Infection, Genetics and Evolution 92 (2021) 104882

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Increased homocysteine mediated oxidative stress as key determinant of hepatitis E virus (HEV) infected pregnancy complication and outcome: A study from Northeast India

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ARTICLE INFO

Keywords:
HEV
Homocysteine
Vitamin B12
Oxidative stress
Pregnancy complications
Folate pathway

ABSTRACT

With the background of association of oxidative stress and Hepatitis E virus (HEV) infection in pregnancy complications the present novel study aimed to evaluate the significance of changes in maternal homocysteine levels and the related mechanism(s) in the pathophysiology of HEV related pregnancy complications and negative outcomes. Term delivery (TD, $N = 194$) and HEV-IgM positive pregnancy cases [$N = 109$] were enrolled. Serum and placental homocysteine levels were evaluated by ELISA and immunofluorescence and in turn correlated with serum Vitamin B12 levels. Distribution of variant *MTHFR C➔T* and *TYMS1494del6bp* genotyping were studied by PCR-RFLP. Differential folate receptor alpha (FR-α) expression in placenta was evaluated by real-time PCR and immunofluorescence respectively. The HEV viral load was significantly higher in both FHF and AVH cases. Higher serum homocysteine levels was associated with preterm delivery (PTD) and fetal death in HEV infected cases and was significantly inversely correlated with serum VitaminB12 levels in HEV cases. Placental homocysteine expression was upregulated in HEV cases, and in cases with negative pregnancy outcome. A Homocysteine level was associated with MTHFR C677T status. Genetic alterations in folate pathway was associated with increased risk of PTD in HEV infected pregnancy cases, disease severity, and negative pregnancy outcome in AVH and FHF groups. FR-α expression was downregulated in placental tissues of HEV infected pregnancy.Placental stress caused by HEV inflicted increased homocysteine due to alterations in maternal vitamin B12 levels and folate pathway components is detrimental mechanism in PTD and negative pregnancy outcome in HEV infected pregnancy cases and holds prognostic and therapeutic significance.

1. Introduction

Pathological infections are known to increase the risk of pregnancy complications including preterm delivery (PTD and negative pregnancy outcome (McClure et al., 2010) especially in developing low income countries (Dhaded et al., 2015). Viral infection has emerged as an important contributor of preterm delivery (Klein and Gibbs, 2005), with a deleterious effect in maternal as well as fetal health (Gervasi et al., 2012). Physiologic changes during pregnancy occurs throughout gestation, including the liver, but without changing its histology; except in cases with previously existing liver pathology or viral infections, which may lead to pregnancy complications (Westbrook et al., 2016). The outcome is usually benign, except in Hepatitis E virus (HEV) infection (Labrique et al., 2014), which is associated with high rate of prenatal mortality (Bednar et al., 1999). Studies have reported the association of high risk of preterm delivery, prematurity, and low birth weight (LBW) in HEV infected pregnant cases(Bose et al., 2014; Pérez-Gracia et al., 2015).Severity of HEV infection results in acute viral hepatitis (AVH) or fulminant hepatic failure (FHF) resulting in increased maternal as well as fetal mortality (Patil et al., 2014). The differences in pregnancy with respect to the gestation period and outcome exists in HEV infected AVH and FHF cases, which is suggestive that apart from

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https://doi.org/10.1016/j.meegid.2021.104882
Received 1 December 2020; Received in revised form 19 April 2021; Accepted 23 April 2021
Available online 24 April 2021

HEV genotype and load; the deregulations in host factors may play a critical role in deciding the fate of pregnancy in HEV infected cases. There are no clinical markers available that predicts the course of a pregnancy and the pathophysiologic mechanisms which are far from being understood in HEV infected pregnancy, and needs exploration and addressing.

Oxidative stress is associated in the pathophysiology of many reproductive complications including infertility, miscarriage, pre-eclampsia, fetal growth restriction and preterm labour (Duhig et al., 2016). Viruses including hepatitis viruses have been documented to induce a state of inflammation and cellular oxidative stress and resulting in disease susceptibility and severity of multiple organ aetiology(Saeed et al., 2017). Homocysteine is an amino acid with a free sulphydryl group and is involved in multiple key metabolic processes, but its levels are tightly regulated during pregnancy (Kumar et al., 2017; Walker et al., 1999). It is a critical oxidative stress marker and is associated with inflammation and vascular diseases (Codoñer-Franch and Alonso-Iglesias, 2016). Hyperhomocysteinemia has been reported to be associated with both pregnancy complications and negative outcomes (Vollset et al., 2000). Blood concentrations of homocysteine are dependent on dietary factors (Cavallé-Busquets et al., 2020) and impaired folate pathway including maternal vitamin B12 and folate deficiency (Mishra et al., 2020) or genetic alterations in key folate pathway genes coding for the enzymes 5-Methylenetetrahydrofolate reductase (*MTHFR*) (Summers et al., 2008) and thymidylate synthase (TYMS) (Ulrich et al., 2002) responsible for homocysteine metabolism (Selhub, 1999). These mechanisms are associated with various vascular-related complications of pregnancy including preeclampsia, placental abruption, recurrent pregnancy loss, fetal growth restriction (FGR) and stillbirth (Ray and Laskin, 1999; Vollset et al., 2000); as well as preterm delivery, negative pregnancy outcome and LBW in preterm delivery from cases from northeast India (Tiwari et al., 2015a). Vitamin B12 and folate plays a significant role in placentation and fetal growth during pregnancy (Greenberg et al., 2011), levels negatively correlates with serum homocysteine concentration (Mishra et al., 2020; Solanky et al., 2010). Folate receptors in placenta like Folate receptor alpha (FR-α) transfers folate through the placenta for proper growth and development of fetus (Kamen and Smith, 2004) and its expression holds importance since they block embryo toxicity mediated by different mediators including homocysteine (Piedrahita et al., 1999).

The liver is the major organ for the metabolism of homocysteine and its regulation is altered in chronic liver diseases. Many excretory and detoxification functions of the fetal liver are assumed by the placenta. Studies suggest that homocysteine can accumulate within the syncytiotrophoblast and has been predicted to influence its function in several ways (Tsitsiou et al., 2011; Tsitsiou et al., 2009) and may be detrimental to the fetus under suboptimal Vitamin B12 and folate status during pregnancy (Sibley, 2009). Apart from the liver, placenta is an extrahepatic site of replication of HEV in humans. We therefore hypothesize that increased homocysteine mediated oxidative stress and deregulation in folate pathway may critically influence pregnancy in HEV infected cases. Presented herein is a novel study from northeast Indian population to elucidate the role of elevated homocysteine resultant oxidative stress in deciding the fate of HEV infected pregnancy.

2. Materials and methods

2.1. Patient enrolment and sample collection

The study included 303 subjects, including healthy term delivery ($N = 194$) and clinically proven HEV infected pregnancy cases ($N = 109$) enrolled from Gauhati Medical College Hospital, Central Hospital NF Railway and GNRC Hospitals, Guwahati, Assam; between 2012 to January 2019 under the supervision of registered clinicians and with informed consent. The *Raosoft* sample size calculator predicted a sample size of 101 at 95% CI on the basis of 7% distribution. All the subjects were clinically free of any underlying chronic kidney diseases, non-smokers and non-alcoholic, and were not under any previous medications. Whole blood in overnight fasting stage were collected from all the cases and controls, and representative number of placental tissue (maternal-facing microvillus plasma membrane [MVM] of the syncytiotrophoblast) from the pregnancy cases [Healthy Term delivery = 40, HEV infected pregnancy cases = 38 (AVH = 33, FHF = 5)] were collected, and a part of the HEV-AVH cases placental tissue was formalin fixed and paraffin embedded. The present study was conducted with ethical permission from the institutional ethics committee of all the participating institutions. The inclusion and exclusion criteria used for the enrolment of HEV cases are stated below.

2.1.1. Inclusion criteria

Patients screened positive for anti-HEV IgM and negative for other hepatotropic virus were firstly included. Based on the clinical and biochemical diagnosis, the pregnancy cases were categorized as HEV infected acute viral hepatitis (AVH) and fulminant hepatic failure (FHF). HEV viral RNA was extracted from 140 µl serum using QIAamp Viral RNA Kit (*Qiagen, Germany*), followed by conversion to cDNA using c-DNA synthesis kit (*Applied Biosystems, US*). HEV RNA detection was performed by Real time PCR using primers based for ORF1 region. HEV RNA positive cases [AVH = 59, FHF = 17] were only further included for the present study.

2.1.2. Exclusion criteria

Pregnant women of less than 18 years and more than 45 years of age were excluded. Pregnancy cases with history of tuberculosis, urinary tract infection (UTI), any other viral infection, gestational diabetes, preeclampsia and vascular disorders were excluded from the study.

2.2. Detection of HEV RNA, Viral load analysis and Genotyping

The Geno-Sen"s HEV PCR reagent (9,111,012 Geno-sen's; Genome Diagnostics Private Limited India) was used for the detection and quantification of HEV using realtime PCR in the Rotor gene 6000 (*Corbett Research*). The PCR amplification for the detection and quantification of HEV using viral RNA was performed according to the manufacturer protocol, and the viral load estimation was done based on the standard curve generated based on the viral standard dilutions provided in the kit and using rotor gene software.

HEV RNA positive cases were further subjected to HEV genotyping analysis by sequencing, followed by comparative phylogenetic analysis with standard HEV genotype sequences from the GenBank using MEGA4.0 software.

2.3. Estimation of homocysteine serum levels and placental expression

The serum homocysteine levels were measured using a commercially available Homocysteine ELISA kit (*ITEH4011, G-Bioscience*). All samples were run in duplicate, and the two measurements were averaged for statistical analysis. The sensitivity of the ELISA was 0.092 nmol/ml. To validate the results, randomly selected 10% cases were analyzed through the chemiluminescence method commercially and compared with the ELISA-based data. Differential protein expression of homocysteine in a representative number of placenta tissues was studied by immunohistochemistry which involves the detection of antigen in tissues and cells in two stages. The first stage includes the binding of the primary antibody to the specific epitope of the particular antigen and the second stage is the detection of the bound antibody to antigen by the colorimetric reaction. So for the primary antibody, we used a commercially available homocysteine antibody (*ab15154, Abcam*) and for detection purposes, the super-sensitive one-step polymer-HRP detection system (BioGenex) based on non-biotin polymeric technology was used. The slides were examined and evaluated for Homocysteine expression by a senior pathologist. The difference in placental expression in HEV-

infected preterm delivery cases with different pregnancy outcomes was studied by immunofluorescence microscopy using the same antibody.

2.4. Estimation of Vitamin B12 serum levels

Serum vitamin B12 levels were analyzed by ELISA using the Immunotag human Vitamin B12 ELISA kit by following the manufacturer's protocol. The final reading was taken at 450 nm within 10 min after adding stop solution in ERBA ELISA reader. All samples were run in duplicate, and the two measurements were averaged for statistical analysis.

2.5. Screening of alterations in important folate pathway genes

Genomic DNA was extracted from whole blood by the standard phenol-chloroform method, and two critical polymorphism *MTHFR C677T* (rs1801133, A222V) and *TYMS3′1494del6 (*rs869066439*)* were analyzed by PCR-RFLP method with the help of the enzymes *Hinf*I (*MTHFR C677T)* and *Dra*I (*TYMS3′1494del6)*, using the methodology reported previously (Frosst et al., 1995; Ulrich et al., 2000). The restriction digested RFLP product was run in 3% Agarose gel. The Randomly selected 20% of case or control cases were re-genotyped for validation by another lab member in a blinded manner.

2.6. Differential folate receptor alpha (FR-α) expression

Total RNA was extracted by standard trizol method from placental tissues, and was converted to cDNA using commercially available High-capacity cDNA conversion kit (ABI, US). Differential fold change in FR-α mRNA expression was studied by realtime PCR using Sybr-green chemistry and FR-α specific primers (5′GCATTTCATCCAGGACACCT3′ and 5′GCTGTAGGAGGTGCGACA3′) and *β-actin* as the internal normalization control. Differential FR-α protein expression in placenta in representative number of cases was studied by immunofluorescence using specific antibody (*Abcam, UK*).

2.7. Statistical analysis

Statistical analyses were performed using SPSSv13.0 software (*Chicago, IL, US*). Results were expressed as means± standard deviations (SD). The Mann–Whitney non-parametric test was used to analyse the differences, and the significance was described as Pearson *p*-value. Student's unpaired *t*-test was used to evaluate differences between groups and the two-sided Pearson and Spearman's rho correlation coefficient was used to determine the relationship between variables.

3. Results

3.1. Demographical profile and stratification of enrolled cases

Healthy Term delivery cases [$N = 194$] and HEV-IgM positive pregnancy cases [$N = 109$] were initially enrolled for the present study with complete clinical and biochemical profile. Screening of the HEV mRNA positive cases amongst the HEV-IgM positive cases were performed by Realtime PCR; and the 76 HEV mRNA positive cases were further included for the present study, which consisted of 59 AVH and 17 FHF cases. In the HEV infected pregnancy cases the fetal death was significantly higher in FHF compared to AVH cases ($p < 0.001$). Majority of the HEV cases were in their third trimester of pregnancy (73/76, 96.05%). Preterm delivery was significantly associated with increased risk of fetal death in AVH cases [OR = 7.792 (2.215–27.415) at 95% CI, $p = 0.001$]. The details of the finally included cases are provided in Table 1.

Table 1
Demographical profile of enrolled pregnancy cases.

Parameters	Healthy Term delivery subjects	HEV mRNA positive HEV-IgM positive cases	
		AVH	FHF
N	194	59	17
Maternal age	25.18 ± 4.12	28.64 ± 5.89	26.69 ± 6.49
Gravida	1.53 ± 0.87	1.88 ± 0.78	2.66 ± 1.12
SGPT (IU/ml)	32.08 ± 13.75	1017.16 ± 703.71	1175.43 ± 842.58
SGOT (IU/ml)	34.66 ± 16.78	812.63 ± 503.12	947.57 ± 601.48
Bilirubin (mg/dL)	0.73 ± 0.26	6.49 ± 1.73	11.2 ± 4.09
Grade of encephalopathy	NA	NA	
Grade II			4 [23.53]
Grade III			7 [41.18]
Grade IV			6 [35.29]
Term delivery (≥37 weeks of gestation)	194 [100.00]	42 [71.19]	5 [29.41]
Preterm delivery (<37 weeks of gestation)	0 [0.00]	17 [28.81]	12 [70.59]
Total Fetal death/IUD	0 [0.00]	19 [32.20]	15 [88.24]
Fetal death/IUD in HEV preterm delivery cases	NA	11 [64.71]	12 [100.00]
Maternal death	0 [0.00]	0 [0.00]	13 [76.47]

Cases represented as Numbers [%age].

3.2. HEV viral load profile and genotyping

The HEV high viral load was significantly higher in FHF cases (912,706 ± 603761copies/ml) compared to AVH cases (12,677 ± 9144copies/ml) ($p < 0.001$). Higher viral load was significantly associated with higher encephalopathy grade (Grade IV compared to Grade III or Grade II) in FHF cases ($p = 0.024$). Increased viral load was significantly associated with preterm delivery in AVH ($p = 0.037$), fetal death in total AVH ($p = 0.019$) cases as well as in AVH preterm delivery cases ($p = 0.043$).

To correlate the severity of the disease with genotype, the HEV-RNA positive cases were subjected for genotyping analysis followed by phylogenetic analysis using MEGA4.0 software. The results of the genotyping analysis showed the presence of HEV genotype 1 only (Supplementary Fig. 1).

3.3. Association of differential homocysteine levels in HEV infected pregnancy

The fasting serum-based homocysteine levels were significantly increased in HEV cases compared to healthy term delivery cases (5.724 ± 0.899 μmol/L) ($p = 0.008$). The serum homocysteine levels were significantly increased in both AVH (7.447 ± 2.352 μmol/L) ($p = 0.048$) and in FHF cases (9.904 ± 3.028 μmol/L) ($p < 0.001$) compared to healthy term delivery cases, and in FHF cases compared to AVH cases ($p = 0.022$) (Fig. 1A). In both AVH ($p = 0.002$) and FHF ($p = 0.015$) groups, increased serum homocysteine levels was significantly associated with preterm delivery (Fig. 1A). Higher serum homocysteine levels were associated with fetal death in HEV- preterm delivery cases for AVH ($p = 0.035$), but not for FHF ($p = 0.077$) group or maternal death ($p = 0.105$) in FHF-HEV cases (Fig. 1A). The homocysteine level was positively correlated with the HEV viral load in HEV pregnancy cases [Pearson corrleation = 0.538, $p = 0.047$; Spearman's rho = 0.602, $p = 0.031$].

Next, the differential placental homocysteine expression between healthy term delivery ($N = 40$) and HEV-AVH ($N = 33$) cases were evaluated by immunohistochemistry using specific homocysteine antibody, and was analyzed by a senior pathologist. The FHF cases ($N = 5$) for which placental samples were available, were not included for the IHC based analysis because of its statistically non-significant numbers. The expression was graded as strong, moderate, mild or no expression. The placental homocysteine expression was observed in majority of the

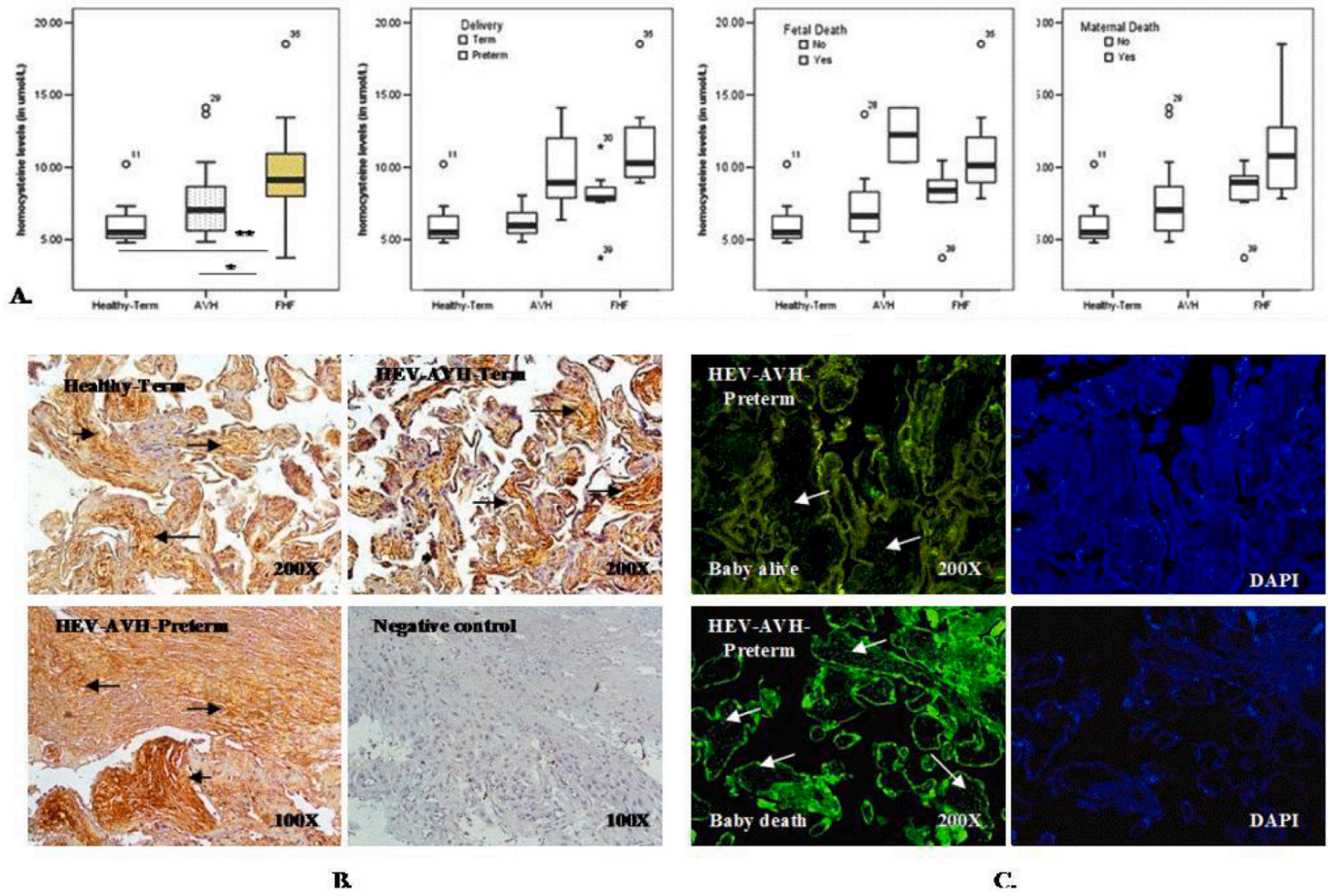

Fig. 1. (A) Difference in serum homocysteine levels in different study cohorts showing a significant gradient increase in expression with increasing severity compared to controls. The significant changes are marked in a single *, whereas the highly significant changes are marked with **. The data clearly reflects the association of increased serum homocysteine with preterm delivery (2nd box-plot), fetal death (3rd box-plot) and maternal death in FHF cases (4th box-blot). (B) Representative images for Immunohistochemistry (IHC) panel showing differences in placental homocysteine expression in AVH cases (term and preterm) compared to healthy term delivery cases. (C) Representative panel of immunoflourescence based analysis showing strong homocysteine expression in HEV-AVH preterm case with negative pregnancy outcome (baby death) compared to case with positive outcome (baby alive status) underlining the significance of placental homocysteine expression in HEV pathophysiology during pregnancy. The DAPI staining was used for counterstaining.

HEV-AVH cases (29/33, 87.88%) compared to healthy term delivery subjects (25/40, 62.50%) (Fig. 1B). Moderate to strong homocysteine expression was observed in HEV-AVH cases compared to healthy term delivery subjects ($p = 0.039$), and in HEV-AVH preterm cases compared to AVH-Term delivery cases ($p = 0.067$). The difference in differential placental homocysteine expression with respect to pregnancy outcome in HEV preterm cases was evaluated by immunofluorescence microscopy (Fig. 1C). The data indicated a strong homocysteine expression in HEV-preterm cases with negative pregnancy outcomes compared to cases with positive pregnancy outcome ($p = 0.187$). The thereby suggests the association of altered homocysteine levels with HEV related pregnancy complications and outcome.

3.4. Serum Vitamin B12 levels and its importance in HEV infected pregnancy

Vitamin B12 level plays a major role in balancing the homocysteine in blood, as it acts as a cofactor in the homocysteine metabolism. The reference range of Vitamin B12 in blood is 118-701 pmol/l. High levels of homocysteine decreases the amount of Vitamin B12 and folate in the body (Devi et al., 2017) which is critical for baby's development, and may also lead to embryological toxicity. The serum Vitamin B12 level studied using commercially available ELISA kit reflected that the average serum Vitamin B12 level was decreased in following group order: Healthy Term delivery (617.870 ± 312.409 pmol/L) > AVH (545.09 ± 258.981 pmol/L) > FHF (337.87 ± 108.41 pmol/L). The serum level of Vitamin B12 in HEV infected pregnancy cases was found to be decreased in both AVH ($p = 0.071$) and FHF ($p = 0.04$) cases compared to healthy term delivery, and in FHF cases ($p = 0.014$) compared to AVH group significantly (Fig. 2A). The serum Vitamin B12 levels were found to be decrease in HEV related preterm delivery compared to HEV term delivery cases ($p = 0.435$) (Fig. 2B). Lower levels of Vitamin B12 were associated with fetal death in HEV- related preterm delivery cases ($p = 0.079$) (Fig. 2C), and maternal death ($p = 0.275$) in FHF-HEV cases. (Fig. 2D). The serum homocysteine levels significantly inversely correlated with serum VitaminB12 levels in HEV related pregnancy cases (p = Pearson correlation = −0.624, $p = 0.013$; Spearman's rho = −0.664, $p = 0.007$].

3.5. Genetic alterations in folate pathway genes and its impact in HEV infected pregnancy

Distribution of variant *MTHFR677*C➔T genotype and *TYMS1494del6* polymorphism analysis was studied in HEV mRNA positive cases only ($N = 76$) compared to term delivery cases (Fig. 3A). The detail genotype distribution data is encrypted in Tables 2 and 3 respectively. The variant *MTHFR* genotype was associated with increased risk of HEV-preterm delivery cases compared to term delivery cases [OR = 7.589, $p <$

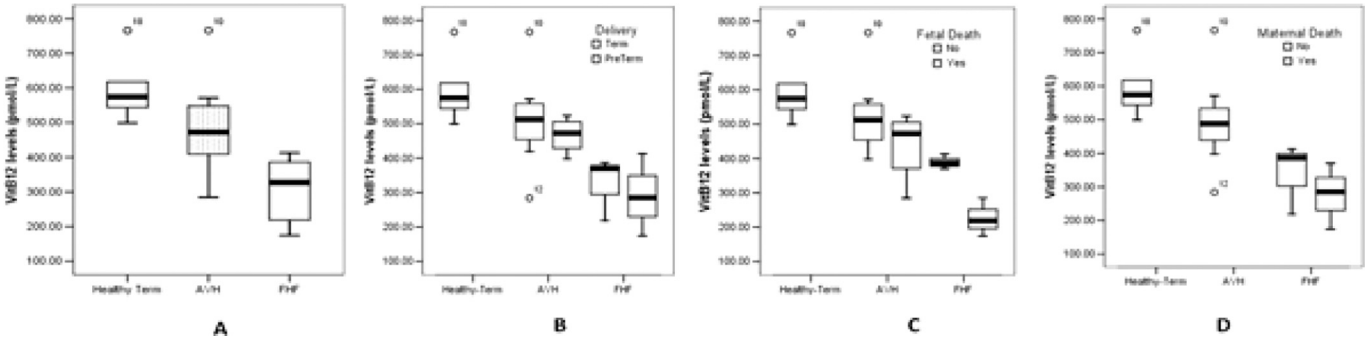

Fig. 2. (A) Decreased levels of serum Vitamin B12 levels in HEV infected cases compared to healthy term delivery cases, the lowest being in FHF cases. (B) Decreased Vitamin B12 levels preterm delivery cases compared to term delivery cases in different HEV cohorts. (C) Decreased Vitamin B12 levels in cases with fetal death in different HEV cohorts. (D) Decreased Vitamin B12 levels in cases with maternal death in HEV-FHF cohort.

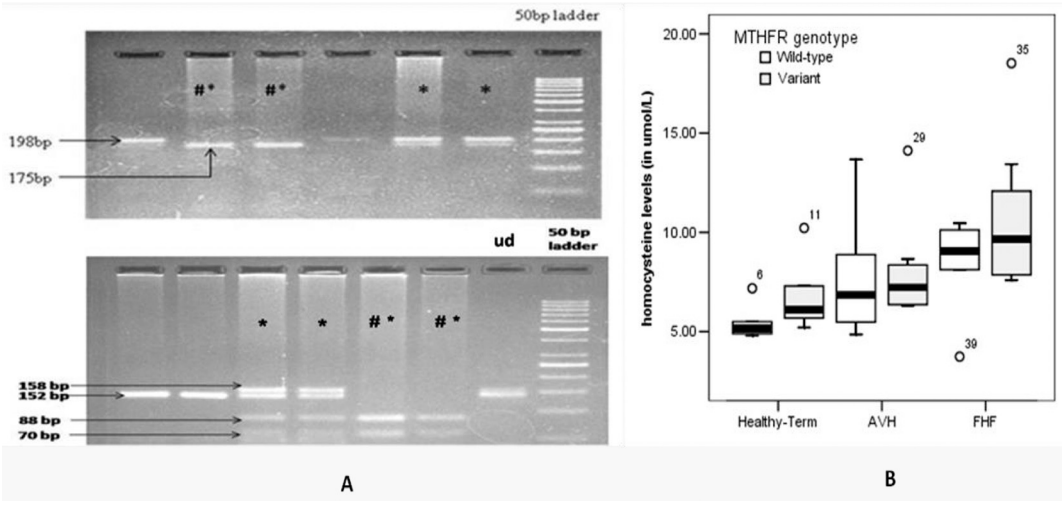

Fig. 3. (A) Representative agarose gel electrophoresis results showing (Upper): *MTHFR* C677T genotyping analysis, the presence of heterozygous (*) and homozygous variant (#*) allele being marked; (Lower) TYMS3′UTRdel6bp polymorphism analysis showing the presence of 6 bp deletion characterised by presence of 152 bp, 6 bp insertion/deletion characterised by presence of 152 + 158 + 88 + 70 bp for heterozygous state marked as (*) and 88 + 70 bp for homozygous 6bpins condition marked as (#*) and undigested marked as ud. (B) Difference in homocysteine levels based on MTHFR C677T genotype in different cohorts (1) term delivery (2) AVH-E and FHF-E pregnancy cases.

Table 2
Distribution of *MTHFR* C677T genotype in term and HEV infected pregnancy cases.

Cases	N	*MTHFR* genotype			variant allele	*p* value	ODDS ratio
		Wildtype	Heterozygote	Homozygote			
Term	194	170 [87.62]	20[10.30]	4[2.06]	24[12.37]	Ref	7.589{3.262–17.658}
HEV Infected Preterm	29	14[48.27]	10[34.48]	5[17.24]	15[51.72]	<0.001*	
HEV term	47	29[61.7]	12 [25.53]	6[12.76]	17[38.29]	Ref	1.726{0.677–4.402}
HEV preterm	29	14[48.27]	10[34.48]	5[17.24]	15[51.72]	0.254	
HEV AVH	59	34[57.62]	16[27.11]	9[15.25]	25[42.37]	Ref	1.209{0.409–3.572}
HEV FHF	17	9[52.94]	6[35.29]	2[11.76]	8[47.05]	0.733	
HEV AVH Term	42	25[59.52]	11[26.19]	6[14.28]	17[40.47]	Ref	1.307 {0.420–4.064}
HEV AVH preterm	17	9[52.94]	5[29.41]	3[17.64]	8[47.05]	0.646	
HEV FHF Term	5	4[80.00]	1[20.00]	0[0.00]	1[20.00]	Ref	5.6 {0.472–66.447}
HEV FHF preterm	12	5[41.66]	5[41.66]	2[16.66]	7[58.33]	0.234	

Cases presented as Numbers [%].

0.001] and HEV- term delivery cases [OR = 1.726, p = NS] (Table 2). The presence of *TYMS1494del6* variant genotype was found to be associated with increased risk of (i) HEV-Preterm delivery compared to healthy term [OR = 1.381] and (ii) Preterm delivery in HEV infected pregnancy cases [OR = 4.629] compared to HEV term. On the basis of del/del allele as homozygote variant when the distribution between healthy term and HEV preterm cohorts were compared then it was found that the distribution of del/del homozygote variant allele was almost significantly higher in HEV preterm cohort $p = 0.052$ and was also associated with increased risk of HEV preterm delivery (OR = 2.901

Table 3
Distribution of *TYMS1494del6* genotype in term and HEV infected pregnancy cases.

Cases	N	*TYMS1494del6* GENOTYPE			Variant allele	p value	ODDS ratio
		Ins/ins	Ins/del	Del/del			
Term	194	18[9.27]	163[84.02]	13[6.70]	90.72	Ref	1.381 {0.303–6.288}
HEV Infected Preterm	29	2[6.89]	22[75.86]	5[17.24]	27[93.10]	0.676	
HEV term	47	12[25.53]	29[61.7]	6[12.76]	35[74.46]	Ref	4.629 {0.954–22.447}
HEV preterm	29	2[6.89]	22[75.86]	5[17.24]	27[93.10]	0.043	
HEV AVH	59	10[16.94]	42 [71.18]	7[11.86]	49[83.05]		1.531{0.302–7.77}
						Ref	
HEV FHF	17	4 [23.52]	9[52.94]	4[23.52]	13[76.47]		
						0.608	
HEV AVH Term	42	8[19.04]	32[76.19]	2[4.76]	34[80.95]	Ref	1.765 {0.334–9.321}
HEV AVH preterm	17	2[11.76]	10[58.82]	5[29.41]	15[88.23]	0.503	
HEV FHF Term	5	2[40.00]	3[60.00]	0[0.00]	3[60.00]	Ref	3.33 {0.319–34.830}
HEV FHF preterm	12	2[16.66]	6[50.00]	4[33.33]	10[83.33]	0.506	

Cases presented as Numbers [%].

{0.950–8.853}0 at 95% CI $p = 0.066$).

In HEV cases, *MTHFR* variant genotype was significantly associated with higher serum homocysteine levels ($p < 0.001$). The presence of *MTHFR* variant genotype has been found to be significantly ($p = 0.050$) associated with the higher homocysteine level in all the study groups (Fig. 3B). The association of *TYMS1494del6* polymorphism with serum homocysteine levels was non-significant in all the case cohorts and sub-cohorts.

3.6. Differential Folate Receptor alpha (FR-α) expression analysis

Folate receptors holds key for transfer of bio-available folate across the placenta, FR-α being one of the most important and well studied one. Differential placental FR-α mRNA expression studied by realtime PCR showed a downregulated expression in both AVH (0.1077 ± 0.0676 folds) and FHF (0.0481 ± 0.0108 folds) cases compared to healthy term delivery cases (Fig. 4A). FR-α mRNA expression was significantly down-regulated in FHF-E cases compared to AVH-E ($p = 0.015$) cases. The FR-á mRNA expression was down regulated in preterm delivery cases compared to term cases in AVH cohorts ($p = 0.166$) and FHF cohorts ($p = 0.212$). Within the HEV-AVH preterm delivery sub-cohort the down-regulation in the expression of FR-α mRNA expression was found to be associated with negative outcome ($p < 0.001$). Protein profile data based on immunofluorescence analysis also showed a deregulated FR-α expression profile in HEV infected pregnancy cases. The expression was more deregulated in HEV pregnancy cases with fetal death compared to live birth in HEV-AVH preterm delivery cases (Fig. 4B).

4. Discussion

HEV infection is a major health problem in developing countries including India (Kar et al., 2008) and is associated with high maternal and fetal mortalities (Bednar et al., 1999) preterm delivery and LBW during pregnancy (Patra et al., 2007; Pérez-Gracia et al., 2015) Placenta has been proved to be an extrahepatic site of HEV replication (Bose et al., 2014). Although sporadic literature has shown the association of endocrinological (Jilani et al., 2007) and molecular endocrinological factors along with differential immunomodulation (Bose et al., 2011) literature falls short of characterizing the molecular events that critically influences differences in HEV infected pregnancy cases. Further there are studies showing viral infection accompanying by oxidative stress (Alfredo Ríos-Ocampo et al., 2020; Liu et al., 2017) With the background of the concern of oxidative stress with the pregnancy complications including PTD as well as viral infection, the present study targeted to evaluate the role of increased serum levels homocysteine and the other component such of deregulated folate pathway including vitamin B12 and folate deficiency in influencing HEV infected pregnancy and outcome of different severity grade. This is the first study of its kind as there is no data on the role of increased homocysteine mediated oxidative stress on HEV infected pregnancy and outcome.

Global data indicate that high viral load influences diseases severity in hepatitis infection (Borkakoti et al., 2013; Bose et al., 2011) In the present study, HEV viral load estimation data shows the significant increase in both FHF and AVH cases. Further the increase viral load was also associated with the preterm delivery as well as fetal death thus indicating its association in the progress to disease severity. Our findings

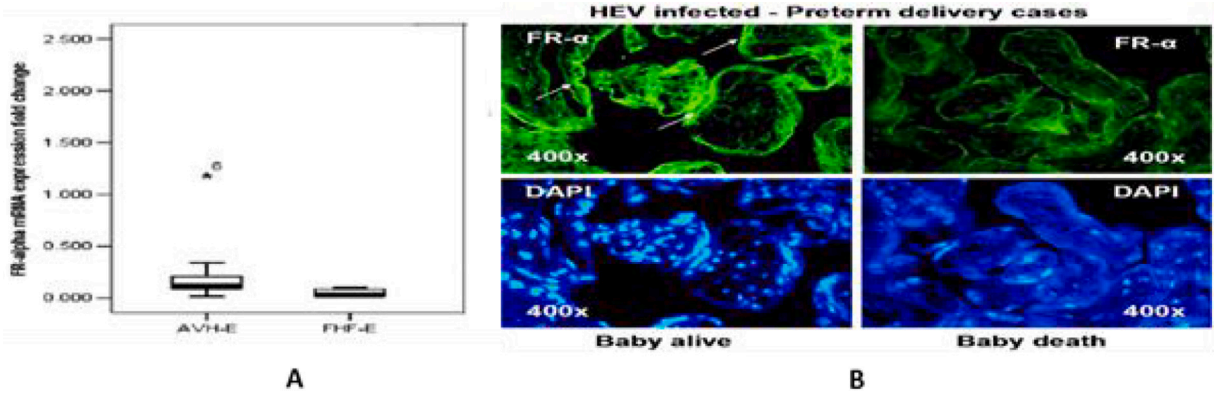

Fig. 4. (A) Box-plot analysis showing a downregulated mRNA expression profile of FR-á in both AVH and FHF cases compared to healthy term delivery cases (B) Panel of IF based photographs representing the increased down-regulation of placental FR-á expression in HEV –AVH preterm cases in baby death cases compared to live birth cases suggesting an association of downregulated FR-á expression with pregnancy complications and outcome in HEV infected cases.

was in concordant with the study of (Borkakoti et al., 2013; Bose et al., 2011) who reported the severity in pregnancy complications with the increase in viral load in HEV infected cases especially.

Pregnancy has been suggested to be a risk factor for viral replication which may be detrimental to both the fetus and the mother (Kar et al., 2008). Reports suggest that risk of preterm delivery is high in hepatitis E infected pregnant women cases (Patra et al., 2007). These studies are in concordant with our study which shows that in the HEV infected pregnancy cases the fetal death ($p = 0.009$) and maternal death ($p = 0.012$) were significantly higher in FHF compared to AVH. Further, the incidence of preterm delivery was also higher in case of FHF compared to AVH.

Oxidative stress is present in HEV infection during pregnancy, as shown by low GSH, and is associated with adverse pregnancy outcomes (Bhatnagar et al., 2016). Deficiency of micronutrient such as Vitamin B12 and folate during pregnancy may lead to elevated maternal plasma levels of homocysteine, affecting pregnancy by increasing the oxidative stress and vasculo-toxicity implicating complications such as pre-eclampsia, intrauterine growth, preterm delivery etc. (Refsum, 2001). Serum homocysteine levels were found to be significantly elevated in HEV infected delivery cases compared to healthy term, as well as HEV-PTD cases compared to HEV infected term delivery cases. Serum homocysteine levels were significantly increased in FHF ($p = 0.022$) compared to AVH. Correlation analysis within AVH and FHF group showed that the higher homocysteine levels was significantly associated with preterm delivery (AVH ($p = 0.002$) and FHF ($p = 0.015$). Apart from association with PTD the higher serum homocysteine levels were also associated with fetal death in HEV-PTD cases (both AVH and FHF group). Higher serum homocysteine level was associated with maternal death in FHF group. The placental level expression analysis also showed that placental homocysteine levels was associated with preterm delivery and negative pregnancy outcome in HEV related cases. The results on the association of elevated homocysteine level with the HEV-PTD and fetal death in our study is similar to findings in non-infected preterm delivery case earlier(Micle et al., 2012; Vollset et al., 2000) which thus signifies the importance of homocysteine levels in pathogenesis of HEV-PTD. These findings definitely underlines the prognostic and biomarker significance of homocysteine in predicting the severity and outcome of HEV infected pregnancy cases.

The defeciency of micronutrient vitamin B12 shows harmful effect during pregnancy as it is involved in the regulation of homocysteine in folate pathway. Serum Vitamin B12 level was decreased in HEV infected pregnancy cases for both AVH ($p = 0.071$) and FHF ($p = 0.04$) cases compared to term delivery and in FHF cases ($p = 0.014$) compared to AVH group significantly. Decreased serum Vitamin B12 level was also found to be associated with both fetal and maternal death. Thus insufficiency of vitamin B12 levels in maternal serum is attribute for the pregnancy complications in our study and similar data has been reported from several studies (Mishra et al., 2020; Rogne et al., 2017; Youssry et al., 2017).

Further our correlation data showed that the elevated homocysteine levels was inversely and significantly associated with the decreased levels of serum Vitamin B12 in HEV infected pregnancy cases (p = Pearson correlation = −0.624, $p = 0.013$; Spearman's rho = −0.664, $p = 0.007$] implicating the toxic effect and increased oxidative stress resulting in various pregnancy complications. Low vitamin B12 level is thus considered to affect the biochemical pathway and hence contributing for elevated homocysteine during pregnancy. Our finding is concordant with the study of (Ray and Laskin, 1999 and Vollset et al., 2000) who validated that lower vitamin B12 level is associated with the hyperhomocysteinemia resulting in pregnancy complications.

Various pregnancy complications have been associated with folate deficiency, which is essential for fetal growth and development and maternal well-being (van der Molen et al., 2000) *MTHFR C677T* polymorphism is responsible for the loss of enzyme activity, and leads to a decreased pool of methyl-THF and hyperhomocysteinemia, particularly in folate deficiency (Frosst et al., 1995) which influences common pregnancy outcomes (James, 2009) and can increase the risk of spontaneous abortion, preterm delivery, or IUGR (Baker, 1993). The variant *MTHFR* genotype contributes to chronic decidual vasculopathy and ultimately PTD (Kramer et al., 2001). Our group has earlier documented the critical association of *MTHFR* C677T polymorphism with the increased risk of PTD as well as negative pregnancy outcome (Tiwari et al., 2015b). The novel findings from the present work also shows that the variant genotype is significantly associated with increased risk of preterm delivery in HEV infected pregnancy cases compared to both Healthy term delivery [OR = 7.589, $p < 0.001$] and HEV-infected term delivery cases [OR = 1.726, $p = 0.354$]. *MTHFR* variant genotype was also associated with increased risk of developing FHF in HEV cases, and increased risk of preterm delivery within the AVH and FHF sub-cohorts.

TYMS1494del6 (rs869066439) polymorphism is a 6 bp sequence (TTAAAG) deletion/insertion in 3′UTR region (Mandola et al., 2004; Mandola et al., 2003) associated with the stability and translation of *TYMS* mRNA thus affecting the protein level expression of TYMS (Ulrich et al., 2000). Previous findings on the polymorphism have shown equivocal documentation either showing its association or no role during pregnancy complications(Choi et al., 2016; Kim et al., 2013; Wang et al., 2015). The *TYMS1494del6* variant genotype was associated with increased risk of HEV-PTD compared to Healthy term delivery cases [OR = 1.381] and HEV infected term delivery cases [OR = 4.629].

Studies suggested that alterations in gene associated with folate metabolism (*MTHFR C677T* and *TYMS1494del6bp* polymorphism) resulting in hyper-homocystenemia likely influence the pregnancy by affecting embryogenesis and fetal growth (Kim et al., 2009; Solanky et al., 2010). Similarly our study also reflects that the differential expression of homocysteine was significantly associated with the *MTHFR C677T* status, and therefore the *MTFHR C677T* genotype is a detrimental factor deciding the fate of HEV infected pregnancy.

Increased expression levels of FR-α in the placenta is critical for catering the increased need for folate for the placenta and fetus during development with the progress of gestation (Yasuda et al., 2008). Reduced expression of FR-α has been shown to be associated with pre-eclampsia (Williams et al., 2012) but not with IUGR (Bisseling et al., 2004). FR-α expression is highly polarised to the MVM of the syncytiotrophoblast (Solanky et al., 2010), and its correlation of its differential expression in PTD has been reported (Castaño et al., 2017). The FR-α expression analysis in the present study showed that the differential mRNA expression of FR-α was downregulated in AVH and FHF cases compared to healthy term delivery cases; in FHF-E cases compared to AVH ($p = 0.015$) and in AVH and FHF preterm delivery cases compared to AVH and FHF term delivery cases respectively. The downregualtion in FR-α mRNA expression was also significantly associated with negative pregnancy outcome in both AVH and FHF groups of HEV-PTD cases ($p < 0.001$). The placental FR-α protein expression profile was concurrent with the mRNA expression profile. IF based data clearly underlined the association of downregulated placental FR-α expression in HEV-PTD cases compared to TD cases and negative regnancy outcome in HEV-PTD cases.

Till date there is no study pertaining to the oxidative stress mediated pregnancy complications due to increased homocysteine levels in HEV infected pregnancy cases. Besides the limitation of present study to perform the model based study pertaining to HEV infection to established the cause and effect of increased oxidative stress in pregnancy complications, the present novel study for the first time has been able to provide crucial insights on the role of increased homocysteine mediated oxidative stress linked with deregulation(s) in key folate pathway components in deciding the fate of HEV infected pregnancy cases. To conclude, increased homocysteine facilitated oxidative stress due to genetic alterations in folate pathway genes in a FR-α deficient condition along with decreased Vitamin B12 level plays a critical role in HEV infected pregnancy complications like preterm delivery and negative pregnancy outcome. Serum homocysteine levels in combination with

MTHFR C677T polymorphism screening has prognostic significance, and probably may be considered as biochemical and genetic biomarkers respectively after more elaborative scientific studies. Based on previously available literature, supplementation of micronutrients, Vitamin B12 and folic acid may be a useful alternative in combating increased homocysteine in HEV infected pregnancy cases and controlling the associated fetal and maternal morbidity and mortality.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104882.

Authors' contributions

Dr. Diptika Tiwari has done the sample collection and processing, experimental analysis and wrote the paper. Dr. Rizwana Sultana has performed the immunofluorescence analysis along with Dr. Diptika Tiwari. Ms. Natasha Kashyap and Mr. Mafidul Islam have performed the HEV RNA detection, viral load analysis and ELISA alongwith Dr. Diptika Tiwari. Dr. Purabi Deka Bose have done the sample processing and regenotyping work for validation. Dr. Chandana Ray Das and Dr. Anjan Kumar Saikia have provided the samples and clinical data. Dr. Sujoy Bose have conceived, designed and supported the work, performed the statistical analysis, and wrote the paper along with Dr. Diptika Tiwari.

Funding information

This work was funded by Indian Council of Medical Research (ICMR) Govt. of India. Award No. Tribal/CFP/17/2018-ECD-II.

Ethical statement

The present study was conducted with ethical permission from the institutional ethics committee of all the participating institutions.

Declaration of Competing Interest

Authors have no conflict of interest.

Acknowledgments

The authors would like to thank Indian Council of Medical Research (ICMR), Govt. of India for providing the necessary infrastructure to carry out all the experimental work smoothly. The authors are thankful to all the Doctors and Nurses of the hospital for their support and cooperation during sample collection for the study. The authors would like to thank all the volunteers who participated in the study.

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