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Original Article

Genetics Section

Assessment of Glu504 Lys Genotype and Single Nucleotide Polymorphisms in Exon 12 and 13 of ALDH2 Gene in Alcoholic Liver Cirrhosis Patients in Northern Karnataka, India: A Cross-sectional Study

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ABSTRACT

Introduction: Alcoholic Liver Disease (ALD) is one of the most significant issues affecting the world today and is the principal cause of atleast 60 of the most significant forms of systemic disorders. The metabolic breakdown of alcohol into acetaldehydes is catalysed by Alcohol Dehydrogenase (ALDH).

Aim: To find out the Glu504 Lys genotype and Single Nucleotide Polymorphisms (SNPs) in the exon 12 and 13 of ALDH2 gene in alcoholic liver cirrhosis patients.

Materials and Methods: This cross-sectional study was conducted at the Department of General Medicine and Genetics Laboratory of the BLDE (Deemed to be University) Shri BM Patil Medical College Hospital and Research Centre (SBMPMC), Vijayapura, Karnataka, India. The study period was from January 2021 to June 2022. ALD patients were recruited. For the present study, 32 patients with ALD symptoms were recruited, and a total of 32 age- and sex-matched controls were included. Patients with existing or past co-infections with Hepatitis B or C and other causes of chronic liver disease were excluded. Blood samples were collected from all patients and subjected to genetic analysis; Polymerase Chain Reaction (PCR) products were then analysed using Sanger-based Deoxyribonucleic Acid (DNA) sequencing.

Results: The mean age of all patients and controls in the present study was 44.06 and 52.09 years, respectively. Out of 32 cases, two mutations were found in exon 13: g.47794 A>T (heterozygous) and g.47854 T>G (heterozygous). These mutations occurred in patients who were younger (mean age 29.5 years) and consumed less alcohol (108 g/day) for a shorter duration (5.5 years) compared to the remaining cases. No SNPs were found in exon 12 of the ALDH2 gene.

Conclusion: Mutations in the exon 13 regions of the ALDH2 gene may be responsible for early predisposition to the disease. Early genetic analysis in selected populations to identify these mutations may help prevent the occurrence of the disease. An association study of the ALDH2 gene with ALD will be conducted in larger samples, along with biochemical and other clinical investigations, to determine the association of these gene polymorphisms.

Keywords: Alcohol dehydrogenase, Alcoholic liver disease, Heterozygous mutation

INTRODUCTION

According to the World Health Organisation (WHO), approximately three million deaths are attributed to alcohol consumption annually. Around 48% of these deaths are due to liver cirrhosis based on global data. The development of Alcohol-related Liver Disease (ALD) is higher in men who consume ≥14 drinks per week compared to women who are at an increased risk for liver injury by consuming ≥7 drinks per week [1]. Alcoholic steatosis, which develops in more than 90% of drinkers, can be reversed by abstinence [2]. Between 10-15% of heavy drinkers may develop alcoholic cirrhosis, severe fibrosis, or alcoholic hepatitis if they continue to consume alcohol excessively [3].

Alcohol is metabolically degraded in the body into acetaldehyde primarily through ALDH in the liver. ALDH then converts acetaldehyde into acetate. Due to acetaldehyde's low molecular weight, mitochondrial ALDH2, among ALDH1 and ALDH2, is crucial for human acetaldehyde metabolism [4]. Alcohol dependency and alcohol-induced liver disease were previously believed to be hereditary conditions that could be influenced by the ALDH2 gene [5].

In recent years, many people have been affected by liver diseases, which is significantly low compared to the high number of alcohol consumers. This points to specific pathomechanics that may play a role in certain individuals. From a research perspective, there are close interactions between genetics and the environment, in addition to other factors. Genetics play a role in the form of genotypes, variants, and their associated genes to understand the mechanisms such as biochemical processes and pathogenicity involved in the development of ALD at the molecular level. An SNP in exon 12 of the ALDH2 gene predicts Lysine instead of Glutamic acid at position 504. The 504 Lys allele restricts the acetaldehyde metabolism of an isoenzyme, creating a catalytically inactive isoenzyme [6]. Individuals carrying the Lys gene have a reduced ability to excrete acetaldehyde and often experience side effects such as nausea, flushing, and vomiting after alcohol consumption [7]. People with this gene may be less likely to consume alcohol excessively and may experience slower disease progression due to these negative effects [8].

In light of this background, a present study has been undertaken to identify the Glu504 Lys genotype and SNP in exon 12 and 13 of the ALDH2 gene in alcoholic liver cirrhosis. The objective of the present study was to determine the significant association of Glu504 Lys in exon 12 and exon 13 SNP in alcohol liver disease and controls within the study population.

MATERIALS AND METHODS

The cross-sectional study was conducted in the General Medicine and Genetics Laboratory of the BLDE (Deemed to be University) Shri BM Patil Medical College Hospital and Research Centre in

Vijayapura, Karnataka, India. The study period was from January 2021 to June 2022, and it involved 32 patients admitted to the hospital with alcohol liver disease. Before conducting the study, an Institutional Ethical Committee Certificate (IEC) (IEC/NO-09/2021 dated 22/01/2021) was obtained from the Institutional Ethical Committee of the study institute. The detailed procedure and purpose of the study were explained to the patients and their families, and informed consent was obtained before collecting the blood samples.

Inclusion and Exclusion criteria: The inclusion criteria for the present study were a history of excessive alcohol consumption and a diagnosis of alcoholic cirrhosis. Patients with existing or past co-infections with Hepatitis B or C and other causes of chronic liver disease were excluded from the study. The control group included individuals with no history of excessive alcohol consumption, no liver diseases, and normal Liver Function Tests (LFT).

Sample size calculation: The sample size for the study was calculated based on the Anticipated Proportion of the ALDH2 gene in Non alcoholic Fatty Liver Disease (NAFLD) (23.7%) and in ALD (4.5%) [9]. The present study required a sample size of 32 per group (a total sample size of 64 assuming equal group sizes) to achieve a power of 80% for detecting a difference in proportions between the two groups at a two-sided p-value of 0.05.

Formula used: $n=(z\alpha+z\beta)^2$ 2 p*q

MD2

Where, Z=Z statistic at a level of significance

MD=Anticipated difference between two proportions

P=Common proportion

q=100-p

Study Procedure

Laboratory Investigations including liver function tests, Haemoglobin Levels, Platelet Counts, Mean Corpuscular Values (MCV), Ultrasonography (USG) abdomen and pelvis, upper GI endoscopy if indicated were conducted for all the patients. A peripheral blood sample of 1 mL was collected in K2 Ethylenediamine Tetraacetic Acid (EDTA) Coated vacutainer and stored in a 4-degree refrigerator from each patient for analysing ALDH2 polymorphism.

Molecular analysis: DNA was extracted from 200 µL of blood samples using a DNA isolation kit (NucleoSpin Blood, Mini kit from Machery Nagel Duren Germany). All the extracted DNA samples were quantified using a Multimode reader (Teckon). Primers were designed as shown in [Table/Fig-1]. (Reference Sequence: NG_059281.), and the amplicon size was appropriate for the Genetic Analyser (ABI 3500xl) using Primer-3 (Bioinformatics tool). The designed primer was confirmed through the Primer Quest Tool for the amplification and also confirmed in Basic Local Alignment Search Tool (BLAST) (Bioinformatics tool) for the specificity of primer binding in genomic DNA. The PCR reaction was performed in a 20 µL reaction volume containing 0.5-1 µL of genomic DNA (75 ng/µL to 100 ng/µL), 0.4 µL of each primer (5 pmol), 10 µL readymade master mix (Takara, Japan), and the total volume was adjusted to 20 µL using milli q water. These PCR Products were subjected to a Thermocycler (Biorad, USA) under the following Cycle conditions: An initial denaturation at 95°C for one minute, followed by 35 cycles at 95°C for 30 seconds (cycle denaturation), primer annealing temperature was 58.50 and 60°C for one minute, 68°C for one minute (primer extension) and a final extension at 68°C for five minutes. Polymerase Chain Reaction (PCR) results were confirmed by agarose gel electrophoresis with a 100 bp ladder. PCR products were subjected to Sanger-based sequencing (ABI_3500xl). For this, forward and reverse primers were used for the samples. Sequence and Electropherograms quality were assessed by Sequence Analysis Software (ABI). Sequence alignment was carried out by Variant Reporter Software (ABI).

S. No.	Name of the primer	Oligo sequence	Product length	Annealing temp
1	ALDH2 12F	5'-TTTGGTGGCTAGAAGATGTC-3'	187	58.5°C
2	ALDH212R	5'-CACACTCACAGTTTTCTCTT-3'		
3	ALDH2-EX-13-F	5'-ATCATGCAAGCTTCCTCCCT-3'	213	60.0°C
4	ALDH2-EX-13- R	5'-ACTCTTACCCTCAGCCAACC-3'		

[Table/Fig-1]: Primer sequences and annealing temperatures used for PCR reaction.

STATISTICAL ANALYSIS

The obtained data was documented in a Microsoft Excel sheet, and statistical analysis was conducted using statistical analysis software Statistical Package for Social Sciences (SPSS) (Version 20.0). The results were presented as Mean (Median)±SD. For normally distributed continuous variables between two groups, an independent t test was used, while for not normally distributed variables, the Mann-Whitney U test was employed. Categorical variables between the two groups were compared using the Chi-square test. The correlation coefficient was utilised to determine the correlation between quantitative variables. A p-value of <0.05 was considered statistically significant. All statistical tests were performed using a two-tailed approach.

RESULTS

The mean age of all patients and controls in the present study was 44.06 and 52.09 years, respectively. The duration of alcohol consumed was 173.88±60.441 grams/day. Other parameters such as Basal Metabolic Index (BMI), total Bilirubin, Aspartate aminotransferase (AST), Alanine Transaminase (ALT), Haemoglobin (Hb), Mean Corpuscular Volume (MCV), and Platelets are tabulated in [Table/Fig-2].

The mean age of patients with mutations is lower compared to other patients (29.5±6.364 years). AST and ALP levels were higher in patients with exon 13 mutations, indicating more liver injury in mutated patients (182.5 and 200 U/L for AST and ALP, respectively) [Table/Fig-3]. The duration of alcohol intake is significantly lower in patients with exon 13 mutations than the control group (5.5 years vs 13.467 years). The amount of alcohol consumption is also lower in patients with exon 13 mutations compared to the control group (108 grams/day vs 178.267 grams/day) as shown in [Table/Fig-3,4].

Sequencing analysis of Exon 12 and 13 of the ALDH2 gene was conducted in 32 alcoholic liver cirrhosis patients and 32 controls. Mutations in exon 13 were found in two patients, while no mutations in exon 12 were identified. The exon 13 mutations were located in the Untranslated Region (3'UTR) as shown in [Table/Fig-5]. The 3'UTR of the messenger Ribonucleic Acid (mRNA) region is transcribed from DNA but will not be translated into protein. The 3'UTRs regulate mRNA-based processes such as mRNA stability, mRNA localisation, and translation. Electropherograms and sequence quality were assessed using software provided by ABI, namely Sequence Analysis (ABI). Alignment of these sequences was performed using Variant Reporter Software (ABI) as shown in [Table/Fig-6,7].

DISCUSSION

Alcohol is a psychoactive drug and a dependence-producing substance. In today's generation, strong spirits are a common part of social gatherings for many populations. Approximately 3 million deaths occur every year due to alcohol consumption, contributing to worldwide disabilities and poor health for millions of people. Overall, alcohol consumption is highly detrimental and is considered one of the global burden diseases [10].

The ALDH2 gene is a mitochondrial enzyme that catalyses the oxidation of acetaldehyde, an intermediate in ethanol metabolism [11]. In East Asian subpopulations, the Glu504Lys SNP of the ALDH2 gene has an incidence of 35-57%, which leads to defects

Clinical parameters	Case/controls	N	Mean	SD	Mann-Whitney U test/ t-test value	p-value	Normal range
Age (years)	Alcoholic Liver Disease (ALD)	32	44.063	11.345	281.5	0.002	---------
	Control	32	52.094	11.203			
Total bilirubin (mg/dL)	ALD	32	9.969	15.017	967	<0.001	1.71-20.5 µmol/L
	Control	32	0.941	0.405			
AST (U/L)	ALD	32	110.156	115.428	914	<0.001	8-33 U/L
	Control	32	31.938	19.536			
ALT (U/L)	ALD	32	43.25	32.277	732.5	0.003	7-56 U/L
	Control	32	25.938	19.351			
ALP (U/L)	ALD	32	166.031	76.442	958	<0.001	30-130 U/L
	Control	32	63.156	30.808			
Platelets (thousands)	ALD	32	150.813	60.236	86.5	<0.001	150,000-450,000 per microlitre
	Control	32	302.406	110.427			
BMI (kg/m^2)*	ALD	32	30.209	2.709	11.694	<0.001	18.5-24.9 kg
	Control	32	22.844	2.314			
Hb (g/dL)*	ALD	32	9.553	1.749	-8.451	<0.001	13.2-16.6 g/dL
	Control	32	13.059	1.566			
MCV (fL)*	ALD	32	95.478	4.229	3.948	<0.00	80-100 fL
	Control	32	89.362	7.675			

[Table/Fig-2]: Demographic data of alcoholic liver cirrhosis patients and controls from study cohort.

Parameters	Group	N	Mean	Normal range
Age	ALD	2	29.5±6.364 years	------
AST (U/L)	ALD	2	182.5	8-33U/L
ALP (U/L)	ALD	2	200	30-130 U/L

[Table/Fig-3]: Data showing clinical association of two patients with mutations.

Parameters	Group	N	Mean	SD	Mann-Whitney U test value	p-value
Hb (g/dL)	ALD	2	11.6	1.273	51.500	0.120
	Controls	30	9.417	1.704		
MCV (fL)	ALD	2	95.7	1.556	29.500	1.000
	Controls	30	95.463	4.362		
Platelets (thousands)	ALD	2	105	21.213	11.500	0.161
	Controls	30	153.867	60.899		
Duration of alcohol intake (years)	ALD	2	5.5	2.121	0.00	0.020
	Controls	30	13.467	4.125		
Amount of alcohol per day (gm)	ALD	2	108	50.912	11.00	0.129
	Controls	30	178.267	59.131		

[Table/Fig-4]: Data showing clinical association of two patients with mutations.

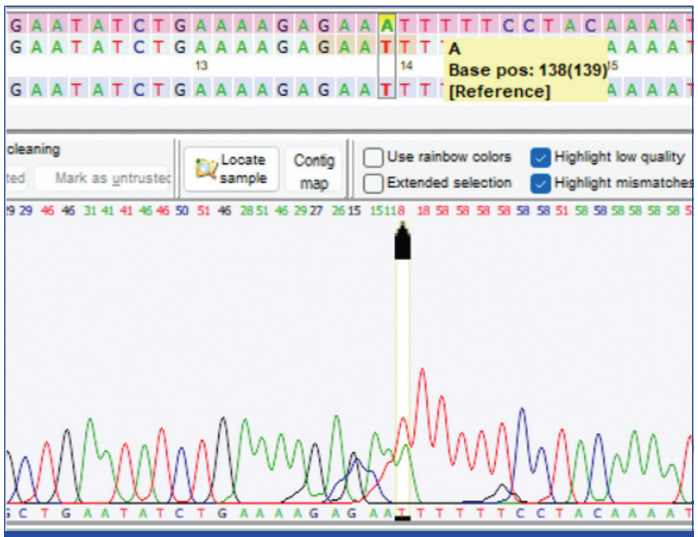
S. No.	Base position in genomic DNA	Nucleotide change	Mutation type	gDNA (NG_012250.2)	Variants
1	g.47794 A>T	A-T	Transition	Heterozygous	3'UTR
2	g.47854 T>G	T-G	Transversion	Heterozygous	3'UTR

[Table/Fig-5]: Details of ALDH2 polymorphism analysis.

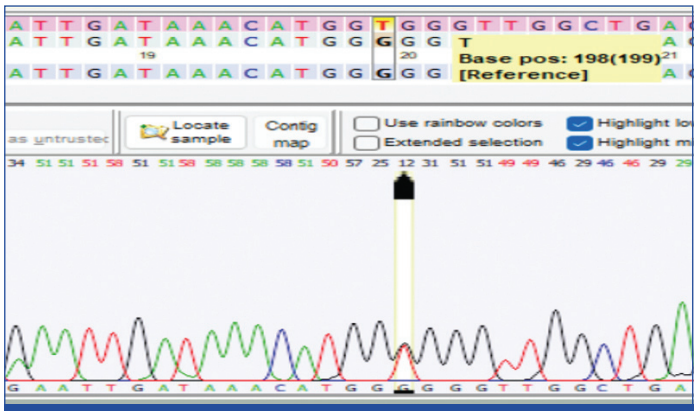
in ALDH2 enzyme activity, altering acetaldehyde metabolism and reducing alcohol tolerance [12].

Chang B et al., studied ALDH2 with Glu504Lys variants and ALD, and their results suggested that the 504 Lys variant is negatively associated with ALD compared to those with ALDH2 504 Glu variants [1]. Another study from the Taiwan population suggested that the ALHD2 rs671 (G>A, missense variant Glu504Lys, exon 12) genotype is a risk factor for spontaneous deep intracerebral haemorrhage [13].

Li D and Zao H investigated the effect of the ALDH gene with the ALDH2 504 Lys allele on alcoholism and alcohol-induced diseases. Their results concluded that the ALDH2 504 Lys allele could significantly lower the risk for alcohol dependence [14]. Hao X and



[Table/Fig-6]: Nucleotide base adenine replaced by thymine leading to 3'untranslted region.



[Table/Fig-7]: Nucleotide base thymine replaced by guanine leading to 3'untranslted region.

Zeng Q stated that AA and GA genotypes of ALDH2 rs671 are factors associated with an increased probability of Non Alcoholic Fatty Liver Disease (NAFLD) among Chinese subjects [15]. In the current context, multiple studies have been conducted in the East Asian population, including China and Japan, while there is limited data on genes/polymorphisms that confer susceptibility to ALD in the Indian community. A study was conducted to investigate the

association between the Glu504Lys genotype and ALDH2 gene polymorphism with ALD [16]. In the present study, the authors observed no association of ALDH2 Glu504 Lys polymorphisms with alcohol liver disease.

Mansoori AA and Jain SK conducted a study on ALDH2, ADH1B, GSTT1, and GSTM1 genes. Their results concluded that the Central India population is at risk for liver disorders due to the association of GSTM1, GSTT1, and ALDH2 gene polymorphisms [17]. Another study from All India Institute of Medical Sciences (AIIMS) New Delhi focused on ADH1B and ALDH2 gene polymorphisms with alcohol dependence, suggesting that the ALDH2 genotype has a higher frequency in alcohol-dependent subjects and is a risk factor for alcohol dependence [18].

Limitation(s)

There were no exon 12 (ALDH2 Glu504 Lys) polymorphisms found in the 32 cases. However, during the sequence analysis of exon 13 of the ALDH2 gene, the authors identified two mutations in exon 13 out of the 32 cases: g.47794 A>T (heterozygous) and g.47854 T>G (heterozygous). These mutations occurred in patients who were younger and had consumed less alcohol compared to the remaining cases. Additionally, based on the present study findings, with a limited sample size due to the non availability of data from an ethnically matched age and sex control population, the sample size was limited. The present study spanned one year and five months for patient recruitment, and their willingness to participate in the study was a contributing factor to the limited sample size.

CONCLUSION(S)

Alcohol consumption is a causative factor for several health problems, including injuries, liver cancers, and other associated diseases, depending on the amount and duration of alcohol intake. In the present study, the prevalence of Glu504Lys variants in the ALDH2 gene with ALD is absent. However, authors identified mutations in exon 13 of the ALDH2 gene, where patients had consumed less alcohol for a shorter duration. Hence, they concluded that mutations in these regions may be responsible for predisposing individuals to early disease onset. Early genetic analysis in selected populations to identify these mutations could help prevent the occurrence of the disease. The present study highlights that polymorphisms in the ALDH enzyme influence the development of AD. Further screening will be conducted on a larger sample size, along with the need for detailed clinical investigations associated with candidate gene polymorphisms.

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