequent as CT genotype in both patients and controls rather than TT genotype (Table3). The G allele frequency of rs7865131C/G was found only 4% in patients but not in controls and their genotype only as CG rather than GG (Table 3). On the other hand, the A allele frequency of rs1978741G/A were found 100% in both patients and controls only as AA genotype rather than AG or GG (Table 3). Statistically these three SNP have no significant differences between T2DM patients and healthy controls.

Bioinformatics analysis revealed that target rs17584499 C/T and newly identified rs1978741G/A might be help to reduce the alternative splicing efficiency by disrupting or creating cryptic splice donor site/enhancer/silencer motif/ Exonic splicing regulatory site (Table 4). The target rs17584499 C/T SNP deleted SC35binding site, two enhancer motif site one silencer motif3 and Exonic splicing regulatory site on PTPRδ gene. Another mutation rs1978741G/A deleted two cryptic splice donor sites and two Exonic Splicing regulatory motifs and two Exonic Splicing regulatory motifs and it created a powerful splicing Silencer Motif2 site.

On the other hand, rs7865131C/G might be lead to aberrant splicing of PTPRδ by creating a cryptic acceptor splice site; many splice enhancer site and one Exonic Splicing Regulatory site (Table 4). This cryptic acceptor splice site, splicing enhancer motif site and Exonic Splicing Regulatory site that may help the alternative splicing. It also deleted Silencer Motif3 site but it is a weak motif [25]that might be not fall any effect on alternative splicing.

**Table3:** Frequency distribution of rs17584499 C/T, rs7865131C/G and rs1978741G/A in T2DM patients and healthy controls

| SNPs | T2DM patients n=50 | Healthy controls n=25 | $\chi 2$ | P value |
|---|---|---|---|---|
| *rs17584499 C/T* | | | | |
| **Genotype** | | | | |
| CC | 34 (68%) | 19 (76%) | 3.6429 | 0.162 |
| CT | 15 (30%) | 6 (24%) | | |
| TT | 1 (2%) | 0 (0%) | | |
| **Allele** | | | | |
| C | 83(83%) | 44 (88%) | 3.4904 | 0.062 |
| T | 17 (17%) | 6 (12%) | | |
| *rs7865131 C/G* | | | | |
| **Genotype** | | | | |
| CC | 46 (92%) | 25 (100%) | 2.1127 | 0.1460 |
| CG | 4 (8%) | 0 (0%) | | |
| GG | 0 (0%) | 0 (0%) | | |
| **Allele** | | | 2.0548 | 0.152 |
| C | 96 (96%) | 50 (1000%) | | |
| G | 4 (4%) | 0 (0%) | | |
| *rs1978741 G/A* | | | | |
| **Genotype** | | | | |
| GG | 0 (0%) | 0 (0%) | - | - |
| GA | 0 (0%) | 0 (0%) | | |
| AA | 50(100%) | 25(100%) | | |
| **Allele** | | | | |
| G | 0 (0%) | 0 (0%) | - | - |
| A | 100 (100%) | 50(100%) | | |

*P values are determined by Pearson $\chi 2$ test*

Table-4: Bioinformatics analysis of rs1978741G/A, rs17584499C/T (target) and rs7865131C/G by HSF (v.2.4.1)

| SNP | Cis elements in intron | Method | Motif Wild type (Value 0-100) | Motif Mutant allele (Value 0-100) | Threshold | Result |
|---|---|---|---|---|---|---|
| **rs1978741G/A** | | | **G allele** | **A allele** | | |
| | Potential splice sites | HSF Matrices | ACAGTTGGT (70.35) | - | - | Two Cryptic splice Donor site delete |
| | | | TTGGTTGCA (65.56) | - | - | |
| | Splicing Silencer Motifs | Silencer motifs (Sironi et al.) | - | TGATTGCA 62.22 (+5.55 %) | 60 | Silencer motif2 created |
| | Other Splicing Motifs | Exonic Splicing motif (Goren et al.) | GTTGGT | - | - | 2 Exonic Splicing motif delete |
| | | | TTGGTT | - | - | |
| **rs17584499C/T Target** | | | **C allele** | **T allele** | | |
| | Splicing Enhancer Motifs | ESE Finder | GTATTCCA 77.01 (+7.87 %) | - | 75.05 | SC35 binding site delete |
| | | PESE Octamers (Zhang &Chasin) | ATTCCAGA (27.1) | - | - | Enhancer motif site delete |
| | | EIEs (Zhang et al) | CCAGAG | - | - | Enhancer motif site delete |
| | Splicing Silencer Motifs | Silencer motifs (Sironi et al.) | TCTCCCAA 70.92 (+27.29 %) | | 60 | One motif3 site delete |
| | Other Splicing Motifs | Exonic Splicing Regulatory Sequences (Goren et al) | TTCCAG | - | - | Exonic Splicing regulatory site deleted |
| **rs7865131 C/G** | | | **C allele** | **G allele** | | |
| | Potential splice sites | HSF Matrices | - | acacgcattcagAC 79.83 | - | Acceptor site created |
| | Splicing Enhancer Motifs | PESE Octamers (Zhang &Chasin) | - | CAGACTTC 28.27 | - | enhancer motif site create |
| | | EIEs (Zhang et al.) | - | TCAGAC | - | enhancer motif site create |
| | | | - | GACTTC59.46 (+0.53 %) | 59.245 | enhancer motif created for 9G8 |
| | Splicing Silencer Motifs | Silencer motifs (Sironi et al.) | ATTCACAC 63.56 (+8.90 %) | - | 60 | motif3 site delete |
| | Other Splicing Motifs | Exonic Splicing Regulatory Sequences from Goren et al. | - | GACTTC | - | one Exonic Splicing Regulatory site created |

## DISCUSSION

Type 2 diabetes (T2D) is a multifactorial disease and caused by a combination of lifestyle and genetic factors [26, 27]. Several studies have identified that more than 40 different susceptible genetic loci had been contribute to the risk of type 2 diabetes [28]. The aim of study is to investigate the association of protein tyrosine phosphates receptor type delta gene (PTPRδ) rs17584499 C/T polymorphism with development of type 2 diabetes mellitus (T2DM) and its therapeutic efficacy on metformin in Bangladeshi T2DM patients. This is the first report on the association of SNP with development of type 2 diabetes mellitus (T2DM) through sequencing method and its therapeutic efficacy on drug in Bangladeshi population**.**

SNP in exonic sequences cause genetic diseases by disrupting the correct pattern of pre-mRNA splicing [29]. However there are an increasing number of new pathogenic variants located in introns [30, 31]. Most pathogenic intronic mutations are highly conserved donor and acceptor sites, polypyrimidine tract and the branch-point sequence. Recently, it was reported that some point mutations that located in splicing-regulatory elements (SREs) such as Exonic splicing enhancer (ESE), Exonic splicing silencers (ESSs), Intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs) in deep intronic sequences can have much more severe effects on the structure of the encoded protein [29]. The target SNP rs17584499 C/T in this study is located at deep intronic sequences in intron 11 of PTPRδ.

In present study, the target rs17584499C/T polymorphism may inactivate splicing enhancer SC35 binding site and two other splicing enhancer motifs [32-34]. This polymorphism also inactivates a weak silencer motif3 predicted by [25]. Therefore this rs17584499C-T may reduce the alternative splicing efficiency of intron 11 of PTPRδ by the inactivating enhancer/weak silencer motifs. T allele frequencies of target rs17584499 C-T polymorphism were found 17.0% in T2DM patients and the heterozygous form (CT) is more frequent (30%) than homozygous form (TT). Almost equal genotyping and allelic frequency of rs17584499C/T polymorphism were also observed in healthy controls. Most interestingly, all T2DM patients having metformin only for 3.62±2.97 years and contained rs17584499 CC, C/T or TT genotype managed their glucose level and lipid profile to within the limits. So the probability of the association of this SNP with development of types 2 diabetes mellitus (T2DM) and its therapeutic efficacy of metformin in Bangladeshi T2DM patients is very low.

One of the two additional SNPs, rs1978741G/A was identified in all (100%) T2DM patients and healthy controls that deleted two cryptic splice donor sites [35]. Therefore it may reduce the aberrant splicing. This SNP also deleted two Exonic Splicing regulatory motifs [34] and Exonic Splicing Regulatory Sequences [36]. On the other hand, it created a powerful splicing Silencer Motif2 site. Previously it was reported that motif 2, which acts as a powerful splicing silencer to suppress the aberrant splicing [25]. Statistically no significant differences were found in allelic frequencies and genotype frequency of rs1978741G/A between T2DM patients and healthy control (Table4). So the probability of the association of this SNP with development of type 2 diabetes mellitus (T2DM) and metformin therapeutic efficacy in Bangladeshi T2DM patients is very low. This SNP might have prevalence in Bangladeshi population in process of natural selection [37].

Another additional SNP rs7865131C/G was identified only in T2DM patients located only 32bp upstream of target SNP rs17584499C/T that created a cryptic acceptor splice site acacgcattcagAC with a very high score (79.83) [35]. Cryptic acceptor splice site and Cryptic donor splice site in deep intronic region may lead to aberrant splicing [38]. This SNP also created many splicing enhancer motif and deleted a weak silencer motif3. Silencer motif3 failed to induce skipping of a constitutive exon, indicating that they might act as weak repressors[25]. Therefore this SNP may lead to aberrant splicing of intron 11 of PTPRδ by creating a cryptic acceptor splice site and many splice enhancer site. G allelic frequencies of this rs7865131C/G polymorphism were 4% only in T2DM patients but not in healthy controls. It's genotype was only as C/G (heterozygous form) rather than GG (homozygous form) and statistically no significant differences were found in allelic frequencies and genotype frequency between theT2DM patients and healthy control.

Bioinformatics analysis showed that the rs7865131C/G SNP may lead to aberrant splicing but its effect was not found in Bangladeshi T2DM patients; one of the causes may be the C/G heterozygous form of this polymorphism. It was previously reported that heterozygote have a survival advantage than a mutating homozygote, because of heterozygote have subnormal functional activities [39]. Therefore it can be hypothesized that this mutation might have a pathogenic effect with T2DM having metformin as a monotherapy when it will be a homozygous mutant. This hypothesis however needs to be investigated further with newly discovered sensitive methods like RNA sequencing which can measure the absolute concentration of specific RNA type [40]. It is also necessary to be replicated in larger T2DM patient samples with only metformin treated, without metformin and healthy control to prove this hypothesis. Our study also suggested that susceptibility genes of T2DM development need be detected in different populations may confer different risks and different drug response, which lead to a better understanding of the molecular pathogenesis of T2DM and provide the new targets for prevention, diagnosis and treatment in T2DM.

## CONCLUSIONS

Considering the findings of the basic clinical characteristics of patients having only metformin for 3.62±2.97 years managed their glucose level and lipid profile to within the limits. Form the molecular, statistic and bioinformatics analysis it was revealed that the probability of the target SNP rs17584499C/T and one additional SNP rs1978741G/A association with development of T2DM and their effect on metformin therapeutic efficacy in Bangladeshi T2DM patients is very low. But carriers of another additional SNP rs7865131C/G: the G allele might be having higher susceptibility to type 2 Diabetes as well as might be presented a bad effect on the therapeutic efficacy of metformin in patients with T2DM. However, more details experiments are required for the precise and evidence based therapeutic evaluation.

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