Microsomal Epoxide Hydrolase Polymorphisms and Haplotypes as Determinants of Hepatitis B Virus- and Hepatitis C Virus-related Liver Disease in Indian Population

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Background: Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of death worldwide. Main causes of HCC are hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. *mEPHX*, a xenobiotic metabolizing enzyme, exhibits a dual role of procarcinogen detoxification and activation, hence considered as a cancer risk factor as well as a protective factor. Two known polymorphic forms of *mEPHX*, exon in exon 3 and 4, are associated with the development of HCC. **Objective:** To determine the association of genotypes and haplotypes of *mEPHX* with risk of HCC developments separately in HBV- and HCV-infected carriers and patients with hepatitis. **Methods:** Polymerase chain reactions (PCR) were carried out using primers to amplify exon 3 (113 Tyr → His variant) and exon 4 (139 His → Arg) polymorphic sites. To distinguish the wild and variant forms, PCR amplification products were digested with restriction endonucleases EcoRV and RsaI for exons 3 and 4, respectively. **Result:** Exon 3 genotypes, Y113H and H113H, shared a protective association with HBV-chronic hepatitis infection ($P < 0.001$ and $P < 0.01$, respectively) as well as HBV-HCC development ($P < 0.001$) among HBV-carrier group, while Y113H acts as a risk factor for HCV-chronic hepatitis development ($P < 0.001$) as well as for HCC development ($P < 0.01$) with HCV-carrier group as reference. Both H139R and R139R, exon 4 genotypes, acted as a risk factor for HBV/HCV-chronic hepatitis infection and for HBV/HCV-HCC development (P ranges from < 0.05 to < 0.001) with HBV/HCV carriers as reference. 113His–139His and 113His–139Arg haplotypes shared a significant negative and positive association, respectively, with HBV hepatitis and HBV-HCC risk. 113Tyr–139Arg haplotype acted as a risk for HCV-HCC development. **Conclusion:** Polymorphic and haplotypic variant forms of *mEPHX* exon 3 and 4 variably determine the susceptibility to develop HCC in HBV- and HCV-carrier subjects. (J CLIN EXP HEPATOL 2012;2:104–111)

Hepatocellular carcinoma (HCC) is one of the most common malignant tumor worldwide, representing the fifth most common cancer, while being the third leading cause of death due to cancer. It shows an annual incidence of over 600,000.¹ The incidence of HCC is the highest in Asia, followed by Africa, Europe, and North and South America. It varies among ethnic groups, and in the last two decades, increasing trends in the

incidence of HCC have been noted in Australia, Central Europe, UK, Japan, and North America.² In the US alone, overall age-adjusted incidence rates of HCC tripled between 1975 and 2005, rising from 1.6 per 100,000 to 4.9 per 100,000.^{3,4} Hepatocellular carcinoma occurs more often in men than in women and is usually seen in people of age 50 or older. However, the age varies in different parts of the world.

Major risk factors for HCC include chronic viral infection due to hepatitis B virus (HBV) and hepatitis C virus (HCV), and alcoholic liver disease. The distribution of these risk factors among patients with HCC is highly variable depending on the geographic region, race, or ethnic group. A review of the literature reveals that 75–80% of cases of HCC are attributable to persistent viral infections with either HBV (50–55%) or HCV (25–30%).² Most of the cases of HBV- and HCV-related HCC occur in the setting of cirrhosis. The risk of HCC in persons with HBV increases from asymptomatic carriers, inactive carriers, and chronic hepatitis without cirrhosis, all with an incidence $< 1/100$ person years to 2.2–4.3/100 person years in

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Abbreviations: AFB1: aflatoxin B1; AFP: alfa-fetoprotein; ALP: alkaline phosphatase; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus

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compensated cirrhotics. In areas of high HBV endemicity (i.e., Asian countries), persons with cirrhosis have an approximately 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis and a 16-fold higher risk of HCC than the inactive carrier. The corresponding figures in low endemic areas (i.e., Europe) for the risk are 16- and 17-fold higher for HCC, respectively.⁵ In the Japanese studies, the HCC incidence rate for patients with chronic HCV hepatitis was 1.8/100 person years and 7.1 in those with compensated cirrhosis. In the US and Europe, the summary incidence rate was 3.7/100 person years.^{5,6} Hence, only a small portion of viral carriers develops HCC, which implies the influence of host factors on individual susceptibility. These inter-individual differences in susceptibility to HCC may be attributed to genetic polymorphisms which have emerged in recent years as an important determinant of disease susceptibility and severity. Genetic polymorphisms in some critical genes, including those involved in xenobiotic metabolism, have been studied in this regard. *mEPHX*, a xenobiotic metabolizing enzyme, exhibits a dual role of procarcinogen detoxification and activation⁷ and may be considered as a cancer risk factor as well as a protective factor, depending on the carcinogens.⁸ Two relatively common genetic polymorphisms are characterized within the exon 3 rs1051740(Tyr113His) and exon 4 rs2234922(His139Arg) of *mEPHX* and these variants are then referred to as 'slow' and 'fast' alleles because they, respectively, have decreased (40–50%) and increased (25%) enzyme activity.⁹ These variant enzymes are known to influence the metabolism of both exogenous and endogenous metabolites produced in response to oxidative stress caused by chronic viral infection.

mEPHX has been found to be polymorphic, with the type and distribution frequencies varying in different ethnicities. In this regard, there are only a few reports suggesting significant associations between *mEPHX* polymorphism and hepatitis virus-related HCC risk.^{10,11} Results from our previous studies have shown that the variant forms of *mEPHX* exons 3 and 4 were, respectively, protective and risk factors for viral-related HCC in Indian population.¹² Earlier, we have studied the association of various genotypes¹² and haplotypes¹³ of *mEPHX* and hepatitis virus-related HCC risk. These association studies were carried out by considering HBV- and HCV-related HCC patients together and risk was evaluated with respect to healthy controls. However, recent estimates suggest that there are approximately 400 million and 170 million chronic carriers of HBV and HCV, respectively.^{14,15} Considering these facts, we designed our case-control study to determine the association of genotypes and haplotypes of *mEPHX* with risk of HCC developments separately in HBV- and HCV-infected carriers and hepatitis patients. The screening of these genotypes/haplotypes might be useful in clinical practice to stratify the carriers into lower and higher risk groups for development of HCC.

MATERIALS AND METHODS

Subjects

A total of 347 patients/subjects, coming to the Liver Clinic of the Department of Hepatology and Department of Transfusion Medicine, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India, were included in the study for genotype analysis. A detailed clinical history and examination was done in all the patients. The patients were routinely investigated for HBV/HCV viral markers; routine hemogram, biochemistry, and alfa-fetoprotein (AFP) levels; ultrasound to determine the characteristics of the space occupying lesions; computed tomography (CT) scan to see for small satellite lesions and vascular involvement; and endoscopy to see for esophageal varices. An informed consent was taken prior to the start of the study. The protocol of the study was approved by the Institute Ethical Committee. In this study, following groups of subjects were included: Groups 1 and 4 included 61 and 60 patients, with inactive hepatitis B and C infection, respectively (HBV carriers and HCV carriers) who are hepatitis B virus surface antigen (HBsAg)-positive in the former case and anti-HCV-positive in the latter case for a period of 6 months or more. These patients had normal levels of transaminases and no clinical symptoms of liver disease along with no radiologic evidence of cirrhosis or varices on endoscopy. Groups 2 and 5 included 107 and 61 patients, respectively (recruited from the previous study)^{12,13} who were HBsAg-positive in the former case and anti-HCV-positive in the latter case for a period of 6 months or more along with raised transaminases $> 1.5 \times \text{ULN}$ and imaging techniques showing no evidence of cirrhosis or no varices on endoscopy. Groups 3 and 6 include 30 and 28 patients, respectively (recruited from the previous study)^{12,13} who were diagnosed with HCC as established by fine needle aspiration cytology (FNAC) or histopathology and the concomitant finding of two imaging techniques, showing a nodule $> 2 \text{ cm}$ with arterial hypervascularization, or by one positive imaging technique, showing hypervascularization associated with AFP concentration $> 400 \mu\text{g/L}$.¹⁶ Hepatocellular carcinoma was HBV-related if the patient is HBsAg+ or anti-HBc+ while it was HCV-related if the patient was anti-HCV+. Groups 1–6 excluded the patients who were human immunodeficiency virus (HIV) positive, or had any other viral marker in the blood or had evidence of hemochromatosis and Wilson's disease. Anti-HCV+ were excluded from groups 1–3 while anti-HBsAg+ were excluded from groups 4–6. Hepatocellular carcinoma was HBV-related if the patient was confirmed for HBsAg positivity. Further double positives, that is, the patient positive for both anti-HBc/HBsAg and anti-HCV, were excluded from the study. The demographic parameters of the three groups of HBV/HCV subjects are described in Tables 1 and 2.

Table 1 Demographic characteristics of hepatitis B virus-hepatocellular carcinoma, chronic viral hepatitis, and carrier subjects.

Parameters	HBV-HCC	HBV-chronic viral hepatitis	HBV carriers
No. of subjects (n)	30	107	61
Age range (yr)	32–77	6–70	14–51
Mean age $\pm$ SD	56.89 $\pm$ 14.03* [†]	32.29 $\pm$ 14.15	31.03 $\pm$ 10.35
Median age (yr)	60	29	30
Males, n (%)	28 (93.33)*	83 (77.57)	47 (77.05)
Females, n (%)	2 (6.67)*	24 (22.43)	14 (22.95)

* $P < 0.001$ with respect to HBV-carrier subjects; [†] $P < 0.001$ with respect to HBV-chronic viral hepatitis subjects.
HBV: hepatitis B virus; HCC: hepatocellular carcinoma; SD: standard deviation.

Table 2 Demographic characteristics of hepatitis C virus-hepatocellular carcinoma, chronic viral hepatitis, and carrier subjects.

Parameters	HCV-HCC	HCV-chronic viral hepatitis	HCV carriers
No. of subjects (n)	28	61	60
Age range (yr)	35–85	10–70	18–54
Mean age $\pm$ SD	62.11 $\pm$ 10.64* [†]	37.39 $\pm$ 11.8	30.77 $\pm$ 7.67
Median age (yr)	65	37.5	30
Males, n (%)	25 (89.29)*	53 (85.48)	55 (91.67)
Females, n (%)	3 (10.71)*	9 (14.52)	5 (8.33)

* $P < 0.001$ with respect to HCV-carrier subjects; [†] $P < 0.001$ with respect to HCV-chronic viral hepatitis subjects.
HCV: hepatitis C virus; HCC: hepatocellular carcinoma; SD: standard deviation.

Methodology

Five milliliters of venous blood was collected from all the patients, in acid citrate dextrose anticoagulant from all the subjects and stored at -80°C for further use.

Deoxyribonucleic Acid Isolation

High-molecular-weight genomic deoxyribonucleic acid (DNA) was isolated from the peripheral blood samples using the standard sodium perchlorate method as described by Daly et al¹⁷ and was suspended in TE buffer (pH 8.0; 10 mM Tris and 1 mM ethylene diamine tetra acetic acid [EDTA]) and finally used for *mEPHX* genotyping.

Genotyping Analyzes

mEPHX Genotyping

The *mEPHX* polymorphisms in exons 3 and 4 were separately determined by PCR-RFLP-based method. The primers used for exons 3 and 4 were as follows: Exon 3 forward primer: 5'-GATCGATAAGTTCGGTTTCACC-3', exon 3 reverse primer: 5'-ATCTTAGTCTTGAAGTGAGGAT-3', exon 4 forward primer: 5'-ATTTATGGAGGCCTGATGTG-3', exon 4 reverse primer: 5'-CTTCTTGAGGATGCCTCTG-3'.

Briefly, the PCR reactions were carried out in 50 μL volume consisting of 100 ng genomic DNA, 1 $\times$ PCR buffer, 1.5 U Taq DNA polymerase, 1 $\mu\text{mol/L}$ primers, and 200 μM each dNTP. The PCR conditions consisted of initial denaturation at 94°C for 5 minutes, 30 amplification cycles

with denaturation at 93°C for 1 minute, annealing at 58°C (exon 3) or 65°C (exon 4) for 1 minute, and elongation at 72°C for 1 minute. The final extension was carried out at 72°C for 10 minutes. The PCR-amplified product was first electrophoresed on agarose gel to check the product quality before treatment with restriction endonuclease. To detect the 113Tyr $\rightarrow$ His variant, (exon 3) and 139 His $\rightarrow$ Arg variant (exon 4), PCR amplification product was digested with 5 U of restriction endonucleases EcoRV for exon 3 and RsaI for exon 4 (MBI Fermentas, Glen Burnie, MD) at 37°C for 12–16 hours and then electrophoresed on 3% agarose gel and the size of the bands was looked for after digestion so as to distinguish wild and variant types.

Haplotype Analyzes of *mEPHX*

The analyzes of haplotypes were done using PHASE v2.1.1 software. Also, the association of each of the haplotype with the risk of hepatitis and HCC was assessed.

Statistical Analysis

Genotype frequencies were calculated as the number of participants with a particular genotype divided by the total number of participants. The statistical significance was depicted by using Pearson P value. Deviations of the *mEPHX* genotype frequencies among different groups, from those expected under Hardy-Weinberg equilibrium (HWE), were assessed by χ^2 -test. Associations between the *mEPHX* polymorphic variants and HCC risk were

Table 3 *mEPHX* genotype distribution (%) among hepatitis B virus carrier, chronic viral hepatitis, and hepatocellular carcinoma subjects and their association with hepatocellular carcinoma.

Genotype	HBV carrier ^a (n=61)	HBV-chronic viral hepatitis ^a (n=107)	HBV-HCC ^a (n=30)	OR ^b (95% CI)	OR ^c (95% CI)	OR ^d (95% CI)	OR ^e (95% CI)
Exon 3							
Tyr113Tyr (Y113Y)	4 (6.56)	27 (25.23)**	10 (33.34) [†]	Ref	Ref	Ref	Ref
Tyr113His (Y113H)	31 (50.82)	41 (38.32)	13 (43.33)	0.20 ^{##} (0.075–0.51)	0.16 [#] (0.048–0.501)	0.17 ^{##} (0.07–0.42)	0.27 (0.063–1.2)
His113His (H113H)	26 (42.62)	39 (36.45)	7 (23.33)*	0.22 [#] (0.085–0.58)	0.27 [†] (0.087–0.88)	0.11 ^{##} (0.04–0.29)	0.13 [#] (0.028–0.61)
Exon 4							
His139His (H139H)	40 (65.57)	38 (35.51) [†]	10 (33.33)**	Ref	Ref	Ref	Ref
His139Arg (H139R)	18 (29.51)	47 (43.93)*	14 (46.67)	2.75 [#] (1.48–5.11)	2.95 [#] (1.36–6.4)	3.11 ^{##} (1.67–5.79)	4.2 [#] (1.32–13.5)
Arg139Arg (R139R)	3 (4.92)	22 (20.56)**	6 (20)*	7.72 ^{##} (2.66–22.39)	12.7 ^{##} (3.26–49.49)	7.99 ^{##} (2.74–23.33)	11.53 [#] (1.97–67.25)

^aGenotype frequency represented as number of subjects (percent distribution), ^bOR for HBV hepatitis with HBV carriers as reference, ^cOR for HBV-HCC with HBV carriers as reference, ^dage and globulin level adjusted OR for HBV hepatitis with HBV carriers as reference, ^eage and globulin level adjusted OR for HBV-HCC with HBV carriers as reference. * $P < 0.05$, ** $P < 0.01$, [†] $P < 0.001$ with respect to carriers, [†] $P < 0.05$, [#] $P < 0.01$, ^{##} $P < 0.001$ by χ^2 -test. n =no. of subjects.

CI: confidence interval; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; OR: odds ratio.

calculated as odds ratios (ORs) with 95% confidence intervals (CIs), and their statistical significance was determined by χ^2 -test; further logistic regression was used to adjust for covariate effect of age, sex, liver disease severity, and exon 3 and 4 polymorphisms using SPSS v15 software. The power of the study was calculated using web-based power calculator. The power of the study came around 80% at 95% CI, but it was lower for exon 3 in the case of HCV group. However, when we compared mutant genotype only the power of the study for exon 3 in the case of HCV group came around 70% at 95% CI.

RESULTS

Demographic characteristics of HBV/HCV-HCC, chronic viral hepatitis, and carrier subjects (Tables 1 and 2) showed that the mean age of HBV/HCV-related HCC subjects was much higher (56.89 and 62.11 years, respectively) than that of HBV/HCV-related chronic viral hepatitis subjects and carriers, while the mean age of HBV/HCV-related chronic viral hepatitis subjects and carriers was comparable. These values show that HCC is a disease of later age. There is higher percentage of males than the percentage of females in HBV/HCV-related HCC, chronic viral hepatitis, and carrier groups. The polymorphisms known in the *mEPHX* exons 3 and 4 are known to, respectively, decrease and increase the enzyme activity. In exon 3, at codon 113, a C has been substituted for T, resulting in the replacement of tyrosine by histidine and loss of *EcoRV* restriction site. The second polymorphism known is in exon 4 at codon 139, where a G to A transition replaces histidine by

arginine and creates *RsaI* cleavage site. The products obtained after the restriction digestion of the exon 3 amplification product by *EcoRV* and exon 4 by *RsaI* as obtained on agarose gel are shown in our previous study.¹²

The *mEPHX* exon 3 and exon 4 genotype distributions among HBV-related groups are depicted in Table 3. It is evident that in the case of exon 3, wild genotype showed significantly higher frequency in HBV-chronic viral hepatitis and HBV-HCC subjects ($P < 0.01$ vs $P < 0.001$), with reference to HBV carriers. Further among the HBV-carrier subjects, >50% of the subjects (50.82%) showed the presence of Y113H genotype, which is higher than that present in the HBV-chronic viral hepatitis (38.32%) and the HBV-HCC subjects (43.33%). Also, the presence of H113H mutant genotype was more (42.62%) in HBV carriers which was higher than that in hepatitis (36.45%) and significantly higher in HCC subjects (23.33%; $P < 0.05$). The data when evaluated to study the association of these genotypes with development of HBV-related HCC revealed that Y113H shared a negative or a protective association with HBV-chronic hepatitis infection (OR=0.20; $P < 0.001$) as well as HBV-HCC development (OR=0.17; $P < 0.001$) among HBV-carrier group. Further, the H113H variant genotype also showed a protective association with HBV-chronic hepatitis development (OR=0.22; $P < 0.01$) and HBV-HCC development (OR=0.11; $P < 0.001$) with reference to HBV-carrier group.

Regarding *mEPHX* exon 4, HBV-carrier subjects had maximum frequency (65.57%) for H139H genotype while H139R genotype and R139R variant genotypes were present at a frequency of 29.51% and 4.92%, respectively. Wild

Table 4 *mEPHX* genotype distribution (%) among hepatitis C virus carrier, chronic viral hepatitis, and hepatocellular carcinoma subjects and their association with hepatocellular carcinoma.

Genotype	HCV carrier ^a (n=60)	HCV-chronic viral hepatitis ^a (n=61)	HCV-HCC ^a (n=28)	OR ^b (95% CI)	OR ^c (95% CI)	OR ^d (95% CI)	OR ^e (95% CI)
Exon 3							
Tyr113Tyr (Y113Y)	22 (36.7)	15 (24.6)	10 (35.71)	Ref	Ref	Ref	Ref
Tyr113His (Y113H)	7 (11.7)	29 (47.54) [†]	10 (35.71)**	6.06 ^{##} (2.67–13.74)	8.05 (2.37–27.35)	3.14 [#] (1.4–7.02)	6.10 [†] (1.17–31.7)
His113His (H113H)	31 (51.6)	17 (27.86)**	8 (28.53)*	0.8 (0.4–1.6)	0.93 (0.33–2.6)	0.57 (0.29–1.09)	0.69 (0.16–3.02)
Exon 4							
His139His (H139H)	39 (65)	23 (37.71)**	9 (32.14)**	Ref	Ref	Ref	Ref
His139Arg (H139R)	15 (25)	29 (47.54)**	14 (50)*	3.28 ^{##} (1.75–6.15)	4.6 [#] (1.72–12.6)	4.04 ^{##} (2.13–7.67)	3.64 [†] (0.91–14.47)
Arg139Arg (R139R)	6 (10)	9 (14.75)	5 (17.86)	2.54 [†] (1.04–6.24)	2.87 (0.73–11.29)	3.61 [#] (1.49–8.73)	3.57 (0.49–25.5)

^aGenotype frequency represented as number of subjects (percent distribution), ^bOR for HCV-chronic hepatitis with HCV carriers as reference, ^cOR for HCV-HCC with HCV carriers as reference, ^dage, bilirubin, total protein, and albumin levels adjusted OR for HCV hepatitis with HCV carriers as reference, ^eage, bilirubin, total protein, and albumin levels adjusted OR for HCV-HCC with HCV carriers as reference. **P*<0.05, ***P*<0.01, [†]*P*<0.001 with respect to carriers, [#]*P*<0.05, ^{##}*P*<0.01, ^{###}*P*<0.001 by χ^2 -test. *n*=no. of subjects.
CI: confidence interval; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; OR: odds ratio.

genotype, H139H, showed significantly higher frequency in HBV carriers compared with HBV-chronic viral hepatitis and HBV-HCC subjects (*P*<0.001 vs *P*<0.01). Both H139R and R139R genotypes were significantly more (*P*<0.05) in HBV-chronic viral hepatitis and HBV-HCC groups as compared with HBV carriers.

In *mEPHX* exon 4, the H139R heterozygous genotype more than doubled the risk for HBV-chronic hepatitis infection (OR=2.75; *P*<0.01) which further increased to 3.1-fold (*P*<0.001) for HBV-HCC development with HBV carriers as reference. Further, the risk for HBV-chronic hepatitis infection as well as HCC development was >7-fold increased (OR=7.72; *P*<0.001 and OR=7.99; *P*<0.001, respectively) with variant R139R genotype among HBV carriers. Univariate analysis was carried out to check the association of various imbalanced risk factors, i.e., age, sex, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, albumin, globulin, and bilirubin levels with *mEPHX* genotypes in various subgroups (data not shown). It was found that age and globulin levels were significant factors (age *P*<0.001 and globulin *P*<0.01). By applying multinomial logistic regression analysis to adjust these factors for determining the association of various genotypes with HBV-related disease risk, adjusted OR were calculated which are shown in Table 3.

The *mEPHX* exon 3 and exon 4 genotype distributions among HCV-related groups are depicted in Table 4. For *mEPHX* exon 3, the frequency of Y113Y wild genotype was lowest in HCV hepatitis subjects (24.6%). The Y113H

genotype had a significantly higher frequency in the HCV hepatitis subjects (47.54%, *P*<0.001) and in HCV-HCC subjects (35.71%; *P*<0.01) when compared with the HBV carriers, while H113H genotype showed significantly lower frequency in HCV hepatitis and HCV-HCC subjects (27.86%; *P*<0.01 and 28.53%; *P*<0.05, respectively) when compared with HCV carriers (51.6%). Association between the *mEPHX* exon 3 genotypes and the carcinoma progression taking Y113Y genotype as reference showed that Y113H variant genotype acts as a risk factor for HCV-chronic hepatitis development (OR=6.06; *P*<0.001) as well as for HCC development (OR=3.14; *P*<0.01) with HCV-carrier group as reference. However, H113H variant genotype showed a negative association with HCV-chronic hepatitis development (OR=0.8) and HCV-HCC development (OR=0.57) with reference to HCV-carrier group albeit nonsignificantly. For exon 4 of *mEPHX*, wild genotype showed maximum frequency in HCV carriers, it was 65% for H139H, 25% for H139R, and 10% for R139R. H139R genotype showed significantly higher frequency in HCV hepatitis and HCV-HCC subjects (47.54%; *P*<0.01 and 50%; *P*<0.05, respectively) when compared with HCV carriers (25%). Similarly, R139R variant genotype showed higher frequency in HCV hepatitis and HCV-HCC subjects (14.75% and 17.86%, respectively) when compared with HCV carriers (10%). Association between the *mEPHX* exon 4 genotypes and the carcinoma progression taking H139H genotype as reference showed that the H139R heterozygous genotype acted as risk factor for HCV-chronic hepatitis infection (OR=3.28; *P*<0.001) and HCV-HCC infection

Table 5 Association of *mEPHX* exon 3 and 4 haplotypes in hepatitis B virus carrier, chronic viral hepatitis, and hepatocellular carcinoma subjects, with the risk of hepatocellular carcinoma.

Haplotypes	HBV carrier ^a (n=122) (%)	HBV-chronic viral hepatitis ^a (n=214) (%)	HBV-HCC ^a (n=60) (%)	OR ^b (95% CI)	OR ^c (95% CI)
113Tyr-139His ^d	25 (20.5)	64 (29.9)	22 (36.66)	Ref	Ref
113Tyr-139Arg	14 (11.47)	31 (14.48)	11 (18.33)	0.86 (0.39–1.89)	0.89 (0.33–2.36)
113His-139His	73 (59.83)	57 (26.63)	12 (20)	0.3 (0.17–0.54)**	0.18 (0.08–0.43)**
113His-139Arg	10 (8.19)	62 (28.97)	15 (25)	2.42 (1.07–5.45)*	1.7 (0.63–4.5)

^aHaplotype frequency represented as number of alleles (percent distribution), ^bOR for HBV hepatitis with HBV carriers as reference, ^cOR for HBV-HCC with HBV carriers as reference, ^dhaplotype taken as reference (OR=1) to analyze risk for other groups. * $P<0.05$, ** $P<0.001$ with respect to carriers. n =no. of chromosomes studied.

CI: confidence interval; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; OR: odds ratio.

Table 6 Association of *mEPHX* exon 3 and 4 haplotypes in hepatitis C virus carrier, chronic viral hepatitis, and hepatocellular carcinoma subjects, with the risk of hepatocellular carcinoma.

Haplotypes	HCV carrier ^a (n=120) (%)	HCV-chronic viral hepatitis ^a (n=122) (%)	HCV-HCC ^a (n=56) (%)	OR ^b (95% CI)	OR ^c (95% CI)
113Tyr-139His ^d	35 (29.17)	41 (33.6)	13 (23.21)	Ref	Ref
113Tyr-139Arg	16 (13.33)	18 (14.75)	17 (30.36)	0.96 (0.42–2.6)	2.86 (1.12–7.27)*
113His-139His	58 (48.33)	30 (24.6)	19 (33.93)	0.44 (0.23–0.82)**	0.88 (0.38–2.0)
113His-139Arg	11 (9.16)	33 (27.05)	7 (12.5)	2.56 (1.13–5.8)*	1.7 (0.54–5.3)

^aHaplotype frequency represented as number of alleles (percent distribution), ^bOR for HCV hepatitis with HCV carriers as reference, ^cOR for HCV-HCC with HCV carriers as reference, ^dhaplotype taken as reference (OR=1) to analyze risk for other groups. * $P<0.05$, ** $P<0.01$ with respect to carriers. n =no. of chromosomes studied.

CI: confidence interval; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; OR: odds ratio.

(OR=4.04; $P<0.001$) with HCV carriers as reference. Further, R139R genotype also significantly increased the risk for HCV hepatitis and HCV-HCC development (OR=2.54; $P<0.05$ and OR=3.61; $P<0.001$, respectively) with HCV carriers as reference. Further, univariate analysis was carried out to check the association of various imbalanced risk factors, i.e., age, sex, AST, ALT, ALP, total protein, albumin, globulin, and bilirubin levels with *mEPHX* genotypes in various subgroups (data not shown). It was found that age, bilirubin, total protein, and albumin levels were significant factors (age $P<0.001$, bilirubin $P<0.01$, total protein $P<0.01$, and albumin $P<0.001$). By applying multinomial logistic regression analysis to adjust these factors for determining the association of various genotypes with HCV-related disease risk, adjusted OR were calculated which are shown in Table 4.

Comparisons of the observed distributions of *mEPHX* genotypes and those predicted by allele frequencies by χ^2 -analysis showed that the populations studied were in HWE, indicating that the study groups were sufficiently random and representative, except for exon 3 in HCV-carrier subjects, which may be due to low sample size.

Table 5 depicts the haplotype analysis of the *mEPHX* exon 3 and exon 4 genotypes in HBV subjects. The distribution of 113Tyr-139His, the all-wild (reference) haplotype showed maximum frequency in HBV-HCC subjects, (36.66%) followed by HBV-chronic viral hepatitis subjects

(29.9%), and least in HBV carriers (20.5%). However, no significant association of this haplotype was observed with hepatitis and HCC development. The distribution of 113His-139His haplotype was maximum in HBV carriers (59.83%) and thus showed a significant protective association with HBV hepatitis (OR=0.3, $P<0.0001$) and HBV-HCC (OR=0.18, $P<0.0001$) development. Further, both the mutant haplotype 113His-139Arg showed a decreased frequency in HBV carriers than in HBV hepatitis and HBV-HCC subjects and it shared a significant positive association with HBV hepatitis risk (OR=2.42, $P<0.05$) and HBV-HCC (OR=1.7, nonsignificant). Haplotype analysis in HCV subjects (Table 6) revealed that the all-wild (reference) haplotype 113Tyr-139His showed maximum frequency in HCV hepatitis subjects (33.6%) compared with HCV carriers (29.16%) and HCV-HCC (23.21%). 113Tyr-139Arg haplotype with its maximum frequency in HCV-HCC subjects showed no association with HCV hepatitis development (OR=0.96) while it shared a significant positive association with HCV-HCC development (OR=2.86, $P<0.05$). 113His-139His haplotype showed highest frequency in HCV carriers (48.33%) and shared a protective association with HCV hepatitis development. However, decreased frequency of 113His-139Arg haplotype was seen in HCV carriers than in HCV hepatitis and HCV-HCC subjects thus significantly increasing the risk for HCV hepatitis development (OR=2.56, $P<0.05$).

DISCUSSION

Hepatocellular carcinoma (also called malignant hepatoma) is the most common type of liver cancer. Chronic infection with HBV and HCV contributes to major risk factors for HCC.

India alone has nearly 45 million people suffering from chronic HBV infection and approximately 15 million people with chronic HCV infection.^{18,12} Infection from HBV is widespread throughout the world, especially in developing nations. Every year, approximately 1 million people die of HBV infection, 33% caused by HCC and the rest with terminal complications of liver disease.¹⁹ Most recently, chronic HCV infection has been recognized as an important factor in the genesis of HCC. The clinical picture of HCV infection is often an acute stage, followed by chronic infection in >80% of people initially infected. Those with chronic HCV infection may progress to cirrhosis of the liver and finally HCC. Current estimates suggest 2–5% of patients with liver cirrhosis due to HCV develop HCC annually.²⁰

Genetic polymorphisms in the detoxifying and antioxidant enzymes can be important markers as they might affect the function of proteins and significantly influence the detoxification and oxidative stress thereby playing a crucial role in HCC. *mEPHX* being a xenobiotic metabolizing enzyme plays an important role in anticarcinogenesis since it catalyze the irreversible hydration of highly reactive epoxide intermediates to yield metabolites of lower reactivity, which can be readily conjugated and excreted.^{21,22} *mEPHX* is present in high concentration in the liver. The two known polymorphic forms at exon 3 and exon 4 can reduce or increase the activity of *mEPHX*, respectively, thus may play a role in increased or decreased carcinogen activation in the liver. These variant enzymes might influence the metabolism of endogenous metabolites produced in response to oxidative stress caused by chronic viral infection of liver causing hepatocarcinogenesis.

There are studies that have shown the association of *mEPHX* polymorphism and susceptibility to HCC development due to heavy aflatoxin B1 (AFB1) exposure^{23,24} and alcohol consumption.²⁵ However, there are only few studies on the association between specific *mEPHX* alleles and susceptibility to liver cancer in HBV-infected subjects²⁶ and HCV-infected subjects.¹⁰ Previously, we have shown that the polymorphic forms of *mEPHX* at exon 3 and 4 modulate the susceptibility to viral-related HCC development among healthy population.¹²

In this study, we attempted to evaluate the role of *mEPHX* polymorphisms and haplotype in HCC development separately in HBV or HCV viral carriers and hepatitis subjects. Genotype distribution showed an opposite trend for HCV carriers in our study and a study based on HCV subjects in Italian population.¹⁰ In our study, HCV carriers showed the lowest frequency for Y113H genotype and the highest for H113H genotype (11.7% and 51.6%, respectively) while

it was highest for Y113H genotype and lowest for H113H genotype in HCV subjects in Italian population (48.9% and 7.6%, respectively). However, a similar trend was observed in the above-mentioned study and in our study for genotype distribution of the three genotypes of exon 4 in HBV-carrier subjects, with the genotype distribution varying in the order H139H > H139R > R139R.¹⁰ The values for genotype distribution for three genotypes of exon 4 in our study and in Italian study are 65.57% vs 61.9%, 29.51% vs 33.7%, 4.92% vs 4.3%, respectively. Moreover, in both these studies, R139R genotype was least frequent among all three groups studied. Variant genotypes of exon 3 (Y113H and H113H) acted as a protective factor decreasing the risk of HCC development in HBV inactive carriers and HBV hepatitis subjects. While in the case of HCV carriers and hepatitis subjects, Y113H genotype was a risk factor for HCC development, whereas no significant association was observed between H113H genotype and HCC development. In a study reported in Ghanaian population, Y113H acted as a risk factor for Aflatoxin B1-related HCC development among controls.²⁴ In our earlier studies, variant genotypes of exon 3 were found to be protective among normal subjects for development of viral-related HCC whereas a study carried out in Chinese population, these genotypes were found to be a risk for aflatoxin B1-associated HCC.²³ In Italian population, H113H genotype was found as a risk factor in HCV carriers for HCC development.¹⁰

In our study, mutant genotypes of exon 4 (H139R and R139R) increased the risk of hepatitis and HCC development in both HBV- and HCV-infected carrier subjects. This observation is similar to our previous study where H139R and R139R genotypes were found to be a risk for viral-related HCC development in healthy subjects. However, in Italian population, no significant association between H139R and R139R genotypes and HCV-related HCC development was found.¹⁰ Thus, a consequence of different genotypes varies in different ethnic groups and in different diseases. In a study in non-Hispanic whites, the variant allele of *mEPHX* exon 4 increased the lung cancer risk by 56% while the variant allele of exon 3 was found to have no effect on lung cancer risk in older people, but it was 53% protective in younger people.²⁶

Research in genetic field in order to unravel complex diseases like cancer suggests to study haplotypes and linkage studies in addition to single nucleotide polymorphisms. Haplotypes provide increased statistical power to detect association and map etiologic mutations over classical single-marker approach.²⁷ Four haplotypes of *mEPHX* were constructed and their association with hepatitis and HCC risk was analyzed. We observed that the all-mutant haplotype of *mEPHX* (113His–139Arg) significantly increased the risk of hepatitis and HCC development in reference to carriers in both HBV- and HCV-infected subjects while in our previous study 113His–139Arg haplotype

was not significantly associated with HCC development among control group.¹³ Haplotype 113Tyr–139Arg was not associated with HCC development among HBV carriers while it was a significant risk factor for HCC development when studied in HCV carriers. 113His–139His genotype showed a significant protective association in HBV carriers for HCC development ($P < 0.001$) while no significant association in HCV carriers for HCC development was seen, although it was significantly protective for hepatitis development among both HBV and HCV carriers ($P < 0.001$ and $P < 0.01$, respectively). A study conducted on esophageal carcinoma subjects from India found none of the *mEPHX* haplotypes to modulate the risk for squamous cell esophageal cancer.²⁸

In conclusion, variant genotypes and haplotypes of *mEPHX* exon 3 and 4 variably determine the susceptibility to develop HCC in HBV- and HCV-carrier subjects. These studies along with our previous study suggest that susceptibility to develop viral-related HCC varies among viral carriers and healthy subjects with respect to *mEPHX* exon 3 and 4 genotypes and haplotypes and at the same time also highlights the importance of studying these genotypes separately in HBV- and HCV-infected subjects. However, the studies need to be carried out in a larger set of population in order to further confirm the results.

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CONFLICTS OF INTEREST

All authors have none to declare.

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