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ORIGINAL ARTICLE

Alcohol Metabolizing Gene Polymorphisms as Genetic Biomarkers of Alcoholic Liver Disease Susceptibility and Severity: A Northeast India Patient Based Study

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Abstract:

Background: Excessive alcohol consumption is associated with genetic predisposition to Alcoholic Liver Disease (ALD), but there is very limited data on both molecular and genetic aspects of ALD among the Northeast Indian (NEI) population. *Aim and Objectives:* Screening the role of genetic alterations in alcohol metabolizing pathway genes in the pathogenesis of ALD which is prevalent in the ethnically NEI population. *Material and Methods:* Whole blood was collected from ALD patients (n=150) [alcoholic chronic liver disease (CLD, n=110) and alcoholic cirrhosis (Cirr/cirrhosis, n=40)], Alcoholic Without Liver Disease (AWLD, n=93) and healthy controls (HC/controls, n=274) with informed consents along with Fibroscan based liver stiffness measurement (LSM) score and clinical data. *Alcohol Dehydrogenase* 2 (*ADH2*) and *Aldehyde Dehydrogenase* 2 (*ALDH2*) genotyping was studied by Polymerase Chain Reaction with Confronting Two Pair Primers (PCR-CTPP); and *Alcohol Dehydrogenase* 3 (*ADH3*) by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. *Results: ADH2**2 genotype was predominant and associated with increased risk of cirrhosis compared to healthy controls, AWLD and CLD cases; and CLD compared to AWLD cases. *ADH3**1 genotype was associated with significantly increased risk of cirrhosis compared to healthy controls, AWLD and CLD cases ($p<0.001$). Variant *ALDH2* genotype was rare and analysis of the joint effects of genotypes showed that higher variant genotype resulted increased risk of CLD and cirrhosis compared to AWLD, and cirrhosis compared to CLD; thereby confirming the association of the polymorphisms in key alcohol metabolizing genes in the predisposition to ALD susceptibility and severity. Presence of variant *ADH2, ADH3* and *ALDH2* genotypes correlated with higher LSM scores in ALD. *Conclusion*: Alterations in the alcohol metabolizing genes are critically associated with ALD susceptibility and severity.

Keywords: Alcoholic Liver Disease, *ADH2, ADH3, ALDH2,* Fibroscan

Introduction:

Chronic excessive alcohol consumption is associated with the development of Alcoholic Liver Disease (ALD) including steatosis, fibrosis, and advance liver diseases like cirrhosis and liver cancer [1, 2]. ALD develops in a subpopulation of chronic alcohol consumers, irrespective of individuals consuming less or more alcohol than the recommended levels i.e. 0 - 21 units (168 g) a week for men and 0-14 units (112 g) a week for women according to the International Center for Alcohol Policies (ICAP) Reports 14, 2003), and the load of ALD cases differs geographically. Prevalence of alcohol dependence rate is lower in Asian populations while compared to other ethnic groups and within Asians itself, it differs across

ethnic subgroups [3-5]. Alcohol consumption and individual's genetic makeup and interactions between the environmental and genetic factors are the main factors underlined to be associated with the liver disease [5, 7]. ALD is a major health economic problem in Northeast India (NEI), where consumption of indigenously prepared alcohol is customary in majority of the tribal communities who are ethnically distinct and tribal dominated compared to other parts of India, and is a leading cause of mortality in these tribal dominated societies. Although the genetic predisposition to ALD have been studied in some Indian population, but very limited data is available on both molecular and genetic aspects of alcohol-related liver disease among the NEI population which needs evaluation and addressing [8,9]. Along with the quantity and quality of the alcohol being consumed, genetic alterations in critical alcohol metabolizing genes had been previously shown to be associated with the pathogenesis of the disease and progression to severe state [10]. The ethanol is mainly metabolized and eliminated by involvement of two primary enzymes *Alcohol Dehydrogenase* (ADH) and mitochondrial *Aldehyde Dehydrogenase* (ALDH) [11]. The oxidation reactions involved during alcohol metabolism through the action of these genes alter the cellular metabolism with harmful effects on lipid and carbohydrate metabolism. Oxygen derived free radicals may cause direct hepatocyte injury by lipid peroxidation whereas acetaldehyde binds covalently to proteins forming adducts and may serve as neoantigens. A functional polymorphism in the *ADH2* gene (*Arg47His)*, *ADH3* (*Ile349Val*) and *ALDH2* gene (*Glu487Lys)* has been shown to alter enzyme efficiency and thereby alter alcohol detoxification rates leading to liver disease [12-15]. Since no studies are available on the associative role of genetic alterations of *ADH2*, *ADH3* and *ALDH2* genes in susceptibility and severity of alcohol related liver disease from the ethnically distinct tribal dominant NEI population; the present study herein focuses to evaluate the role and prognostic significance of the *ADH* and *ALDH2* genotypes in ALD pathogenesis in northeast India, and may be utilized for early prognosis, screening and clinical interventions of alcoholic cases thereby limiting ALD progression to severity.

Material and Methods:

Patient Enrolment and Stratification:

Three ml of whole blood samples collected from 150 cases of clinically proven ALD enrolled in the Central Hospital, NF Railway, Guwahati and Diphu Civil Hospital, Assam. Ninety three cases of Alcoholic without Liver Disease (AWLD) with alcoholism history and 274 age-sex matched community based healthy controls. All relevant clinical and biochemical data with informed consents were taken under the supervision of registered medical practitioners. The study was approved by the Institutional Ethics Committee of the participating institutes. The diagnosis of ALD was based on the American Association for the Study of Liver Diseases (AASLD) and American College of Gastroenterology (ACG) practice guidelines which included (i) screening for alcohol abuse based on Cut down Annoyed Guilty Eye-Opener (CAGE) criteria (ii) documentation of alcohol excess and evidence of liver disease (iii) physical examination (iv) hepatic imaging and (v) confirmation of cirrhosis by liver biopsy based examination. The alcohol related liver disease cases were further stratified into two groups- (i) alcoholic chronic liver disease (CLD, n=110), based on confirmation by imaging, biochemical profile, clinical profile and history of regular alcohol intake (quantity) and CAGE criteria along

with Liver Stiffness Measurement (LSM); and (ii) alcoholic cirrhosis (Cirr, n=40), which was confirmed on to by liver biopsy based examination besides above diagnostic techniques. Patients with intravenous drug abusers, diabetes, chronic renal failure, liver disease associated with viral infection and co-infection with other viruses and age group below or above 18 to 60 years were excluded from the study. All the clinically stratified cases and healthy controls were subjected to further blood biochemistry analysis, and liver stiffness measurement based analysis performed by the registered medical practitioner using Fibroscan 420 (*Abott*, India). The LSM score was expressed in kPa; and the data obtained for each case was used for correlation with the genotyping results, disease susceptibility and severity.

DNA Extraction and Genotyping:

Genomic DNA was extracted from 200µl of whole blood samples according to the standard phenol-chloroform method and checked by BioSpectrometer basic (Eppendorf, Germany) for quality and concentrations. Variation in allele frequencies of *ADH2 Arg47His* and *ALDH2 Glu487Lys* genotype was carried out as reported by Tamakoshi *et al.*, (2003) on Polymerase Chain Reaction with Confronting Two Pair Primers (PCR-CTPP) method with slight modification. PCR-CTPP is a method for genotyping with given melting temperatures for two sets (four) of primers that is applicable for most Single Nucleotide Polymorphisms (SNPs) [16]. Genotyping for *ADH3* polymorphism was performed by

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method followed by restriction digestion with *SspI* restriction enzyme [17]. The interpretation of genotyping was done as follows on the basis of band patterns in 3% agarose gel run.

(i) *ADH2 Arg47His* genotype determination:

Wild type genotype (*ADH2**1) is marked by the presence of two bands at 459bp and 219bp; heterozygous genotype (*ADH2**2) yields three bands at 459bp, 280bp and 219bp; and homozygous genotype (*ADH2**3) is represented with two bands at 459bp and 280bp.

(ii) *ADH3 Ile349Val* genotype determination:

*ADH3**1 genotype is represented by the presence of a single band of 130bp; *ADH3**2 is represented by three bands at 63bp, 67bp and 130bp; and two bands at 63bp and 67bp represents homozygous *ADH3**3 genotype.

(iii) *ALDH2 Glu487Lys* genotype determination:

The appearance of two bands of 176bp and 119bp represents wild type genotype; three bands at 176bp, 119bp and 98bp represents heterozygous genotype; and two bands at 119bp and 98bp depicting homozygous genotype respectively.

Statistical Analysis:

Data generated were expressed as mean ± SD whenever necessary. All the statistical analysis was performed using the SPSSv13.0 software (SPSS Inc, Chicago, USA). Mann-Whitney non-parametric test was performed for analysis of *ADH2*, *ADH3* and *ALDH2* genotype distributions between cohorts, and a p value of less than 0.05 was considered statistically significant. The Odd Ratio (OR) analysis was performed to analyze the risk of genotype distribution with disease susceptibility and severity. An adjusted two tailed *P* value less than 0.05 at 95% CI was considered statistically significant.

Results:

Demographical, Biochemical and Clinical Profile of the Enrolled Cases

A total of n=517 cases, including healthy controls (HC, n=274), alcoholic without liver disease

(AWLD, n=93), alcoholic chronic liver disease (CLD, n=110) and alcoholic cirrhosis (Cirr, n=40). The majority of the ALD patients were male. Elevated level of AST was found to correlate with progression in alcohol related disease severity. The liver stiffness measurement score was found to be significantly higher in ALD cases compared to AWLD and healthy control counterparts (Table 1).

***ADH2, ADH3* and *ALDH2* genotyping**

The *ADH2* and *ALDH2* genotyping was performed by PCR-CTPP method, and *ADH3* genotyping was evaluated by PCR-RFLP method. The distribution of *ADH2*, *ADH3* and *ALDH2* genotypes is tabulated in Table 2. For validation of results, 10% of the cases were randomly selected and reanalyzed by another lab member in a blinded manner, and was concurrent with the initial genotyping data. Briefly, the highlights of the genotyping results are summarized below:

(i) *ADH2* genotyping results

Presence of *ADH2**2 genotype was predominant in the studied population cohorts AWLD and ALD cases including healthy controls. The analysis of distribution of the variant genotype in different alcoholic cohorts compared to non-alcoholic controls showed that the presence of higher variant *ADH2* genotype frequency resulted in increased risk of cirrhosis in ALD cases {OR=1.336, p=0.705), whereas higher distribution of wild-type *ADH2**1 genotype resulted in decreased risk of development of alcoholic liver disease in AWLD cases {OR=0.433, p=0.027); underlying the significance of ADH2 genotype in ALD pathogenesis in northeast Indian population. In fact, the presence of variant *ADH2* genotype doubled the risk of ALD {OR=2.053, p=0.121}, CLD {OR=1.824, p=0.187} and cirrhosis {OR=3.088, p=0.135} compared to AWLD cases. Moreover, the presence of higher variant *ADH2* genotype also resulted in increased risk of cirrhosis compared to CLD (OR=1.693, p=0.510} cases (Table 3); which clearly signifies the role of ADH2 variant genotype in both ALD susceptibility and progression to severity.

(ii) *ADH3* genotyping results

ADH3 gene gives two alleles, *ADH3**1 and *ADH3**2 which code respective subunits γ1 of higher activity and γ2 of lower activity towards ethanol. *ADH3**1 has been reported to be associated with alcohol dependence. The distribution of *ADH3* genotypes was comparable between the healthy controls, AWLD and CLD cases; but the presence of *ADH3**1 genotype resulted in significant decreased risk of cirrhosis compared to all these groups (Table 2 and Table 3).

(iii) *ALDH2* genotyping results

The distribution of variant *ALDH2* genotype was an uncommon phenomenon in our enrolled study cohorts [4/517, 0.77%], with only alcoholic cirrhosis patients cohort [3/40, 7.5%] showing higher distribution of variant *ALDH2**2 genotype which was significantly higher compared to both control ($p<0.001$) and AWLD ($p=0.008$). The variant genotype also resulted in increased risk of cirrhosis compared to CLD [OR=8.838, p=0.027] in the ALD cases (Table 3); thereby suggesting the role of the variant *ALDH2* genotype in ALD disease severity.

Gene-Gene Interaction and its Association with Susceptibility and Severity of ALD

Table 4 and Table 5 present a comprehensive analysis of the evaluation of the joint effect or interaction among the gene variants, by virtue of various genotype combinations of *ADH2*, *ADH3* and *ALDH2* genes. The statistical analysis on the

combinatorial or joint effects of two gene combination clearly revealed that variation in *ADH2* variant genotype distribution is the most critical factor for the susceptibility and severity of ALD. In combination with *ADH3* variant genotype, presence of *ADH2* variant genotype was associated with increased risk of CLD (1.577 folds) and cirrhosis (2.036 folds) compared to AWLD, and cirrhosis (1.291 folds) compared to CLD. Moreover, in combination with *ALDH2* variant genotype, presence of *ADH2* variant was found to almost double the risk of CLD compared to AWLD (1.824 folds); and more importantly, increased the risk of cirrhosis compared to controls (3.405 folds), AWLD (6.338 folds) and CLD (3.475 folds) (Table 4).

When the joint effects of the genotypes of all the genes were considered and studied, it also showed that the presence of higher variant genotypes combination resulted in increased risk of CLD (1.577 folds) and cirrhosis (2.036 folds) compared to AWLD, and cirrhosis compared to CLD (1.291 folds); thereby confirming the association of the polymorphisms in key alcohol metabolizing genes in the predisposition to alcohol related liver disease susceptibility and severity (Table 5).

Association of Genotype Variation with Disease Severity within the Cohorts

To further assess the importance of genotype variations within the studied cohorts, the genotyping data was correlated with the LSM score (*kPa*) obtained on Fibroscan screening. The presence of variant *ADH2* genotype was associated with higher LSM score in controls (p=0.002), CLD (p=0.571) and cirrhosis (p=0.013) cases.

The presence of variant *ADH3**1 genotype resulted in increased LSM score in healthy controls (p=0.263) and CLD (p=0.091) cases. Joint effects of variant *ADH2* and *ADH3**1 genotypes was found to be associated with higher LSM score in healthy controls (p=0.003), CLD (p=0.569) and cirrhosis cases (p=0.118); whereas the combinatorial effect of variant *ADH2*+ *ADH3**1+variant *ALDH2* genotypes was found to result in increased LSM score in healthy controls (p=0.003), CLD (p=0.527) and cirrhosis patients (p=0.653) indicating the significance of the genotype variations in severity of liver disease, and the prognostic significance of the genotypes (Fig 1).

Table 1: Demographical, Biochemical and Clinical Profile of Enrolled Cases and Healthy Controls

Cohort	N	Sex		Mean age (years)	Mean ALT (IU/L)	Mean AST (IU/L)	P value	Mean LSM score (kPa)	P value
		Male	Female						
HC	274	196	88	39.20 ± 12.60	34.00± 18.00	39.00 ± 45.00	ref	4.89 ± 1.21	ref
AWLD	93	73	20	43.11 ± 11.22	54.80 ± 27.42	44.15 ± 15.61	0.839	5.67 ± 3.60	0.798
CLD	110	94	16	45.02 ± 13.01	68.45± 58.44	90.06 ±44.48	0.026	28.42 ± 17.69	<0.001
Cirrhosis	40	32	8	45.97 ±11.01	51.00 ± 28.38	133.21 ±85.67	<0.001	63.54 ± 22.47	<0.001

**HC- Healthy Controls, AWLD- Alcoholic Without Liver Disease, CLD- Alcoholic Chronic Liver Disease, Cirr or Cirrhosis- Alcoholic Cirrhosis, ALT- Alanine Aminotransferase, AST- Aspartate Aminotransferase, LSM- Liver Stiffness Measurement*

Table 2: Showing Distribution of *ADH2, ADH3* and *ALDH2* Genotypes in Different Cases Cohorts Compared to Healthy Controls

Analysis for Distribution of *ADH2*							
Cohort	***N***	***ADH2* Genotype**			**Less common genotype**	**P value of variant allele**	**ODD ratio at 95%CI**
		***(ADH2*1)* Wild type (*Arg/Arg*)**	***(ADH2*2)* Heterozygous (*Arg/His*)**	***ADH2*3* Homozygous (*His/His*)**			
HC	274	18[6.57]	256[93.43]	0	256[93.43]	Ref.	Ref.
AWLD	93	13[13.98]	80[86.02]	0	80[86.02]	**0.027**	0.433 (0.203 - 0.922)
CLD	110	9[8.18]	100[90.91]	1[0.91]	101[91.82]	0.577	0.789 (0.343 - 1.814)
Cirrhosis	40	2[5]	38[95]	0	38[95]	0.705	1.336 (0.298 - 5.988)
Analysis for Distribution of *ADH3*							
Cohort	***N***	***ADH3* Genotype**			***ADH3**1 active genotype**	**P value of variant allele**	**ODD ratio at 95%CI**
		***ADH3**1**	***ADH3**2**	***ADH3**3**			
HC	274	98[35.77]	62[22.63]	114[41.60]	98[35.77]	Ref.	Ref.
AWLD	93	33[35.48]	40[43.01]	20[21.51]	33[35.48]	0.961	0.988 (0.604 - 1.615)
CLD	110	36[32.73]	53[48.18]	21[19.09]	36[32.73]	0.573	0.874 (0.547 - 1.396)
Cirrhosis	40	2[5.00]	34[85]	4[10.00]	2[5.00]	**< 0.001**	**0.095 (0.022 - 0.400)**
Analysis for Distribution of *ALDH2*							
Cohort	***N***	***ALDH2* Genotype**			**Less common genotype**	**P value of variant allele**	**ODD ratio at 95%CI**
		Wild type (*Glu/Glu*)	**Heterozygous (*Glu/Lys*)**	**Homozygous (*Lys/Lys*)**			
HC	274	274[100]	0	0	0[0]	Ref.	Ref.
AWLD	93	93 [100]	0	0	0[0]	1.000	NA
CLD	110	109 [99.09]	1[0.91]	0	1[0.91]	0.115	NA
Cirrhosis	40	37[92.5]	3[7.5]	0	3[7.5]	**<0.001**	NA

Value represented as no. of case [%], P value <0.05 was considered statistically significant.
NA: ODDS ratio couldn't be computed because none of the healthy controls had ALDH2 variant genotype.
**HC- Healthy Controls, AWLD- Alcoholic Without Liver Disease, CLD- Alcoholic Chronic Liver Disease, Cirr or Cirrhosis- Alcoholic Cirrhosis.*

Table 3: Showing Distribution of *ADH2, ADH3* and *ALDH2* Genotypes in CLD and Alcoholic Cirrhosis Cases Cohorts Compared to AWLD; and in Alcoholic Cirrhosis Cases Compared to CLD

Analysis for Distribution of *ADH2*									
Cohort	**N**	***ADH2* Genotype**			**Variant genotype**	**P value ***	**ODD ratio at 95%CI**	**P value ****	**ODD ratio at 95%CI**
		***(ADH2*1)* (*Arg/Arg*)**	***(ADH2*2)* (*Arg/His*)**	***ADH2*3* (*His/His*)**					
AWLD	93	13[13.98]	80[86.02]	0	80[86.02]	Ref.	Ref.	Ref.	Ref.
CLD	110	9[8.18]	100[90.91]	1[0.91]	101[91.82]	0.187	1.824 (0.742 - 4.481)	0.510	**1.693 (0.350 - 8.195)**
Cirrhosis	40	2[5]	38[95]	0	38[95]	0.135	**3.088 (0.663 - 14.373)**		
Analysis for Distribution of *ADH3*									
Cohort	**N**	***ADH3* Genotype**			***ADH3**1 active allele**	**P value ***	**ODD ratio at 95%CI**	**P value ****	**ODD ratio at 95%CI**
		***ADH3**1**	***ADH3**2**	***ADH3**3**					
AWLD	93	33[35.48]	36[32.73]	20[21.51]	60[64.52]	Ref.	Ref.	Ref.	Ref.
CLD	110	36[32.73]	2[5.00]	21[19.09]	74[67.27]	0.680	0.885 (0.494 - 1.583)	**0.001**	**0.108 (0.025 - 0.474)**
Cirrhosis	40	2[5.00]	*ADH3**1	4[10.00]	38[95.0]	**< 0.001**	**0.096 (0.022 - 0.422)**		
Analysis for distribution of *ALDH2*									
Cohort	**N**	***ALDH2* Genotype**			**Variant genotype**	**P value ***	**ODD ratio at 95%CI**	**P value ****	**ODD ratio at 95%CI**
		Wild type (*Glu/Glu*)	**Hetero-zygous (*Glu/Lys*)**	**Homo-zygous (*Lys/Lys*)**					
AWLD	93	93 [100]	0	0	0[0]	Ref.	Ref.	Ref.	Ref.
CLD	110	109 [99.09]	1[0.91]	0	1[0.91]	0.358	NA	**0.027**	**8.838 (0.892 - 87.596)**
Cirrhosis	40	37[92.5]	3[7.5]	0	3[7.5]	**0.008**	NA		

Values represented as no. of case [%], statistically P value <0.05 was considered significant.
*P value *: genotype distribution between AWLD and ALD cases; p value **: genotype distribution between CLD and cirrhosis cases cohorts. AWLD- Alcoholic Without Liver Disease, CLD- Alcoholic Chronic Liver Disease, Cirrhosis- Alcoholic Cirrhosis.*

Table 4: Distribution of *ADH2+ADH3, ADH2+ALDH2* and *ADH3+ALDH2* Genotype Combinations in AWLD, CLD and Alcoholic Cirrhosis and Healthy Controls and their Statistical Evaluation

Analysis for distribution of *ADH2+ADH3* combined											
Cohort	**N**	***ADH2+ADH3* Genotype**			**Variant genotype**	**P value**	**ODD ratio at 95%CI**	**P value**	**ODD ratio at 95%CI**	**P value**	**ODD ratio at 95%CI**
		***ADH2**1 + *ADH3**2/3**	***ADH2**2+ *ADH3**2/3 or *ADH2**1+ *ADH3**1**	***ADH2**2 +*ADH3**1**							
HC	274	12[4.39]	170[62.04]	92[33.57]	262[95.62]	Ref.	Ref.	-	-	-	-
AWLD	93	9[9.68]	55[59.14]	29[31.18]	84[90.32]	0.058	0.427 (0.174 - 1.050)	Ref.	Ref.	-	-
CLD	11	7[6.36]	69[62.73]	34[30.91]	103[93.64]	0.418	0.674 (0.258 - 1.759)	0.384	**1.577** (0.563 - 4.411)	Ref.	Ref.
Cirrhosis	40	2[5.00]	36[90.00]	2[5.00]	38[95.00]	0.859	0.870 (0.187 - 4.040)	0.371	**2.036** (0.420 - 9.877)	0.757	**1.291** (0.257 - 6.492)
Analysis for distribution of *ADH2+ALDH2*											
Cohort	**N**	***ADH2+ALDH2* Genotype**			**Variant genotype**	**P Value ***	**ODD ratio at 95%CI**	**P Value ****	**ODD ratio at 95%CI**	**P Value *****	**ODD ratio at 95%CI**
		***ADH2**1 + ALDH2*1**	***ADH2**2/3 +*ALDH2**1 or *ADH2**1+ *ALDH2**2/3**	***ADH2**2/3 +*ALDH2**2/ 2**							
HC	274	22[8.03]	252[91.97]	0[0.00]	252[91.97]	Ref.	Ref.	-	-	-	-
AWLD	93	13[13.98]	80[86.02]	0[0.00]	80[86.02]	0.092	0.537 (0.259 - 1.115)	Ref.	Ref.	-	-
CLD	110	9[8.18]	100[90.91]	1[0.91]	101[91.82]	0.960	0.980 (0.436 - 2.200)	0.187	**1.824** (0.742 - 4.481)	Ref.	Ref.
Cirrhosis	40	1[2.50]	36[90.00]	3[7.50]	39[97.50]	0.211	**3.405** (0.446 - 25.981)	0.049	**6.338** (0.800 - 50.206)	0.219	**3.475** (0.426 - 28.344)

Continued...

Analysis for Distribution of *ADH3+ALDH2*											
Cohort	**N**	***ADH3+ALDH2* Genotype**			**Less common genotype**	**P Value ***	**ODD ratio at 95%CI**	**P Value ****	**ODD ratio at 95%CI**	**P Value *****	**ODD ratio at 95%CI**
		ADH3*2/3 + *ALDH2*1	***ADH3*1+ *ALDH2*1* or *ADH2*2+ *ALDH2*2/3***	***ADH3*1+ *ALDH2*2/3***							
HC	274	89[64.96]	48[35.04]	0[0.00]	48[35.04]	Ref.	Ref.	-	-	-	-
AWLD	93	59[63.44]	34[36.56]	0[0.00]	34[36.56]	0.791	1.069 (0.655 - 1.743)	Ref.	Ref.	-	-
CLD	110	74[67.27]	36[32.73]	0[0.00]	36[32.73]	0.667	0.902 (0.564 - 0.442)	0.568	0.844 (0.473 - 1.508)	Ref.	Ref.
Cirr-hosis	40	35[87.50]	5[12.50]	0[0.00]	5[12.50]	0.004	0.265 (0.100 - 0.698)	0.005	0.248 (0.089 - 0.693)	0.014	0.294 (0.106 - 0.813)

Values represented as no. of case [%], statistically P value <0.05 was considered significant.
*P value *: genotype distribution between cases cohorts and healthy controls; p value **: genotype distribution between AWLD and ALD cases; p value ***: genotype distribution between CLD and cirrhosis cases.*
**HC- Healthy Controls, AWLD- Alcoholic Without Liver Disease, CLD- Alcoholic Chronic Liver Disease, Cirrhosis- Alcoholic Cirrhosis.*

Table 5: Detailed Distribution of *ADH2+ADH3+ALDH2* Genotype Combination in AWLD, CLD, Alcoholic Cirrhosis and Healthy Controls and Their Statistical Evaluation

Analysis for distribution of *ADH2+ADH3+ALDH2* combined											
Cohort	**N**	***ADH2+ADH3+ALDH2* Genotype**			**Variant genotype**	**P value ***	**ODD ratio at 95%CI**	**P Value ****	**ODD ratio at 95%CI**	**P Value *****	**ODD ratio at 95%CI**
		1ADH + *ALDH2*1*	**2/3ADH+ *ALDH2*1 or 1ADH + *ALDH2*2/3***	**2/3ADH + *ALDH2*2/3***							
HC	274	6[4.38]	131[95.62]	0	131[95.62]	Ref.	Ref.	-	-	-	-
AWLD	93	9[9.68]	84[90.32]	0	84[90.32]	0.058	0.427 (0.174 - 1.050)	Ref.	Ref.	-	-
CLD	110	7[6.36]	102[92.73]	1[0.91]	103[93.64]	0.418	0.674 (0.258 - 1.759)	0.384	**1.577** (0.563 - 4.411)	Ref.	Ref.
Cirr	40	2[5.00]	35[87.50]	3[7.50]	38[95.00]	0.859	0.870 (0.187 - 4.040)	0.371	**2.036** (0.420 - 9.877)	0.757	**1.291** (0.257 - 6.492)

*Values represented as no. of case [%], 1ADH= ADH2*1+ADH3*2/3; 2ADH=ADH2*2+ADH3*2/3 or ADH2*1+ADH3*1. Statistically P value <0.05 was considered significant. P value *: genotype distribution between cases cohorts and healthy controls; p value **: genotype distribution between AWLD and ALD cases; p value ***: genotype distribution between CLD and alcoholic cirrhosis cases. *HC- Healthy Controls, AWLD- Alcoholic Without Liver Disease, CLD- Alcoholic Chronic Liver Disease, Cirrhosis- Alcoholic Cirrhosis.*

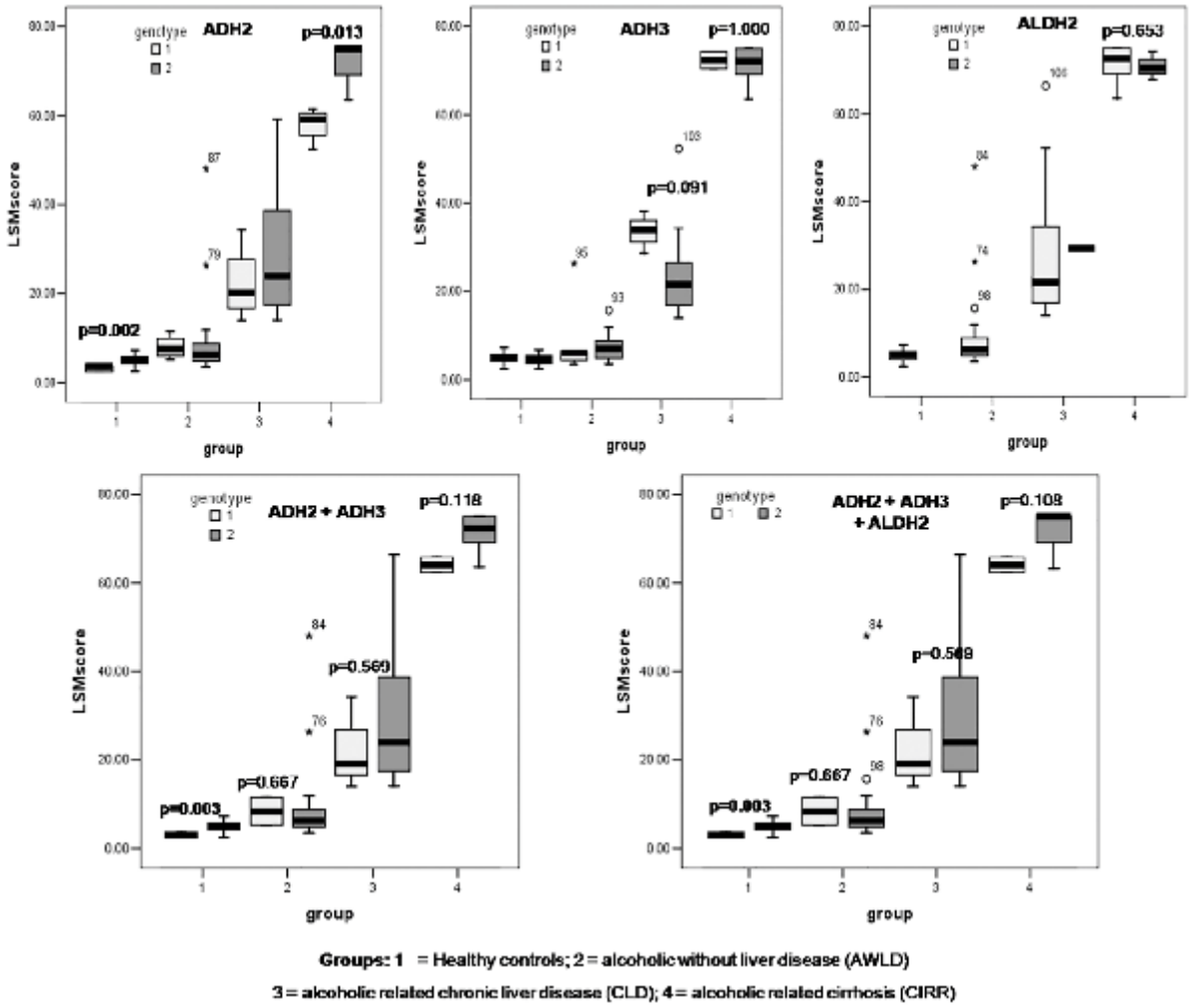

Fig. 1: [Upper panel] Difference in Average LSM Scores (*kPa*) in *ADH21 (genotype 1) v/s *ADH2**2/3 Variant Genotypes (genotype 2), *ADH3**2/3 (genotype 1) v/s *ADH3**1 (genotype 2) and *ALDH2**1 (genotype 1) and *ALDH2**2/3 Variant Genotypes (genotype 2). [Lower panel] Difference in LSM Score Based on Combined ADH genotype (*ADH2+ADH3* genotype) which are Both Present on Chromosome 4, and Based on Combined *ADH* and *ALDH2* genotype showing the Association of Altered Genotypes with Higher LSM Score and therefore the Higher Liver Disease Index.**

Discussion:

Alcoholism remains a major cause of concern of liver disease around the world [18]. ALD resulting due to excessive alcohol consumption is a leading medical burdens of the northeast region in India, where indigenously prepared alcoholic beverages preparation and routine consumption is customary in most of the indigenous tribal communities. Multiple factors have been reported to be associated with ALD susceptibility and severity, including the alterations in genetic factors and gene-environment interactions [19, 20]**.** Although studies on the genetic predisposition to ALD has

been reported from north and south Indians population, but there is very limited data on both molecular and genetic aspects of ALD among the NEI population [21,8,9]. The alcohol metabolization pathway plays a key role in degradation of the consumed alcohol concentration, mediated through the activity of several key genes like *ADH2*, *ADH3* and *ALDH2*. Both *ADH2* and *ADH3* genotype and its polymorphism were highlighted from Asian and east Asian population, and hence it was specifically evaluated in the present study [22, 23]. The present study aimed to identify the associative role of genetic effect(s) of *ADH2*, *ADH3* and *ALDH2* variations in the susceptibility and severity of alcoholic liver disease, considering both the single and joint or additive effects, in the ethnically distinct NEI population.

Amongst the human ADH gene loci, two classes I ADH genes are polymorphic with three alleles existing for either *ADH2* or *ADH3* shows substantially different enzymatic characteristics. According to the differences in the capacity to metabolize alcohol to acetaldehyde, it has been speculated that individuals with the more active *ADH2**2 and *ADH3**1 alleles are at increased risk of developing alcohol-related organ damage due to a higher acetaldehyde exposure. *ADH2**1 genotype is more prevalent in world (90%) and oriental population (30%), and important differences are observed in allelic distribution between oriental people and Caucasian races [21, 24, 25]. *ADH2**2 is detectable in Asians, which encode the low activity β1 and the high activity β2 subunits. The CTPP based PCR amplification analysis for *ADH2* in our studied cohort showed the predominance of *ADH2**2 genotype in the general population, and importantly also resulted in increased risk of cirrhosis development compared to controls, AWLD and CLD cases. Studies have earlier identified that *ADH2**2 allele and genotype *ADH2**2/2 as a risk factor for alcohol related liver diseases [26, 27, 28]. In Asian populations, *ADH2**2 was found higher in controls in comparison to the alcoholics [29, 30, 31, 32, 33]. The frequency of mutant *ADH2**2 allele has been also reported to be rare in north Indian population [8]. This indicates the genetic predisposition of northeast Indian population to ALD severity.

On the basis of kinetic properties of alcohol dehydrogenase gene polymorphism the *ADH3**1 is associated with faster metabolism of alcohol to acetyldehyde. The *ADH3**1 genotype was importantly found to be associated with reduced ALD risk, especially cirrhosis. In this regard, the presence of lower *ADH3**1 genotype distribution in ALD cases from northeast India is beneficial. Higher frequency of *ADH3**1/3*2 in Europeans was earlier demonstrated and most of the studies concluded, that the frequency distribution of allele *ADH3**1 and allele *ADH3**2 are equal in the white race [24]. The study reports of Osier (1999) among the Taiwanese Chinese population stated that the distribution ratio of *ADH3**1 and *ADH3**2 genotype frequency were also not significantly different between alcoholics and healthy controls. But the higher prevalence of *ADH3**2/3 genotype in ALD cases in the NEI population has relevance with both disease susceptibility as well as alcohol dependency since individuals with slow alcohol degradation capacity are more likely to consume alcohol excessively and to develop alcoholism; and it becomes detrimental in a background of *ADH2**2 genotype, which is evident from the gene-gene interactions additive/joint effect analysis, which resulted in increased risk of CLD and cirrhosis compared to AWLD, and cirrhosis compared to CLD [34].

Alcohol gets oxidized into acetaldehyde due to enzyme activity of ADH genes is again oxidized to acetate by the enzyme activity of *Aldehyde Dehydrogenase 2 (ALDH2)* gene and person carrying heterozygous or homozygous due SNP (*Glu487Lys*) has reduced ability to metabolize acetaldehyde. The allelic variation of *ALDH2* results also modifies the drinking behaviour, hence results into the risk of alcoholism in East Asian populations and acetaldehyde metabolism also remarkably becomes different into disease severity in case of inborn error of variant *ALDH2**2 allele homozygosity and heterozygosity [35,36]. The variant *ALDH2* genotype was found uncommon in the studied cohorts, but the presence of higher *ALDH2**2 genotype resulted in significantly increased risk of cirrhosis compared to CLD (p=0.027) by more than eight folds. *ALDH2**2 genotype is also uncommon in north Indian population and its absence has been reported for predisposition towards alcohol dependence and ALD predisposition in north Indian population [8]. Some of the studies found associations between this SNP and alcohol related liver disease in East-Asian population [37].

The distribution of variant *ALDH2**2 varies from 16-35% in the Han Chinese, Koreans, Japanese and Vietnamese, while Mongolians, Tibetans, Thais, Filipinos, Malays and Taiwanese aborigines varies 1-10% and rarely found in black populations, Caucasians and American Indians [36]. The frequency of *ALDH2* alleles varies among the populations in Asian countries [5]. The *ALDH2**2 allele rarely occurs in the white populations. The investigations of the Caucasian race found an almost total homozygous character of *ALDH2**1 [38].

Further combination of *ADH3* with *ALDH2* variant genotype in presence of *ADH2* variant genotype showed almost double the risk of CLD compared to AWLD and moreover increased the risk of cirrhosis compared to controls (3.405 folds), AWLD (6.338 folds) and CLD (3.475 folds). When we considered all the genotypes, the joint affect of variant genotypes of ADH and *ALDH2* genes was found to result in increased risk of susceptibility and severity of ALD compared to AWLD cases. Moreover, on analysing the importance of the genotype(s) on the clinical profile of the cases on the basis of Fibroscan LSM score showed the association of ADH2*2/3, ADH3*1 and ALDH2*2 individually and combinatorially with higher LSM score in ALD cases and end stage liver disease; which suggests that importance and prognostic significance of the genotypes in ALD susceptibility and severity in the NEI population.

Conclusion:

The present study clearly underlines the role of genetic alterations in the alcohol metabolizing genes in the susceptibility and severity of ALD in northeast Indian population. *ALDH2* polymorphism is a rare event in northeast population. When the joint effects of the difference in genotypes were analyzed in association with disease susceptibility and severity, *ADH2* genotype was found to be detrimental factor in both the situation, and may be suitably used as prognostic genetic marker for stratifying alcoholic cases predisposed to ALD and associated severity with possible clinical interventions, thereby limiting ALD related morbidity and mortality specially in populations where alcoholism is a prevalent factor.

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