Polymorphism of Aldose Reductase Gene and Susceptibility for Developing Diabetic Retinopathy among Type 2 Diabetes Mellitus Patients

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Abstract

Diabetic retinopathy (DR) can be defined as damage to microvascular system in the retina due to prolonged hyperglycaemia , the major systemic risk factors for onset and progression of DR is duration of diabetes . In this study we try to study the possible candidate genes that may contributed for developing and progression of diabetic retinopathy, mainly polymorphism of aldose reductase gene and its roles in developing diabetic retinopathy. Polymorphism of C/T at -106 region of aldose reductase gene were studied by conventional polymerase reaction (PCR) and Restrictive fragment length polymorphism by restrictive endunuclase . We have investigated the C / T polymorphism at the promoter region of the *ALR* gene as a candidate gene for susceptibility to diabetic Retinopathy and found that CC genotype was(27%) in non retinopathy , and in diabetic retinopathy was(56%) . The calculating odd ratio for CC genotype as risk factor with developing diabetic retinopathy was (3.5), and for C allele frequency the odd ratio was 2.9 which meaning that CC genotype is associated with increased risk for possibility of developing DRP and represented risk factor for developing DRP among type 2 diabetes mellitus . The prevalence of bad glycemic control as indicated by HbA1c among diabetic retinopathy patients and diabetic control groups are 70% and 58% respectively which proved that poor glycemic control for a long duration has a role in developing and progression of diabetic retinopathy .

Keywords: Diabetic Retinopathy, Aldose Reductase, HbA1c

الخلاصة

اعتلال الشبكية السكري من مضاعفات الأوعية الدموية الدقيقة لداء السكري وان تطور المرض مرتبط بمدة الإصابة بالسكري .في هذه الدراسة تمت دراسة مدى ارتباط التغاير الجيني في الدوز ردكتيز مع تطور مرض اعتلال الشبكية السكري . التغاير في منطقة ألجين تمت دراستها بواسطة جهاز البلمرة المتكاثر لتضخيم قطعة من ألجين التي تحوي على التغاير ثم هضم الناتج بواسطة انزيم القاطع ومن تحليل النتائج بينت ان النمط الجيني نسبته 56% عند مرضى اعتلال الشبكية السكري و27% في مجموعة السيطرة .CC

حساب عامل الخطورة لهذا النمط الجيني أوضح ان مدى الخطورة كانت 3.5 مما يؤكد علاقته بتطور المرض. دراسة مقياس السيطرة للسكري أوضحت ان مرضى السكري الغير مسيطر على أساس استخدام مقياس الهيموكلوبين المتسكر عرضة لتطور مرض اعتلال الشبكية السكري.

الكلمات المفتاحية: اعتلال الشبكية السكري، انزيم الدوز ريديكتيز، الكلوكوز المتسكر

Introduction

Diabetic retinopathy (DR), can be defined as damage to microvascular system in the retina due to prolonged hyperglycemia (Paul,2013) . DR is the most common and specific microvascular complication of diabetes **,** It remains a major cause of visual impairment worldwide among the people in working age and is a leading cause of visual loss in older patients(Marshall ,2006) .

DR is broadly classified as either non proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR).These depend on microvascular changes in the retina as studied by ophthalmoscopy (ETDRS group). Fundus abnormalities in

diabetic patients have a progressive course, from mild retinopathy, non proliferative, where microaneurisms are the main feature(Wilkinson 2002 and Agarwal ,2006*).* to severe proliferative disease with neovascularizationof the disc, retina and iris (Antoniae2007 andO'Doherty2008).

There are four main pathways for developing DRP:

I Polyol/Aldose reductase pathway
II Hexosamine pathway
III Protein kinase C pathway with activation of vascular endothelial growth factor
IV Advanced Glycation End products pathway(Brownlee 2001and Sheetz 2002.*)*

Aldose reductase gene(ALR2) gene encode the enzyme aldose reductase the first and rate-limiting enzyme of polyol pathway , which converts glucose to sorbitol in an NADPH-dependent reaction .ALR2 is expressed in many tissues (Vikramadithya*n* 2005). . Including the retinal pigment epithelial cell ,renal mesangial cells and schwan cells (Dagheret ,2004 .al, Richeti ,2007) . and it may therefore participate in the pathogenesis of diabetic complications affecting the eyes , kidneys and the nervous system, (Oates ,2004) . In humans , the functional ALR2 gene is located on chromosome 7q35 and consists of 10 exons spanning 18 kp of DNA(Cao, 1998*)* .

HbA1c is a form of haemoglobin joins with glucose in the blood. When glucose sticks to these molecules it forms a glycosylated haemoglobin molecule, also known as A1c and HbA1c.The more glucose found in the blood the more HbA1c will be present. Due to the fact that red blood cells survive for 8-12 weeks before renewal, by measuring HbA1c, a good guide to the average blood glucose reading over that period can be returned(*Lind 2008)* . The interaction between glucose and hemoglobin are showed in figure no . 1 (Abbas, 2011).

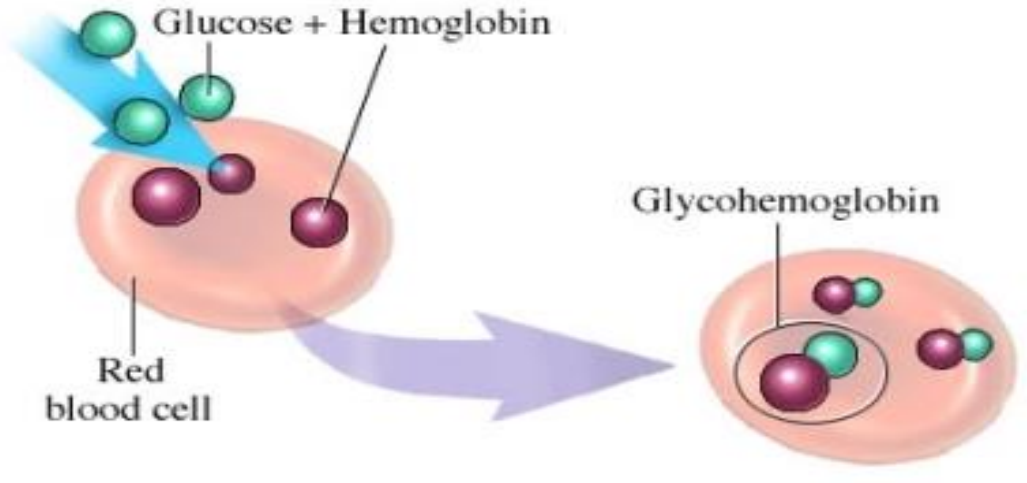

Figure 1: Glycated Hemoglobin (HbA1c) Formation **.**

The American Diabetes Association, a joint statement from the American Association of Clinical Endocrinologists/American College of Endocrinology, and a World Health Organization Consultation each recommend an A1C of 6.5% or higher as a criterion for the diagnosis of diabetes(WHO .2011).

Materials and Methods

Selection groups A case control study was done on 125 patients with NPDRP and PDRP as well as control groups

The subjects participated in this study were classified into following:-

Group one: 75 patients with 2 DM– 75 (46 males and 29 females) with retinopathy ranging from NPDR to proliferative retinopathy. Non proliferative ranging into mild ,moderate and severe diabetic retinopathy

Group two: patient with type 2 diabetes mellitus without retinopathy

Group three: apparently healthy subjects were chosen as healthy controls, they were non smoker, non alcohols, and did not have any history of chronic diseases.

Exclusion criteria

1-Congestive heart failure
2-Urinary tract infection
3-Fever
4-Perepheral neuropathy
6-Renal disease
7 - Patients with type 1 diabetes mellitus
8- Gestational diabetes

Methods

Ophthalmologic examination including visual acuity (by means of snellen charts), intraocular pressure (using Applanation Tonometry),fundoscopy (utilizing slit lamp and contact lenses) were completed by an ophthalmologist and the patients were categorized according to the degree of their retinopathy. .

HbA1C was determined by the colorimetric determination of glycohemoglobin in whole blood by means of a cation-exchange reasin using Stanbio Kit, Texas-USA .

DNA Extraction Protocol From fresh Blood by Geneaid kit .

PCR amplification by using the following primers

The following primer sets combinations were used to determine the C/T polymorphism

Table 1. Sequences of primers used for PCR amplification

Tm	Primers Sequences	genes
65	5- C C T T T C T G C C A C G C G G G G C G C G G G	Aldose reductase gene
	3- C AT G G C T G C T G C G C T C C C C A G	C/T -106 polymorphism

PCR can amplify a small amount of template DNA (or RNA) into large quantities in a few hours. This is performed by mixing the DNA with primers on either side of the DNA (forward and reverse), *Taq* polymerase (of the species *Thermus aquaticus*, a thermophile whose polymerase is able to withstand extremely high temperatures), free nucleotides (dNTPs for DNA, NTPs for RNA), and buffer. The temperature is then alternated between hot and cold to denature and reanneal the DNA, with the polymerase adding new complementary strands each time (Carr AC ,2012*)* .

Conventional PCR were used to amplify –(106) polymorphic region of aldose reductase gene .

Stage	Temperature (C^o)	Time(min)	Function	cycles
1	94	2	Initial denaturation	
	94	2	DNA denaturation	45
2	66	1	Primer anneling	
3	72	1	Template elongation	
4	72	5	Finalelongation	
5	4	4	Incubation	Hold
				-

Products of PCR are analysed by electrophoresis through agarose gels electrophoresis

Polymerase chain reaction –restriction fragment length polymorphysim

Polymerase chain reaction –restriction fragment length polymorphysim (PCR-RFLP) is used for genotyping depending on restriction endunuclase cleavage. present of SNPs that alter the restriction sequence can be genotyping by this method .Amplification for region that surrounding restriction enzyme site by PCR and the digested the product by appropriate enzyme for genotyping *Junhua ,2006)* .. Endunuclase digestion by *BfaI* .This procedure was done by preparing A volume 10 µL of each amplified products (which contained 326fragments) together with 5 units each of restriction enzyme *Bfa1* were put into the tubes and incubated at 37 C^o for 1-3 hour. Digested amplified DNA fragments were electrophoresed on 2% agarose 2 h at 100 V), and the bands visualized after staining with ethidium bromide under UV light. A 100 base-pair ladder (Clever Scientific - UK) (were used as a size marker for estimation of fragment sizes.

Results

1 Descriptive study for DRP patients groups

The demographic and medical characteristics of patients are shown in table2.

Table 2: Patients clinical characteristics .

Characteristics	DRP		No DRP	P Value
Age (Years)	NPR 56±8		55.5 4.5	0.8
	PR 51± 9.4			
Gender	M 46 F 29		M 16 F 9	0.3
BMI(KG/M^2)	NPR	M 27±4.2 F 27±4.2	M 30±2.1 F 24±2.5	0.5
	PR	M 30±4. F 24±**2.**		
HbA1c%	NPR 8.2±o.8		7.6± 0.6	0.005*
	PR 10.2±2.4			
Duration in years	NPR 17±**6**		5.5	0.001*
	PR 12±**6.2**			

P Value <o.o5 significant ,* significant

2. Age distribution of patients are shown in figure 2.

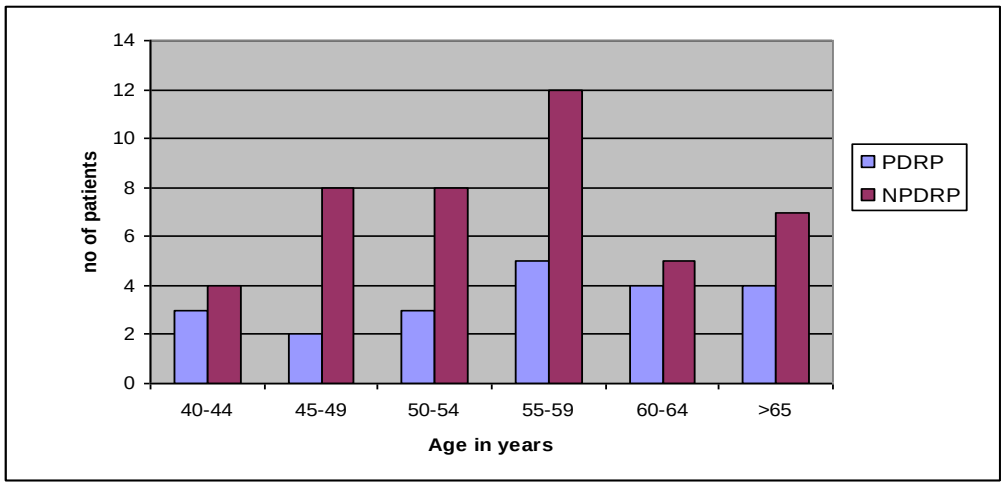

Figure 2: Distribution of cases according to age

3.Gender distribution of patients are shown in figure 3

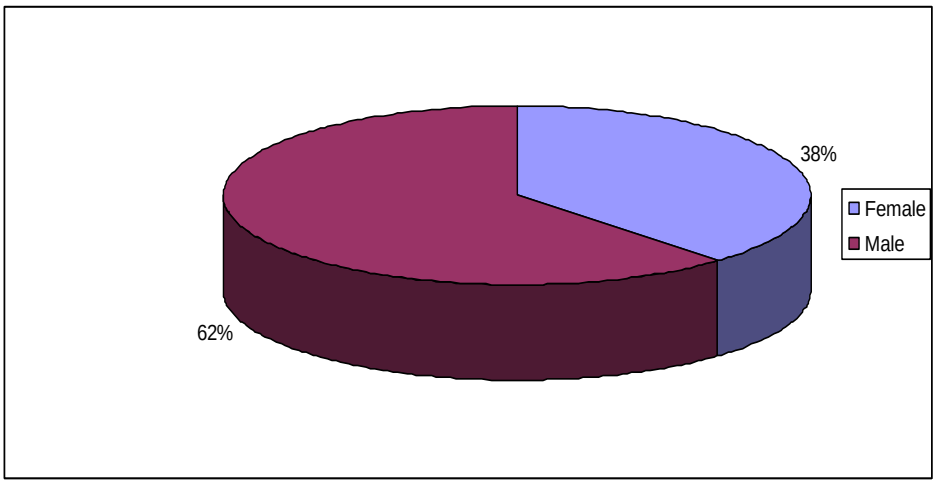

Figure 3: Gender distribution of diabetic retinopathy.

4 .The relationship between duration of DM and retinopathy are show in table 3

Table 3: Duration of DM and DRP **.**

Diabetic retinopathy	Mean ± SD Duration (DM) in years	P-value
Mild DRP	**8.4±5.8**	0.001*
Moderate DRP	9±**6.05**	0.001*
Severe DRP	17±**7.2**	0.001*
Proliferative DRP	12±**6.2**	0.001*

P value of < 0.05* was considered to be statistically significant

5.The relationship between family history and DRP are shown in figure 4

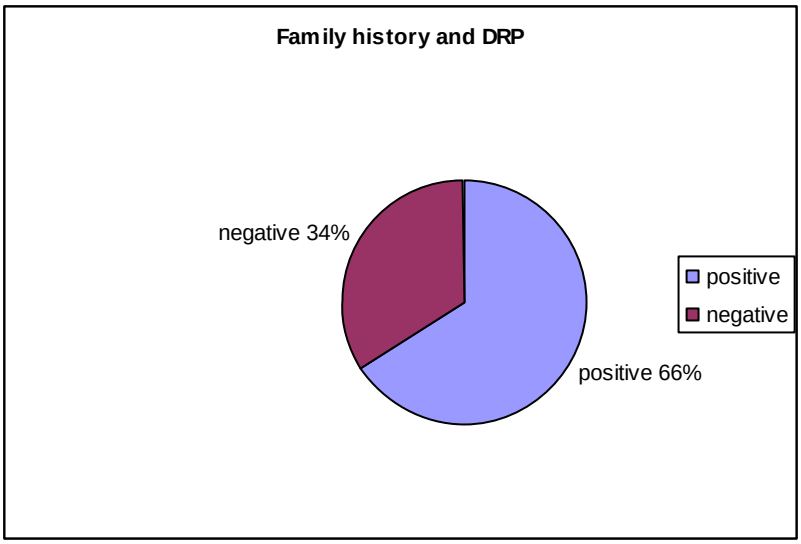

Figure 4: Diabetic retinopathy in relation to family history.

5 .Diabetic retinopathy and treatment type are revealed in figure 5

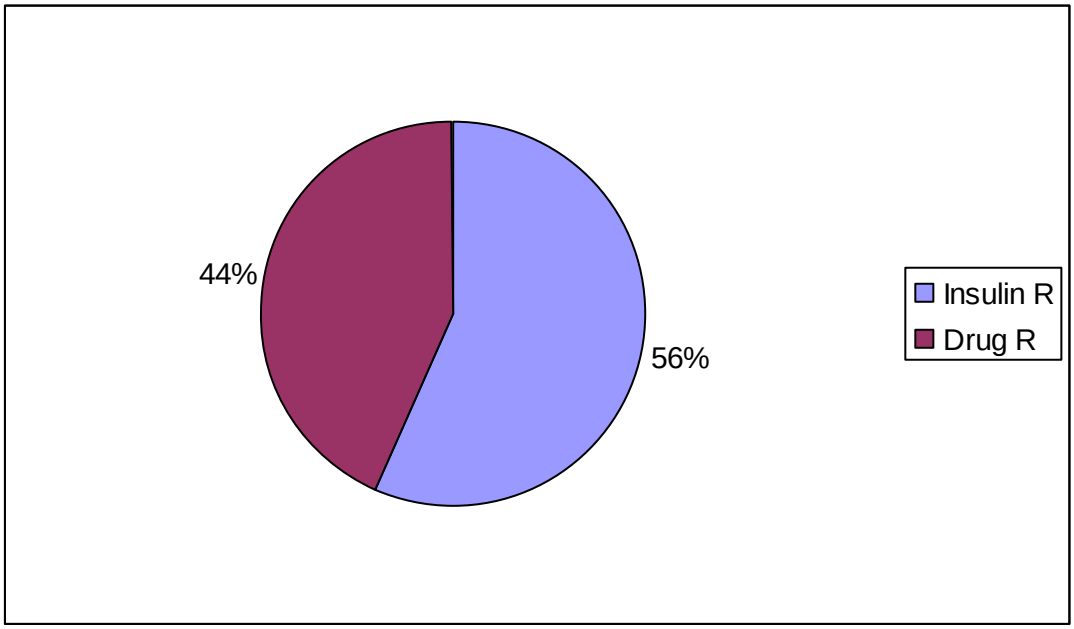

Figure 5: DRP and Treatments types .

Table 4: The odd ratio for independent variables as possible risk factor for DRP .

Independent variable	Odd ratio	95%CI	P-Value
DURATION >5	**5.3**	1.9-13.4	0.001*
INSULIN R AND DRP	4.5	1.6-9.4	0.001*
HbA1c%> 7	3.6	1.5-11.3	0.001*
FAMILY HISTORY	3.2	1.3-6.4	<0.01*
Hyperglycemia and DRP	2.6	1.44.3	<0.001*
Obesity and DRP	1	0.7-0.9	0.3NS

P-value of < 0.05* was considered to be statistically significant *significant NS not significant ,CI confidence Interval

HBA1c and DRP

The percentage of HbA1c among patients with DRP with different degrees and diabetic patients without retinopathy are represented in table 5

Table 5: Mean and SD of HbA1c% in diabetic retinopathy and control .

	GROUPS	Mean ± SD
HbA1c%	**Mild DRP**	**7.9**±0.7
	Moderate DRP	8.5±**1.5**
	Severe DRP	8.2±**0.7**
	ProliferativeDRP	10.2±**2.4**
	Control DNR	7.5±**0.6**

Table6:ANOVAs analysis for HbA1c between control and diabetic retinopathy

Control group	Comparism groups	Sig.
NDRP	Mild DRP	>0. 05
	Mod.DRP	0.005*
	severe.DRP	0.005*
	Proliferative DRRP	0.005*

(P-value of < 0.05* was considered to be statistically significant)

Table 7 :Percentage of HbA1c % among DRP patients.

HbA1c%	DRP+	DRP-
Good control<6.8	**30%**	42%
Fair and poor control >6.8	70%	58%

A positive correlation between hyperglycemia and HbA1c in patients with DRP were shown in figure 6.

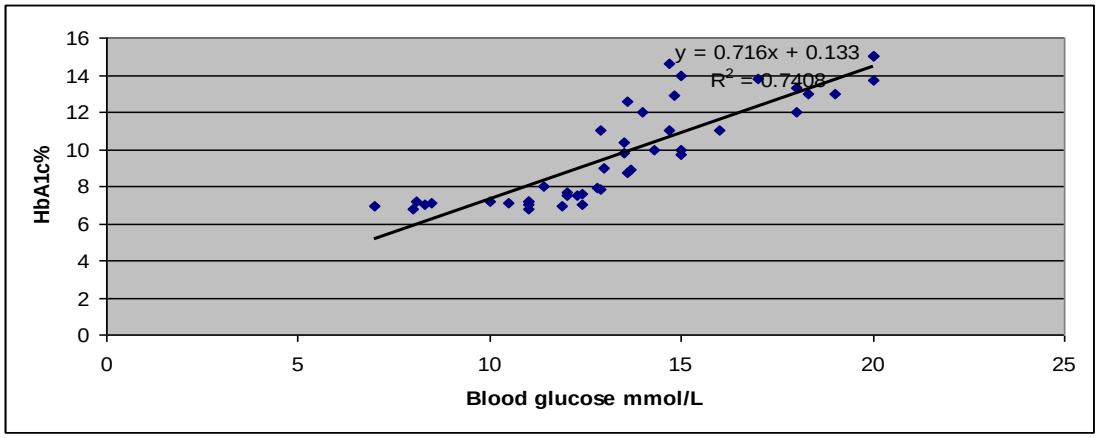

Figure 6: Linear regression for HbA1c% and blood glucose

DNA concentration and purity are examined by nanadrop spectrophotometer and the result were revealed that the mean of DNA were 67±19 ng/ul and DNA purity were 1.7±0.12 ,the concluded results were summarized in Table 6

Table6: DNA concentration and purity .

DNA	Mean ± SD ng/ul
Concentration	**67±19**
Purity	1.8±**0.12**

PCR was done for amplification of –C/T polymorphysim for aldose reductase gene and the product of PCR were run on 2% agarose electrophoresis ,the result for run product on agarose electrophoresis are shown in figure no.7

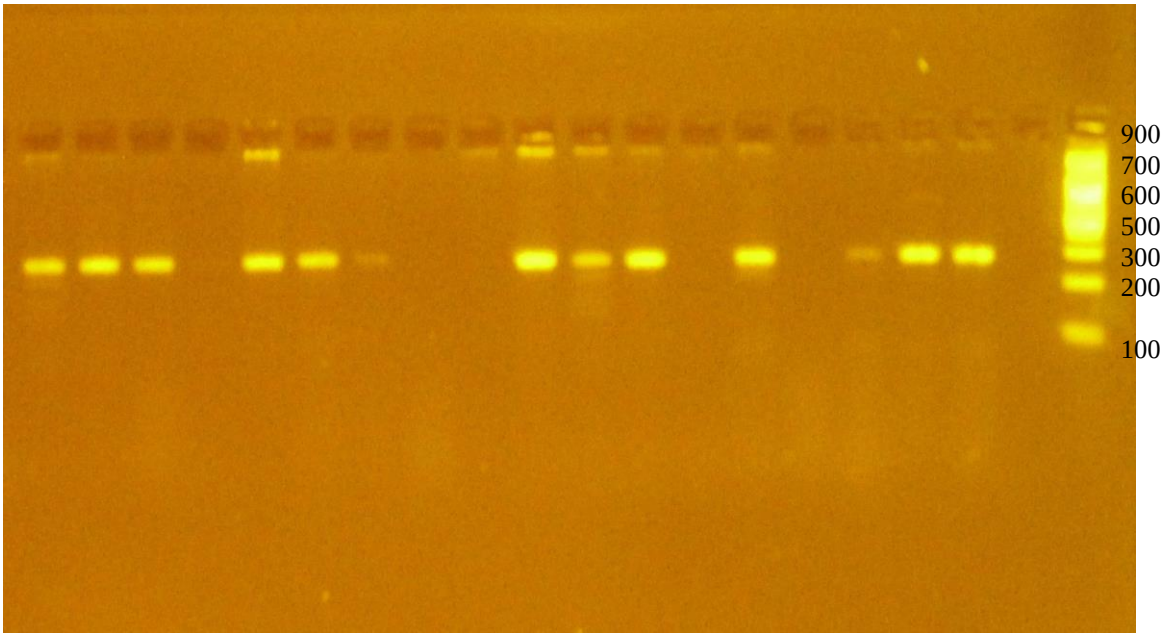

Figure 7: Electrophoretic pattern of amplification products of -106 polymorphic region of aldose reductse gene. Amplified products were electrophoresed in 2% agarose(Promega master mix) gel 70V,20mA for 120 minute and direct visualization with Ethidium Bromide under UV light .
Lane 20: DNA Ladder marker
Lane1-19 bands of amplification for C/T polymorphism at -106 promoter region of Aldose reductase gene . Each well loaded with 10µl of PCR product. Amplification product appeared as band of about 306 bp

PCR – RFLP

The PCR products after amplification were digested by *BfaI* for genotyping the aldose reductase gene according to polymorphic region after digestion specific bands were produce, and separated by agarose electrophoresis and standardized with DNA ladder. The digestion productsgenotyping as following

CC 2 bands 100 ,206 bp

CT 3 bands 100 ,206 ,306 bp

TT 1 band about 306bp

The products of digestion were represented in figure no . 8

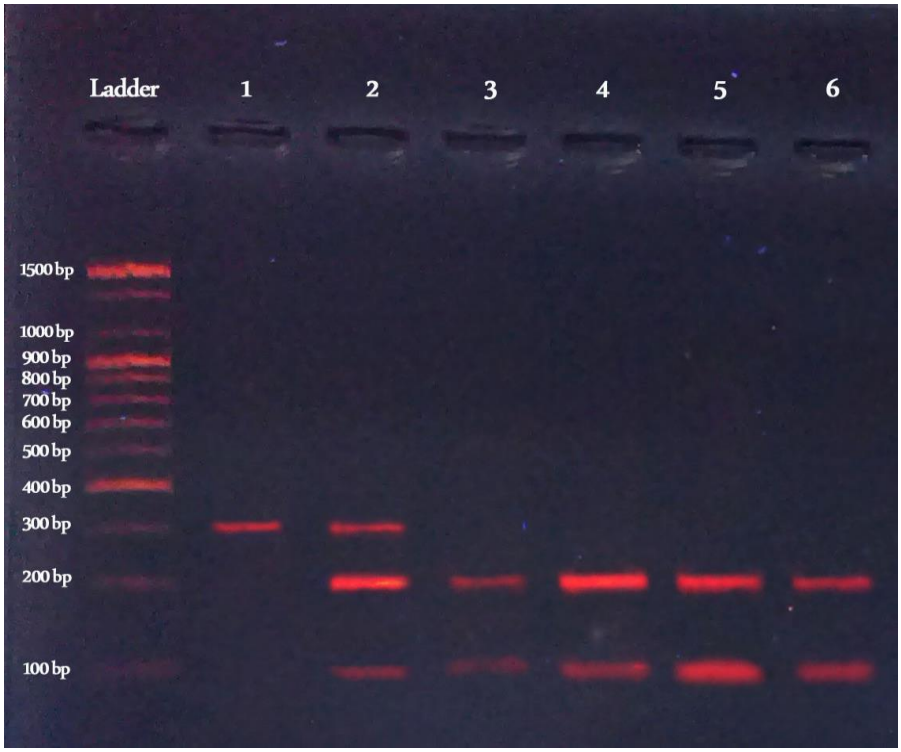

Figure8: RFLP pattern of Aldose reductase gene polymorphysim.

Ladder DNA Marker , Lane 1 TT Genotype, Lane 2 CT Genotype , , Lane 3 4,5.6 CC Genotype

Genotyping of aldose reductase gene

Genotyping of aldose reductase gene according to polymorphism at-106 promoter region were represented in table 7.

Table 7: Genotyping of aldose reductase gene according to C/Tpolymorphysim

Genotype	HC N0. %	Control DNR No. %	DRP No. %	OD CI	p value
CC	**- -**	**8 27**	**42 56**	**3.5 1.4-8.8**	**o.oo1***
CT	**8 30**	**8 27**	**15 20**	**0.6 o.25-1.3**	**NS**
TT	**17 70**	**14 46**	**18 24**	0.5 0.3-1.3	**NS**
Allele frequency					
C	**16 32**	**24 40**	85 57	**2.9 1.6-5.4**	**0.001***
T	**34 68**	**36 60**	65 43	**0.8 0.4-1.3**	**NS**

(P-value of < 0.05* was considered to be statistically significant)
NS non significant .OD odd ratio ,CI confidence interval .

Discussions

According to data in figure 3 which reveals that the diabetic retinopathy, in our study the males to females ratio is(1.6) meaning that according to these data DRP in males is more prevalent than females .A similar preponderance has been reported from the (CURES Eye study Rema *et .al* 2005)

Our result increase in BMI and developing DRP were statistically insignificant as shown in table 2 ,this result were agreed by studies of(Laurence and, Rasmieh *et.al.* 2008) .

The analysis of data obtained in our study indicated that duration of diabetes is risk factor for developing and the progression of diabetic retinopathy , the duration of diabetes is probably the strongest predictor for development and progression of retinopathy this result were conformed by studies of(SotoPedre and Krishna *et .al.* 2007)

Positive relationship between family history and developing diabetic retinopathy was confirmed by another study (Muecke *et .al* 2008). From analysis of data about relationship between treatment and developing DRP indicated that insulin was associated with developing DRP ,this relationship are not because the insulin considered as risk factor for developing DRP ,but the patient with type 2DM with chronic hyperglycemia and poor glycemic control prior insulin use treatment implicated in developing DR.

Insulin therapy and developing DRP proved by other studies while hyperglycemia was a risk factor for the progression of retinopathy in all patients, change of treatment from oral drugs to insulin was associated with increased risk of retinopathy progression to 3-fold increased risk of blindness/visual impairent (Mohan *et .al* 2005).

According to data in table 5 and 6 indicated that poor glycemic control by using HbA1c % has a role on developing diabetic retinopathy ,the relationship between poor glycemic control by using HbA1c % and DRP were cofirmed by other study(Kamran and Long-M *et .al* 2010) .

In this work we try to study the possibility of polymorphism of aldose reductase gene and developing DRP among type 2 DM genotyping of aldose reductase gene according to C/T polymorphism at promoter region We have investigated the C/T 106 polymorphism at the promoter region of the *ALR* gene as a candidate gene for susceptibility to diabetic retinopathy, and found that the CC genotype in diabetic group without retinopathy was(27%), and in diabetic retinopathy was(56%). The calculating odd ratio for CC genotype as risk factor with developing diabetic retinopathy was (3.5), and for C allele frequency the odd ratio was 2.9 which meaning that CC genotype is associated with increased risk for possibility of developing DRP and represented risk factor for developing DRP among type 2 DM

Aldose reductase (ALR) is the first and rate-limiting enzyme in the polyol pathway, The polyol pathway is involved in microvascular damage, the hallmark of diabetic retinopathy and pathogenic vascular and hemodynamic changes contributing to DR ALR has been identified in human pericytes, which exhibit an active polyol pathway as appeared figure no.9. (Brownlee et al and.Das Evcimen *et al. 2001)* .

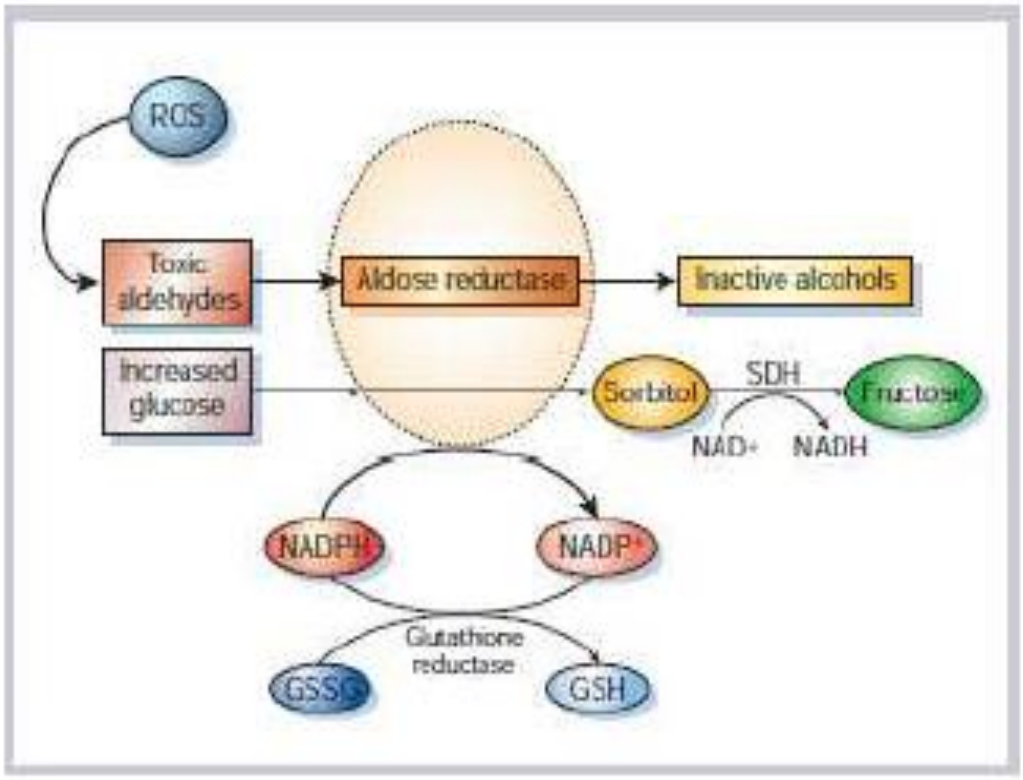

Figure 9: Aldose reductase and polyol pathway .

GSH= Reduced glutathione ,GSSG= Oxidized glutathione,SDH=Sorbitol dehydrogenase ,ROS= Reactive oxygen species .

Candidate genes studies have been reported for DR, and their recent meta-analysis found genetic variation in the ALR gene to be the most significantly associated with DR .

Basal promoter activity of the human ALR2 gene is located between −192 and +31 upstream of the mRNA capsite (Wang *et a l.*1993 *).*

The C-106T polymorphism is located proximal to the CCAAT promoter element and may, therefore, have functional significance. This is supported by a recent study in C/T-106 polymorphisms where found to be double transcription activities of the ALR2 gene 5′ regulatory region *(*Li Q *et al* 2002).

Association between polymorphism at -106 of aldose reductase gene and developing of diabetic retinopathy among patients with type 2 DM were confirmed by other studies for *(*Sotoodeh *et al*, 2007; Katakami *et al* 2011*).*

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