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Amar Deep, Ajay Vardaan, Durgesh Dubey and Ravi Mishra

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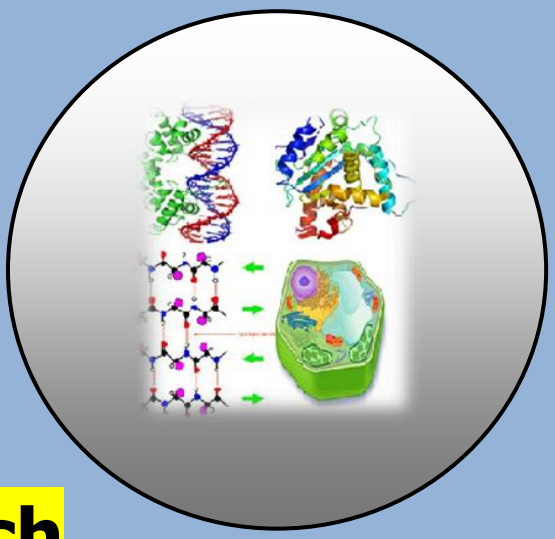

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Dr. Ajay Vardaan Amar Deep
http:// www.jbcr.in
jbiolchemres@gmail.com
info@jbcr.in

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Association of GALT-1 Gene Mutations with Primary Female Infertility

Amar Deep, Ajay Vardaan,*Durgesh Dubey and **Ravi Misra
Department Medical Gastroenterology, King George's Medical University, Lucknow -226003 (U.P.), India
***CBMR, SGPGIMS, Lucknow – 226003, (U.P.), India**
****Department of Internal Medicine, King George's Medical University, Lucknow – 226003, (U.P.), India**

ABSTRACT

Infertility is defined as a couple's inability to achieve pregnancy following one year of appropriately timed and unprotected intercourse. Reproductive endocrinologists, consider a couple to be infertile if the couple has not conceived after 6 months of contraceptive-free intercourse if the female is over the age of 35. Globally, the incidence of infertility is estimated to be about 13–18% (Thonneauet al 1991; Jones and Toner 1993; Irvine 1998) in the human population, regardless of race, ethnic group, etc. Many genes are likely to be involved in the complex process of reproduction. GALT-1 is one of the gene which is responsible for infertility. A GALT (Galactose-1 Phosphate UridylTransferase) catalyze the production of Glucose-1 Phosphate and Uridylphosphate (UDP) Galactose from galactose-1 phosphate and UDP Glucose. This reaction is important in the conversion of galactose to glucose. As glucose is the main energy source in most of the organism, its impairment leads to the disease called Galactosemia. It is an inborn disorder caused by autosomal recessive mutation in GALT gene. Galactosemia also develops primary symptoms which include vomiting, diarrhea and jaundice and long term symptoms which include mental retardation, Premature Ovarian failure, and speech deficits in female. We performed a case control study to determine the prevalence of Q188R, K285N and N314D mutations and to evaluate any association of these mutations with the female infertility.
Keywords: Infertility,Galactosemia, GALT (Galactose-1 Phosphate UridylTransferase), UDP Galactose.

INTRODUCTION

Infertility is defined as a couple's inability to achieve pregnancy following one year of appropriately timed and unprotected intercourse. Reproductive endocrinologists, consider a couple to be infertile if the couple has not conceived after 6 months of contraceptive-free intercourse if the female is over the age of 35 (declining egg quality of females over the age of 35 account for the age-based discrepancy as when to seek medical intervention). Globally, the incidence of infertility is estimated to be about 13–18% (Thonneauet al.,1991; Jones and Toner 1993; Irvine 1998) in the human population, regardless of race, ethnic group, etc. Moreover, 20% of couples consult their general physician because of difficulty conceiving, and half of those couples (10%) require specialist care (Hull et al., 1985; Beurskenset al., 1995). By this criterion it has been estimated that approximately 15-20% of couples attempting to achieve pregnancy are unable to do so. WHO defined infertility as an inability of a sexually active non-contraception couple to achieve pregnancy in one year. It is also defined as the state in which a couple wanting a child cannot conceive after 12 months of unprotected intercourse (Mueller and Daling 1989; Thonneau*et al* 1991). A combination of male and female factors accounts for the remaining 20% - 30% of cases. This suggests that in more than 50% of couples presenting for infertility evaluation, a male factor is equally contributory. In USA their latest figures on infertility indicates that in couples in which the woman is under 35y are: 40% female, 23% male, 17% combined male and female, 10% more than one female factor and 10% unexplained factors responsible for infertility. However, the approach to the infertile couple has started with an evaluation of the female, primarily because it is usually the female partner who has initiated a workup by consultation with her gynecologist (Meschedeet al., 1997). The American Society for Reproductive Medicine (ASRM) states, "...women in their 20's to early 30's are most likely to conceive." an estimated 10.2% of women between the ages of 15 to 44, or about 6.2 million women, have impaired fertility, and the incidence is increasing. About 25% of women experience some period of infertility during their reproductive years. The qualitative diagnosis of infertility requires physical evaluation of female and male abnormalities, endocrine anomalies (their hormonal and genetic background) and genetic conditions of both partners that interfere with reproduction. Many genes are likely to be involved in the complex process of reproduction. GALT-1 is one of the gene which is responsible for infertility. GALT (Galactose-1 Phosphate UridylTransferase) catalyse the production of Glucose-1 Phosphate and Uridylphosphate (UDP) Galactose from galactose-1 phosphate and UDP Glucose (Leloir 1957). This reaction is important in the conversion of galactose to glucose. As glucose is the main energy source in most of the organism, its impairment leads to the disease called galactosemia. It is an inborn disorder caused by autosomal recessive mutation in GALT gene. Galactosemia also develops primary symptoms which include vomiting, diarrhea and jaundice and long term symptoms which include mental retardation, Premature Ovarian failure, and speech deficits in female. GALT Deficiency is most often connected with the Q188R (188- glutamine→arginine in GALT protein), K285N (285-lysine→aspragine) mutation and N314D (314-aspragine→ aspartate in GALT protein) mutations. We performed a case control study to determine the prevalence of Q188R, K285N and N314D mutations and to evaluate any association of these mutations with the female infertility.

PRIMARY V/S SECONDARY INFERTILITY

Couples with primary infertility which have never been able to conceive while, on the other hand secondary infertility is difficulty conceiving after already having conceived (and either carried the pregnancy to term, or had a miscarriage).

Prevalence

Generally, worldwide it is estimated that one in seven couples have problems in conceiving, with the incidence similar in most countries independent of the level of the country's development.In nearly 50% of infertile couples who seek care, a male factor is diagnosed (David M. Nudell etal., 2000).Fertility problems affect one in seven couples in the UK. Most couples (about 84 out of every 100) who have regular sexual intercourse (that is, every 2 to 3 days) and who do not use contraception will get pregnant within a year. About 92 out of 100 couples who are trying to get pregnant do so within 2 years (Clinical guideline 11. London: NICE; 2004).Women become less fertile as they get older. For women aged 35, about 94 out of every 100 who have regular unprotected sexual intercourse will get pregnant after 3 years of trying. For women aged 38, however only 77 out of every 100 will do so. The effect of age upon men's fertility is less clear (Information for Those who are trying for a baby, august 2014).In Sweden, approximately 10% of couples are infertile. In approximately one third of these cases the man is the factor, in one third the woman is the factor and in the remaining third the infertility is a product of factors on both parts (The Columbia Electronic Encyclopedia Copyright©2004. Licensed from Columbia University Press).

Prevalence of infertility in developing countries

Only a limited number of reports are available on the prevalence of infertility in developing countries. According to Boivin et al., (2007), the 12-month prevalence rate ranges from 6.9 to 9.3% in less developed countries.

Even less data are available from Asia and Latin-America, but a report compiled by the World Health Organization (WHO) indicated that the prevalence of infertility in these regions fell within the globally expected range 8–12% of couples of reproductive age and was thus lower when compared with African countries (World Health Organization, 1991).

India

Motherhood is the most important goal for Indian women. Childlessness is associated with stigmatization, social isolation and sometimes violence (Unisa, 1999; Riessman, 2000). Even in the case of male infertility, women usually bear the negative consequences of their inability to conceive. Preventive and curative services for infertility have not yet been a priority in India despite the importance of motherhood. Assisted Reproductive Technology(ART) is only provided in private centers and is only accessible by those who can afford this treatment. Owing to the stigma of infertility, secrecy is an important component of assisted conception, especially where donor gametes are involved (Bharadwaj, 2003).

RISK FACTORS FOR FEMALE INFERTILITY

Worldwide, an estimated 10.2% of women between the ages of 15 to 44, or about 6.2 million women, have impaired fertility and the incidence is increasing. About 25% of women experience some period of infertility during their reproductive years. Between 1982 and 1988 there was a 37% increase of infertile women between the ages of 35 to 44. The number of infertile women is expected to reach 6.3 million in the year 2000, and may be as high as 7.7 million in 2025.

As a woman age her chances for fertility declines. Infertility in older women appears to be mostly due to a higher risk for chromosomal abnormalities that occur in her eggs as they age. Although they have a higher risk for miscarriage given in table-1

Table 1. Chances for Pregnancy by Age.

Age	Fertility %
Up until 34	90%
By age 40	Declining to 67%
By age 45	Declining to 15%

b) Weight Factors and Excessive Exercise: Obesity is highly associated with poly cystic ovarian syndrome (PCOS), which is cause of infertility in some cases. Women at risk include, women with eating disorders, such as anorexia or bulimia. Women on very low−calorie or restrictive diets are at risk, especially if their periods are irregular. Strict vegetarians might have difficulties if they lack important nutrients, such as vitamin B12, zinc, iron, and folic acid. Marathon runners, dancers, and other who exercise very intensely. (Lower body fat contributes to menstrual irregularities in competitive athletes, but other mechanisms are also involved.)

c) Lifestyle Factors*:* Smoking, caffeine, alcohol, veginaldouching and sexual practices which affects the lifestyle.

d) Environmental Risks Factors: Estrogen like chemicals or those that disrupt hormones are of particular concern for infertility in men and for effects on offspring women. Some being studied include: Diethylstilbestrol (DES), an estrogen compound and by this women face a higher risk for cervical cancer, genital tract abnormalities and miscarriage.Bisphenol-Awidely used chemical found in plastic food containers and bottles that has provoked concern.Use of the chemical in female rats has produced prostate abnormalities in their male offspring.Therehas been some concern that plant−based estrogens (phytoestrogens), such as the isoflavones found in soy and other foods, may reduce fertility rates.Exposure to pesticides has caused known reproductive problems in animals and birds. Pesticides with estrogen−like effects include DDT, aldrin, dieldrin, PCPs, dioxins and furans.

c) Stress and fertility: Severely elevated levels of stress hormone can, in fact shut down menstruation.

CAUSES FEMALE INFERTILITY

a) Pelvic Inflammatory Disease: Pelvic inflammatory disease (PID) is the major cause of infertility worldwide. PID comprises a variety of infections caused by different bacteria that affect the reproductive organs, appendix and parts of the intestine that lie in the pelvic area. The sites of infection most often implicated in infertility are in the fallopian tubes, a specific condition referred to as salpingitis.About 20% of women who develop symptomatic PID become infertile. PID also significantly increases the risk of ectopic pregnancy (fertilization in the fallopian tubes).

b) **Endometriosis:** Endometriosis is a common disease of reproductive age group women. It was first described by Dr. Sampson in 1925 as, “presence of ectopic tissue which possesses the histological structure and function of uterine mucosa”.Endometriosis accounts for 10 to 15% of infertility. There is an increasing trend towards treating infertile with endometriosis surgically. This condition, in which endometrial grows outside the uterus, is a major cause of infertility in women.

c) Polycystic Ovarian Syndrome: Patients whose ovaries contain many small cysts have hormone imbalances and do not ovulate regularly.

d) Premature Ovarian Failure (Early Menopause): Premature ovarian failure (POF; OMIM no. 311360) is a cause of female infertility due to the loss of normal ovarian function in women before the age of 40 years. The condition is defined by the absence or cessation of normal menses for at least 6 months (primary or secondary amenorrhea), menopausal level of follicle stimulating hormone (FSH) O40 mIU/ml, hypoestrogenism and infertility.

e) Hypogonadotropic Hypogonadism: Hypogonadotropichypogonadism is a rare condition in which follicle−stimulating hormone (FSH) and luteinizing hormone (LH) are underproduced and prevent the development of functional ovaries.

f) Functional Hypothalamic Amenorrhea (FHA): Functional hypothalamic amenorrhea (FHA) is the absence of menstruation due to disturbances in the thyroid gland and hypothalamus−pituitary−adrenal (HPA) system, which regulates reproduction and other important functions. The eating disorders anorexia and bulimia are most often associated with FHA. FHA may be due to other different factors, most unknown.

g) Luteal Phase Defect (Implantation Failure): Luteal phase defect is a general term referring to problems in the corpus luteum that result in inadequate production of progesterone. Because progesterone is necessary for thickening and preparing the uterine lining, the ovum fails to successfully implant in the endometrium.

h) Elevated Prolactin Levels (Hyperprolactinemia): Prolactin is a hormone produced in the pituitary gland that stimulates breast development and milk production in association with pregnancy. High levels of prolactin (hyperprolactinemia) reduce gonadotropin hormones and inhibit ovulation.

i) Galactosemia: GALT (Galactose-1 Phosphate UridylTransferase) catalyse the production of Glucose-1 Phosphate and Uridylphosphate (UDP) Galactose from galactose-1 phosphate and UDP Glucose (Leloir 1957).This reaction is important in the conversion of galactose to glucose. As glucose is the main energysourse in most of the organism. Where its impairment leads to the disease Galactosemia.Galactosemia is a group of inherited metabolic disorders in which an enzyme deficiency affects the normal metabolism of the sugar galactose into galactose-1-phosphate uridyltransferase (GALT; EC 2.7.7.10; MIM# 230400). Characteristic clinical disorders of metabolism have been described for all three enzymes but “classical galactosemia” is brought about by a deficiency in activity of the GALT enzyme. The most common (and most severe) form, results from a disruption of the galactose-1-phosphate uridyltransferase (GALT) gene.

j) Genetic causes of female infertility: It is often difficult to establish a diagnosis for the cause of infertility. “Idiopathic infertility” still predominates as a diagnosis, which reflects the challenges of defining the pathophysiology of infertility in individual couples. The large number of genes that are potentially involved in sex determination, steroidogenesis,fertility and space limitations preclude a comprehensive review of all genetic abnormalities associated with infertility (*e.g*. chromosomal abnormalities, disorders of steroid biosynthesis, and metabolic disorders). Table -2 show genetic causes of female infertility.

GALT-1 GENE

GALT Gene is located on **9p13** and consists of **11 exons.** GALT Deficiency is most often connected with the Q188R (188- glutamine→arginine in GALT protein), K285N (285-lysine→aspragine) mutation and N314D (314-aspragine→aspartate in GALT protein) mutations. The connection between Q188R, K285N, and N314D mutations and infertility can be confirmed by deoxyribonucleic acid (DNA) analysis of infertile women. For this reason we performed a case control study to determine the prevalence of Q188R, K285N and N314D mutations.

DUARTE VARIANT [GALT, ASN 314 ASP]

This polymorphism was identified by Reichardt and Woo (1991). The substitution is an A-to-G transition at basepair 2744 of exon 10, which adds an AvaII cut site. Elsas et al. (1994) identified 13 N314D alleles.

Table 2. Single Gene Disorders in the Hypothalamic-Pituitary-Gonadal Axis Resulting in Impaired Fertility.

Gene	Locus	Product	Phenotype	
			Sex	Feature
GNRHR	4q21	G-Protein couple receptor	F	Delayed puberty, Variable thelarche, amenorrhea.
HESX1	3p21	Paired like homodomain TF	M/F	Stepto-optic dysplasia, panhypopituitarism.
PROP1	5q35	Paired like homodomain TF	M/F	Variable HHG, GH and TSH deficiency.
FSHβ	11p13	Glycoprotein hormone	F	No thelarche, primary amenorrhea.
FSHR	2p21-p16	G-Protein couple receptor	F	Variable puberty, , primary and secondary amenorrhea.
LHR	2p21	G-Protein couple receptor	F	Normal puberty,amenorrhea or oligomenorrhea.

HHG; Hypogonadotropichypogonadism

GALACTOSEMIA [GALT, GLN 188 ARG]

Reichardt et al. (1991) demonstrated a transition at nucleotide 591 that substituted arginine for glutamine-188 (Q188R). The Q188R mutation was the most frequent galactosemia mutation characterized to 1991; it accounted for one-fourth of the galactosemia alleles studied. Elsas et al., (1994) stated that the Q188R mutation accounts for approximately 70% of Caucasian galactosemia.

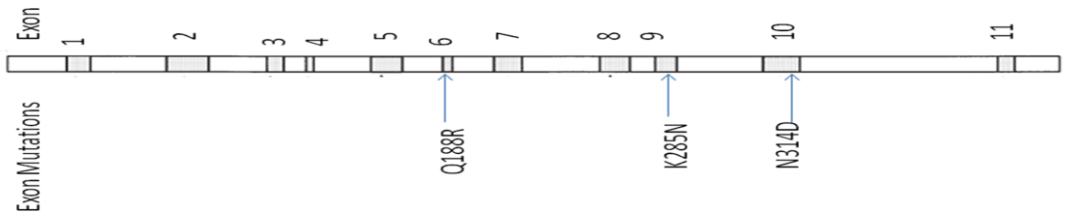
GALACTOSEMIA [GALT, LYS 285 ASN]

In a study of 30 families with classic galactosemia (230400) in Denmark, (Greber-Platzer et al.,1997) found that the second common galactosemia mutation was lys285 to gln (K285N).So we are hypothesized that might be possible that GALT-1 gene mutation may have any association with the infertility.

Mutation	A A Change	Exon	Nucleotide change
Q188R	Glu to Arg	exon 6	C**A**G to C**G**G
K285N	Lys to Asn	exon 9	AA**G** to GA**T**
N314D	Asn to Asp	exon 10	**A**AC to **G**AC

STRUCTURE OF THE GENE

The burgeoning activity in the genetic investigation of classical galactosemia grew from information made available first from cloning an expressible GALT cDNA (Flach et al., 1990; Reichardt and Berg, 1988) and subsequently from cloning the full-length human GALT gene (Leslie et al., 1992). The gene, located at chromosome 9p13 (Shih et al., 1984), spans about 4.3 kb of DNA arranged into 11 exons. The cDNA is 1295 bases in length and encodes a polypeptide of 379 amino acids. The active protein is a dimer with an estimated molecular mass of 88 kDa.(figure-1 chromosome 9p13)



MATERIAL AND METHODS

Total 20 primary female infertility samples were collected from the Krishna Medical Centre (Private Clinic) Lucknow. Subjects that were already evaluated for cause of infertility and where no cause could be ascertained were included in female infertility category. Along with this 35 healthy fertile female were also taken as control for the present study. The written informed consent has been obtained from all the patients and controls.

DNA extraction from whole blood and DNA extraction was done by standard phenol-chloroform method described by Poncz et al (1982) with minor modifications. Following steps were used.

To the whole blood (2 ml), RBC lysis solution (NH4Cl 7 g/lit and NH4HCO3 0.072 g/lit) was added and mixed thoroughly by inverting the tubes several time. The tubes were then centrifuged at 2500rpm at 40C for 10 min, the supernatant was discarded. This procedure was repeated until the pellet becomes colorless. This pellet is enriched with WBC's.The WBC pellet was suspended in 4 ml of STE solution [0.05 M NaCl, 0.01 M Tris Buffer and 0.007M EDTA pH (6.5)]. To this 200Ul of 10% SDS and 20 Ul of (100 Ug/ml) of proteinase K was added. The tubes were kept for overnight incubation at 37ºC. Complete lysis of WBC was judged by progressive increases of viscosity of the solution.An equal volume of Tris saturated phenol [200mM Tris buffer (pH8.0), 0.01% Hydroxyquinoline] was added to the WBC lysate, mixed well and allow standing for 10 min at 40C then centrifuged at 2500 rpm at 40C for 10 minutes. The aqueous phase was recovered for further use. To this an equal volume of a mixture of chloroform: isoamyl alcohol (24:1v/v) and tris saturated phenol was added and allowed to stand at 4°C for 10 minutes and centrifuged at 2500 rpm at 4^0C for 10 minutes. The aqueous phase was recovered for precipitation of DNA.

PRECIPITATION OF DNA

To the aqueous phase, approximately 5 volume of chilled absolute ethanol was added. As ethanol was being added DNA fibers solve out of the solution and become visible to the naked eye. The DNA was recovered by centrifugation at 2500 rpm at 4^0C for 10 minutes. The tubes were kept for 1-2 hours at 4^0C for complete precipitation of the DNA. The precipitated DNA was separated and air dried and was dissolved in 200 µl of HPLC grade water. The high molecular weight DNA was allowed to dissolve completely by keeping it at 37^0C for a night.The quality and quantity of the DNA was checked 0.8% agarose gel electrophoresis.

The polymerase chain reaction (PCR) technique was used for detecting the polymorphisms of selected genes and for amplification of the gene needed for sequencing analysis via enzymatic replication. PCR conditions for mutation analysis of Q188R, K285 and N314D are same.

PCR was performed by using the programs- Initial denaturation at 95^0C for 12 min, for 35 cycle. Denaturation at 94^0C for 30 sec, annealing at 55^0C for 30 sec, extension at 72^0C for 1min, final extension at 72^0C for 7 min. The PCR products were then checked on 1.5% of agarose gel and visualized under UV trans illuminator. 8µL of the PCR products were then aliquoted in separate PCR tube for RFLP to which 10U/tube (PCR product 8µl+1µl R.E./tube means 10U/tube) of the restriction enzyme was added and was kept overnight in incubator at 37°C. The restricted fragments were checked on 1.5 % gel.

MUTATION ANALYSIS OF GALT-1 GENE

Q188R, K285N and N314D mutations of the GALT gene were identified by using the polymerase chain reaction and RFLP analysis (LukacBajalo et al 2002, Karas et al 2003). PCR was performed for Q188R, K285N and N314D mutations after that amplified products were digested with restriction enzymes Hpa II, Ava II and Tsp 509I respectively. Primer sequences for different mutation given in table 3.

Table 3. Oligonucleotide sequences of the primers of GALT-1 gene.

Primer names	Primer sequence (5'-3')
K285N	F- GATGGAGGTTGCTCCCAGTA
	R- AGCACAAGGGCAACAGAAGT
Q188R	F- GGGTCGACGTCGGATGTAACGCTGCCACTCA
	R- GGGGACACAGGGCTTGGCTCTCTCCCA
N314D	F- GGGTCGACGAGATGCTGGGACTGAGGGTGGAGCA
	R- GGGGTCGACGCCTGCACATACTGCATGTGA

RESULT

In The proposed study, we have taken 20 primary female infertility cases and 35 healthy fertile female as control. All the subjects were already evaluated for the known causes of the infertility.

MOLECULAR ANALYSIS

Characterization of the Q188R, K285N and N314D mutations, The PCR-RFLP analysis for the evaluation of Q188R, K285N and N314D mutations of the GALT-1 gene is shown in Figure 2,3 and 4.

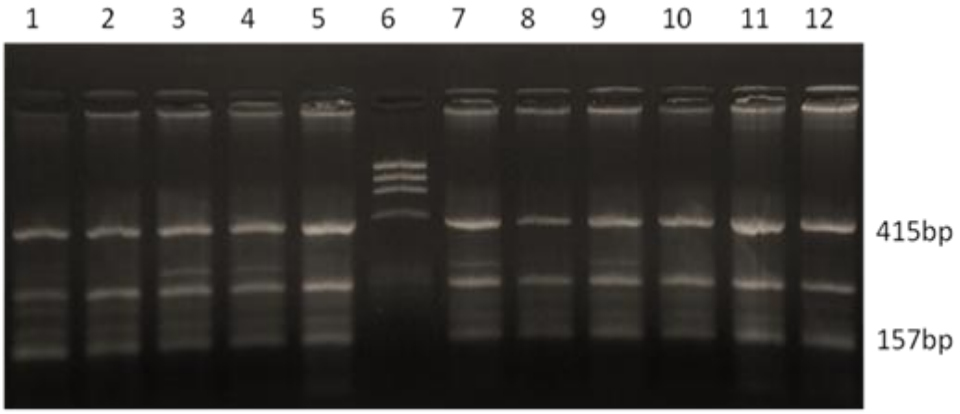


Figure 2. Gel picture showing the RFLP analysis of Q188R mutation, Lane 1-5, 7-12: Normal; Lane 6: Molecular weight marker.

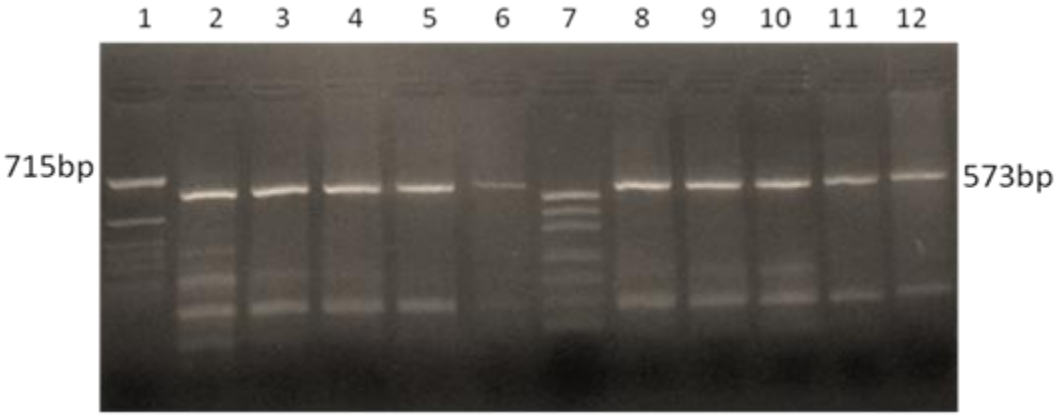


Figure 3 Gel picture showing the RFLP analysis of K285N mutation, Lane 1: Undigested PCR product; 2-6, 8-12: Normal; Lane 7: Molecular weight marker.

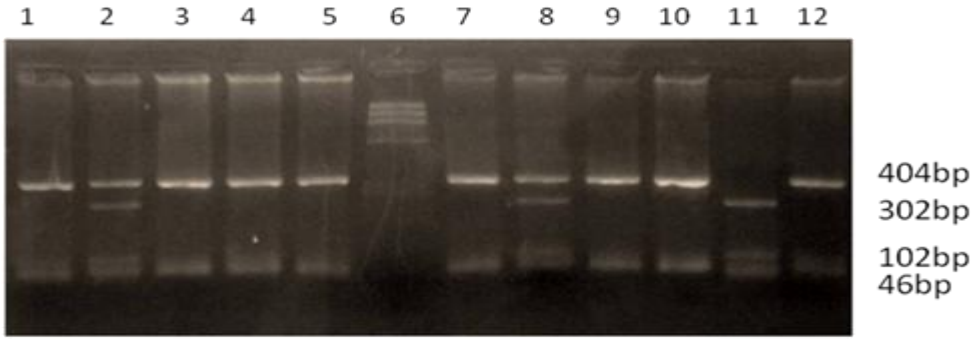


Figure 4 Gel picture showing the RFLP analysis of N314D mutation, Lane 2and 8: Heterozygous; Lane 11: Homozygous, Lane 1,3-5,7,9,10,12: Normal, Lane 6: Molecular weight marker.

ASSOCIATION OF GALT-1 GENE MUTATIONS WITH PRIMARY FEMALE INFERTILITY:

After the molecular analysis of GALT-1 gene mutations among the 20 primary female infertility cases and 35 healthy fertile female; none of the case or control was found to be positive forQ188R and K285N mutations.

In the cases we have found 16/20 (80%) wild type genotype, 03/20 (15%) heterozygous and 01/20 (5%) homozygous mutant genotype. However, in the control group 27/35 (77.1%) having wild type genotype, 06/35 (17.1%) having heterozygous genotype and 02/35 (5.7%) having homozygous mutant genotype. The distribution of wild type (A) and mutant allele (G) frequencies were 35/40 (87.5%) and 05/40 (12.5%) respectively in the cases whereas in the control group the distribution of wild type and mutant allele was 60/70 (85.7%) and 10/70 (14.2%) respectively. The distribution of the N314D alleles in the group of infertile women was not significantly different from that in control (p=0.79). The odds ratio of 0.86, (95% 0.23-3.04) suggests that there is no risk of infertility in the presence of mutant allele.

Table 3. Distribution of N314D mutation genotypes and allele frequency in the cases and controls.

Groups	**Genotype**			**Allele frequency**	
	AA	**AG**	**GG**	**A**(Wild type)	**G** (Mutant)
Cases (20)	16 (80%)	03 (15%)	01 (5%)	35 (87.5%)	05 (12.5%)
Control (35)	27 (77.1%)	06 (17.1%)	02 (5.7%)	60 (85.7%)	10 (14.2%)
P value, OR (95% CI)				0.79, 0.86 (0.23-3.04)	

DISCUSSION

Cramer *et al.* suggested the association of female infertility with the presence of heterozygosity for the Duarte-2 variant or classic galactosemia. Most likely, only the Duarte-2 variant with decreased GALT activity presents a risk for reproductive dysfunction. Using DNA analysis to detect the Q188R, K285N and N314D mutations, no association between these mutations and female infertility was found in our study. We did not found any significant association between the distribution of mutant allele of N314D mutation in cases and control. Our results are in concordance with the findings of Bajalo et al in 2005; they have performed the study in Solvenion population. Although molecular analysis of infertile women shows no connection between the three most common GALT mutations (i.e., Q188R, K285N, and N314D) and female infertility, it is suggestive that these mutations along with the other mutations in the GALT gene could be responsible for the decrease in GALT activity and should therefore be studied in the future with large number of samples.

CONCLUSION

Our data support the conclusion that there is no association between the Q188R and K285N mutations and N314D associated alleles with primary female infertility. One of the oldest human problems with widespread prevalence is infertility. Apart from the social, economic consequences, infertility has a serious impact on husband-wife relationship including their physical and mental health. In the society childlessness is a challenging condition to the couple and has to face many social and family problems. To overcome this, a large number of investigations as well as hormonal assessments are done to diagnose and manage the infertility. So there are still needs further researches on this topic for preventing of this type of social and family problems.

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Corresponding author: Dr. Ajay Vardaan, Assistant Professor, Officiating Head, Dept. of Medical Gastroenterology, King George's Medical University, Lucknow-226003, (UP) India.
Email: drajaymd12345@gmail.com jsa.amardeep@gmail.com durgeshbio7@gmail.com ravi.mishra8@gmail.com
Contact: 91 945551930691 / 9454321122 / 91 9450784933 / 919415027871