TO EXPLORE THE ASSOCIATION OF CYP2C9 ALLELES IN TYPE 2 DIABETIC PATIENTS EXPERIENCING ADVERSE DRUG REACTION DUE TO SULFONYL UREA TREATMENT

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ABSTRACT

Aim: *To explore the association of CYP2C9 genetic polymorphism in Type2 diabetic patient under the treatment of sulfonylurea (glimepiride) and undergoing adverse drug reactions (ADRs) Glimepiride, a sulfonylurea hypoglycemic agent, is metabolized by cytochrome P450 2C9 (CYP2C9) which is known to have genetic polymorphisms. The most frequent polymorphism is the single nucleotide polymorphism (SNP) which describes the occurrence of at least 2 different alleles for one gene differing only at one specific DNA position Studies shows that CYP2C91*,CYP2C9*2 and CYP2C9*3 are the most important known alleles of CYP2C9. The wild type allele CYP2C91* is with normal enzyme activity CYP2C92*allele has a nucleoside change from cytosine to thiamine at gene position 3608 and hence causes a substitution of arginine by cysteine at protein position144; it has only minor effect on substrate affinity. In CYP2C93* allele nucleoside change from adenine to cytosine at gene position 42614 , results in an amino acid substitution of isoleucine by leucine at protein position 359 this substitution results in a loss up to 70 % of the enzyme activity with CYP2C93* allele as compared with the wild type allele CYP2C91* Patient possessing a decreased enzyme activity because of its genetic disposition, will have a higher bioavailability and smaller clearance for drugs metabolized via this specific CYP. Impaired metabolism of sulfonylurea due to gene polymorphism in the metabolic enzyme CYP2C9 might lead to adverse drug reaction like hypoglycaemia, so genotyping of CYP2C9 may thus serve as a useful tool for predicting adverse effects caused by sulfonylurea and thus helps the clinicians for safer prescribing of oral hypoglycaemic agents.*

Material And Methods: *Type2 diabetic patients of Guwahati Medical College Hospital, Assam under the treatment of sulfonylurea (glimepiride) was identified.70 patients with glimepiride treatment at a dose of 2mg once daily and undergoing therapy from day 1(one) to 2 years were possible to be identified in this 2 years of the study.32 patients had no complain for the drug but 38 patients suspected of having at least one adverse drug reaction(ADR) . Blood samples of patients experiencing ADRs as well as from patient without any complain for the drug were collected after written informed consent and prior ethical committee clearance ,reference no.MC/190/2007/Pt11/22 date:30/03/11.DNA was isolated , alleles CYP2C91*(wild type) and CYP2C92*, CYP2C93* variant form was detected by Polymerase chain reaction –Restriction fragment length polymorphism.*

Results: *Variant form CYP2C92*and CYP2C93* allele on both groups with and without ADR experienced during the course of therapy was studied along with the wild type CYP2C91* from the variant form. Allele CYP2C9 1*(wild type, having 80% enzyme activity) predominates in the group experiencing no any adverse drug reaction. No variant form CYP2C92* allele was able to be identified in any of the two groups whereas variant form CYP2C93* allele was identified in few samples of the group with ADRs. A test for significance (chi square test) for occurrence of allele in parameter like adverse drug reaction was done. The p value (<0.05) obtained was found to be statistically significant.*

Key Words: *Single nucleotide polymorphism, CYP2C9, Polymerase chain reaction, Adverse drug reaction.*

INTRODUCTION

Following administration of any medication, it is not always possible to predict its effects in the individual patient. Due to the major inter-individual variability in response to pharmacotherapy, in some patients, adverse drug reactions (ADRs) or therapeutic failure instead of therapeutic success are observed. (1)According to world health organization ADR is any response to a drug which is noxious, unintended and occurs in doses normally used for prophylaxis diagnosis or therapy of disease or for modification of physiological function (WHO 1972). ADRs occur in 5% of all hospital admission, occur in 10-20% of all hospital inpatient, causes death in 0.1% of medical inpatient. (3)Adverse drug reaction adversely affect patient quality of life preclude use of drug in most patient although they may occur in few patient and also mimic disease resulting in unnecessary investigation and delay in treatment.

The prevalence of diabetes is increasing at an alarming rate affecting more than 250 million individual worldwide. In adult, type 2 diabetes is the most prevalent form accounting for 90-95% of worldwide cases of diabetes (4). Type2 diabetes is characterized by decreased insulin secretion from pancreatic beta cells and decreased tissue responsiveness to the normal action of insulin (Insulin resistance). There are numerous pharmacological and non-pharmacological treatments for type 2 diabetes. Life style intervention such as diet and exercise is effective, however long term adherence is typically poor, as such most type2

diabetic patient will require one or more drug therapies. To manage hyperglycemia available pharmacological agents include biguanides, sulfonylurea, insulin, thiazolidinediones, glucagon like peptide 1 analogue, alpha glucosidase inhibitors, non-sulfonylurea insulin secretagogues, amylin agonist, dipeptidyl dipeptidase (DPP4) inhibitors.(1,4)

The American diabetes association and European diabetic association recently published a consensus statement regarding the management of hyperglycemia. According to this statement the management of type2 diabetes is divided in tiers. Tier 1 includes lifestyle intervention, Biguanides (metformin), Sulfonylurea and Insulin. Tier 2 represents less well-validated therapies such as thiazolidinediones, pioglitazone and the glucagon-like peptide 1 analog, exenatide. Medications that fall into the 'other therapy' category are those not recommended in tier 1 or 2 approaches, and include α-glucosidase inhibitors, non-sulfonylurea insulin secretagogues, pramlintide and DPP-4 inhibitors.(12)

The tiered drug treatment approach serves as a useful guidance for the pharmacotherapeutic management of type2 diabetes. However, it does not solve the problem that substantial inter individual variability exist in anti-diabetic drug disposition and response. The field of pharmacogenomics has recently applied to the treatment of type2 diabetes. The objective of pharmacogenomics is to find out the relationship between variants in the human genome and the variability in the effects of drugs. The field of pharmacogenonics has been applied to the sulfonyl urea clinical studies in order to elucidate its variability in response, disposition and adverse affect (2, 4, 10). Sulfonyl urea acts by binding to ATP dependent potassium channels (KATP) on pancreatic beta cell. KATP channels exist as a complex comprised of SUR1 (sulfonyl urea receptor 1) which is the regulatory subunit and inward rectifier potassium ion channel (Kir6.2) which forms the core of the channel. Four SUR1 receptor and four Kir6.2 subunits make up the KATP channels. The binding of sulfonyl urea with SUR1 receptor result in the closure of KATP channel and hence resulting in the increase of intra cellular potassium concentration and hence depolarization of beta cells and subsequent opening of calcium channels. As the calcium moves into the beta cells it stimulates the insulin is containing secretary granules to the cell surface and hence these insulin containing secretary granules are ultimately released from the beta cells into the circulation (4).

Sulfonyl urea class of drugs is classified into two generations. The first generation includes the Tolbutamide, Chlorpropamide, Tolazamide and Acetohexamide. The second generation sulphonyl urea includes Glyburide, Glipizide, Gliclazide and Glimipiride. All has the same mechanism of action. The second generation sulfonyl ureas are metabolized by CYP450 (CYP2C9). CYP2C9 isoenzyme is highly polymorphic. Till date more than 34 different alleles for CYP2C9 have been discovered of which CYP2C91*, CYP2C92* and CYP2C93* seems to be important because of their high allele frequency. (5)The CYP2C9 gene is located within a cluster of CYP2C sub family genes in the order CYP2C18—2C19—2C9—2C8, mapped on chromosome 10q24. CYP2C9 consists of 50,734 bp, has 9 Exon and its open reading frame is 1,473 bp long It encodes for an enzyme with 490 amino acids (NCBI Reference Sequence). CYP2C9*2 and CYP2C9*3 differ from the wild type CYP2C9*1 by a single nucleotide substitution i.e. C430>T in exon 3 and A1075>Cin exon 7 respectively. This leads to an amino acid substitution of arginine by cysteine at position 144 and isoleucine by leucine at position 359 of the enzyme encoded by CYP2C9*2 and CYP2C9*3 respectively. The wild type allele CYP2C91* is of normal enyme activity CYP2C92* has a minor effect on the substrate affinity but the rate of metabolism is reduced to approximately 50%. A reduction of 70% of enzyme activity with CYP2C93* as reported by Anderson in in -vitro studies, 2004. (5,6,13)

OBJECTIVE

To explore the association of CYP2C9 alleles CYP2C91*, CYP2C92* and CYP2C93* in diabetic population on sulfonyl urea with and without adverse drug events experienced during the course of therapy.

MATERIALS AND METHODS

A study was undertaken in the Guwahati Medical College Hospital Assam, with prior Ethical Committee clearance (reference number MC/190/2007/Pt11/22 date: 30/03/11) of Guwahati Medical College Hospital. Around 300 type2 diabetic patient in both in- patient and out- patient department of endocrinology and medicine department of Guwahati Medical college hospital was possible to come across in the 2year study, Out of which only 70 were found to be taking glimipiride 32 patient had no complain for the drug but 38 patient happen to experience at least one adverse drug reaction after taking the drug. Blood was withdrawn from both types of patient with written informed consent and was analyzed for detection of allele CYP2C91*, CYP2C92* and CYP2C93*. Patient of both sexes and all age group are included in the study, patient adverse event history, history of medication and its course, duration (day1 to-2 years) with dosage 2mg once daily, concomitant medication details were recorded in the ADR analysis format followed by Indian pharmacovigilance programme. 3ml of blood from identified type 2 diabetic patients under sulfonyl

urea (glimepiride) therapy was collected having no complain against the drug as well as from the patient experiencing adverse drug reaction of the drug, with their written informed consent and analyzed for detection of allele CYP2C91*, CYP2C92* and CYP2C93*. DNA was isolated from the blood samples by DNA isolation kit (Hipure blood genomic DNA mini preparation kit, Himedia). Extracted DNA was kept at -80^{0}C PCR-RFLP (restriction fragment length polymorphism) technique was used for detection of variant form, CYP2C9*2 (Arg144Cys) and CYP2C93* (Ile359Leu) allele and the wild type allele CYP2C91* from the variant form by digestion with restriction enzyme. (9)CYP2C9*2, primers used are forward primer5' –TACAAATACAA TGAAAATATCATG–3' and reverse primer 5' – CTAACAACCAGGACTCATAATG–3'. For CYP2C9*3, primers used are two forward F1and F2and one reverse primer R
F1 (5'–AATAATAATATGCACGAGGTCCAGAG ATGC–3) F2 (5'-AATAATAATATGCACGAGGTC CAGAG GTAC–3') and R (5'–GATACTATGAA TTTGGGGACTTC–3').

A 20 ***µl*** reaction mixture (1 µl forward primer, 1 µl reverse primer, 10 µl reaction buffer and nuclease free water to make up the volume***)*** was prepared and the reaction conditions followed with an annealing temperature of 55 °C. This mixture was then heated to 94 °C to separate the DNA strands, cooled to the primer specific annealing temperature to allow primer binding to the DNA (annealing temperature 55 °C, brought to 65 °C to elongate the primers. The three steps, denaturation (94 °C), hybridization (annealing temperature, Tanneal) and synthesis (65 °C) are repeated 34 times followed by a longer synthesis step (7 min) and a cooling step of the reaction mixture at 4 °C. The PCR product with a length of 690 bp was subsequently digested with the restriction endonuclease AvaII, 10 µl PCR product mixtures, 12 µl water, 1x concentrated buffer (NEB buffer). 4 and 2 units AvaII enzyme (10units/µl) were mixed and incubated at 37 °C for 3 h. The products were separated by 2 % agarose gel electrophoreses (7, 8). After separation by gel electrophoreses allele showed the cut 690 bp PCR product at 520 bp indicating the wild type allele. CYP2C9*2 can be determined as the wild type allele shows the uncut 690 bp PCR product, For the detection of the polymorphic allele CYP2C9*3, two forward primer F1 and F2 and one reverse used. (9)Forward primer F1 and F2 lead together with the reverse primer R to two different amplicons for the wild type allele and two different amplicons for the CYP2C9*3 allele in two separate restriction reactions the PCR products are then digested with either NsiI or KpnI restriction enzyme. The amplicon of the wild type allele defined by primer F1 carries the recognition site for NsiI and can therefore be cut by this enzyme, whereas the amplicon defined by primer F2 has no complete recognition site for KpnI and cannot be cut. Digestion with NsiI leads to a product (120bp) only for the wild type allele (both samples). Digestion with KpnI leads to a product (120 bp) only for the CYP2C9*3 allele. No CYP2C9 2* allele was detected as. Only 690 base pair product was obtained after digestion with Avail digestion which indicates wild type allele CYP2C1*. Allele for CYP2C93* was detected, this sample showed kpn1 digestion at120 base pair, others showed Nsil digestion at 120 base pair which indicates wild type CYP2C91*.

RESULT

ADR most frequent with glimepiride were hypoglycaemia, abdominal discomfort, visual disturbance, hyponatremia, edema (table1). Allele CYP2C91*(wild type) with normal enzyme activity predominates in the subjects. No variant CYP2C92* detected. Allele CYP2C93* was detected in 5 patient. (Table-2) All alleles negative for the nucleotide substitutions at position 3608 (*2) and 42614 (*3) were presumed to be wild type CYP2C9*1.

Table 1: Spectrum of suspected adverse drug reactions noted among 38 patients

S. No.	ADR of Glimipiride	% of all ADRs:n=38
1	Hypoglycaemia	4(10.5%)
2	Vomiting	0
3	Urinary Retention	3(7.8%)
4	Abdominal Discomfort	4(10.5%)
5	Diarrhea	0
6	Elevation of Liver Enzymes	1(2.6%)
7	Cholestasis	0
8	Jaundice	0
9	Allergic Reaction	0
10	Edema	3(7.8%)
11	Agranulocytosis	0
12	Anaemia	1(2.6%)
13	A plastic Anaemia	0
14	Paresthesia	9(23.6%)
15	Hyponatremia	1(2.6%)
16	Visual Disturbances	8(2i%)

Table 2: CYP2C9 allele frequencies

Allele	Cyp2c91*	Cyp2c92*	Cyp2c93*	Total
Sex (male)	42 (60%)	0	4 (5.71%)	46 (65.71%)
Sex (female)	23 (32%)	0	1 (1.42%)	24 (34.28%)
Total	65 (92.8%)	0	5 (7.14%)	70 (100%)

n= 70(total number of subjects), ADR*ALLELE (Chi square test).p value <0.05(0.025).

DISCUSSION AND CONCLUSION

In this study we identified the adverse drug reaction due to sulfonyl urea (glimepiride). Glimepiride was found to be most frequently

prescribed drug by the clinicians for the control of blood glucose in type2 diabetic patients.70 patient taking glimepiride was selected for the study, out of which 38 patient were suspected of having at least one ADR and 32 patient had no complain for the drug. ADR most frequent with glimepiride were hypoglycaemia, abdominal discomfort, visual disturbance, hyponatremia and edema. Our prime objective of the study is to investigate the presence of variant form CYP2C92* and CYP2C93* allele in the ADR experienced diabetic subjects on glimepiride of Guwahati Medical College Hospital, Guwahati (Assam) by PCR-RFLP method. Allele CYP2C9 1* (wild type allele having 80% enzyme activity) predominates in the subjects without adverse drug reaction. Variant form CYP2C92*allele was not able to be identified in any of the groups. In the group experiencing ADR, allele CYP2C93* was identified in four patient experiencing ADR (hypoglycaemia) and in one patient experiencing ADR (acute visual disturbances).

For CYP2C9*3, a loss of up to70 % of the enzyme activity is published (13). Presence of allele CYP2C93* in the subjects with hypoglycaemic events explains us about the reduced metabolism of glimepiride resulting in higher bio availability and lower clearance of the drug in the subjects and resulting in adverse drug reactions. The test for significance (chisquare test) for occurrence of allele in parameter like adverse drug reaction was done. The p value obtained was 0.025(<0.05) which is considered to be statistically significant. Polymorphisms of CYP2C9 gene significantly affect the pharmacological response of diabetic patients to sulfonyl ureas because of its reduced metabolism followed by increase in drug bioavailability and risk of adverse drug reaction. In allele CYP2C93* there is a nucleoside change from adenine to cytosine at gene position 42614 which results in the amino acid substitution of isoleucine by leucine in protein position 359 and thus results in a loss of 70% enzyme activity compared to wild type(6). This suggest that among other factors individuals with genetically determined low CYP2C9 activity can be at a risk for sulfonylurea associated adverse drug reactions.

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