

Basic Study

Estrogen receptor expression in chronic hepatitis C and hepatocellular carcinoma pathogenesis

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Author contributions: Kaul A and Kaul R designed and coordinated this study; Iyer JK, Kalra M and Kaul R acquired and analyzed the data after performing the molecular investigations; Payton ME performed the statistics on acquired data; Iyer JK, Kalra M, Kaul A and Kaul R contributed to writing and final approval of the article.

Supported by Cancer Sucks, Bixby, Oklahoma Research grant to Kaul R.

Institutional review board statement: The research study protocol was approved by expedited review procedure by the Institutional Review Board of University of Minnesota (FWA 00000312) and Oklahoma State University-Center for Health sciences (2003012-OSU-CHS).

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: All available data can be obtained by contacting the corresponding author.

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Manuscript source: Unsolicited manuscript

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Received: March 20, 2017
Peer-review started: March 22, 2017
First decision: April 21, 2017
Revised: May 12, 2017
Accepted: July 22, 2017
Article in press: July 24, 2017
Published online: October 7, 2017

Abstract**AIM**

To investigate gender-specific liver estrogen receptor (ER) expression in normal subjects and patients with hepatitis C virus (HCV)-related cirrhosis and hepatocellular carcinoma (HCC).

METHODS

Liver tissues from normal donors and patients diagnosed with HCV-related cirrhosis and HCV-related HCC were obtained from the NIH Liver Tissue and Cell Distribution System. The expression of ER subtypes, ER α and ER β , were evaluated by Western blotting and real-time RT-PCR. The subcellular distribution of ER α and ER β was further determined in nuclear and cytoplasmic tissue lysates along with the expression of

inflammatory [activated NF- κ B and I κ B-kinase (IKK)] and oncogenic (cyclin D1) markers by Western blotting and immunohistochemistry. The expression of ER α and ER β was correlated with the expression of activated NF- κ B, activated IKK and cyclin D1 by Spearman's correlation.

RESULTS

Both ER subtypes were expressed in normal livers but male livers showed significantly higher expression of ER α than females ($P < 0.05$). We observed significantly higher mRNA expression of ER α in HCV-related HCC liver tissues as compared to normals ($P < 0.05$) and ER β in livers of HCV-related cirrhosis and HCV-related HCC subjects ($P < 0.05$). At the protein level, there was a significantly higher expression of nuclear ER α in livers of HCV-related HCC patients and nuclear ER β in HCV-related cirrhosis patients as compared to normals ($P < 0.05$). Furthermore, we observed a significantly higher expression of phosphorylated NF- κ B and cyclin D1 in diseased livers ($P < 0.05$). There was a positive correlation between the expression of nuclear ER subtypes and nuclear cyclin D1 and a negative correlation between cytoplasmic ER subtypes and cytoplasmic phosphorylated IKK in HCV-related HCC livers. These findings suggest that dysregulated expression of ER subtypes following chronic HCV-infection may contribute to the progression of HCV-related cirrhosis to HCV-related HCC.

CONCLUSION

Gender differences were observed in ER α expression in normal livers. Alterations in ER subtype expression observed in diseased livers may influence gender-related disparity in HCV-related pathogenesis.

Key words: Estrogen receptor α ; Estrogen receptor β ; Hepatitis C virus-related cirrhosis; Hepatitis C virus-related hepatocellular carcinoma; Sex and gender; Normal liver

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Core tip: Our study, for the first time, demonstrates gender-based differences in basal expression of estrogen receptor α (ER α) in the liver of normal males and females. Altered nuclear and cytoplasm liver ER subtype protein expression was also observed in hepatitis C virus (HCV)-cirrhosis and HCV-related hepatocellular carcinoma (HCC) that correlate with the inflammatory and oncogenic markers, activated NF- κ B and cyclin D1, implicating their role in chronic cirrhosis and malignant transformation. These findings may have an impact on future planning strategies for the development of novel treatments or prognostic markers by targeting ERs or their signaling pathways during chronic HCV-related cirrhosis and HCC development.

Iyer JK, Kalra M, Kaul A, Payton ME, Kaul R. Estrogen receptor expression in chronic hepatitis C and hepatocellular carcinoma pathogenesis. *World J Gastroenterol* 2017; 23(37): 6802-6816 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i37/6802.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i37.6802>

INTRODUCTION

Hepatitis C virus (HCV) infection is a major health problem in the United States^[1]. HCV-infection tends to be asymptomatic for long periods of time during which fibrosis and cirrhosis can develop, resulting in end-stage liver disease^[2,3]. No viable vaccine is currently available for HCV prevention and, while newer and expensive direct acting antivirals are available for treating early HCV-infection, transplantation is the only effective treatment for patients with advanced or end stage liver disease^[4,5]. HCV-infection related fibrosis and cirrhosis are associated with the development of chronic liver disease that eventually leads to hepatocellular carcinoma (HCC), the most common type of liver cancer^[6-8]. The latest epidemiological data shows that HCC has the fastest growing death rate in the United States^[9]. Thus, there is an urgent need to develop novel, effective and affordable therapeutics to treat chronic HCV-infections.

Understanding the contribution of host factors that directly or indirectly interact with HCV is central to finding new treatment modalities^[10]. Striking gender disparity in HCV and HCC pathogenesis has led to the investigation of estrogen as one of the key host players in modulating the course of HCV-pathogenesis. Epidemiological and clinical studies have shown that chronic HCV-infections are more prevalent in males and progress more rapidly to cancer development as compared to females^[11,12]. Similar to males, postmenopausal females lacking circulating estrogen show accelerated progression of HCV-infection to fibrosis and HCC development^[12,13]. In contrast, physiological estrogen levels seen in premenopausal females, have been associated with less severity and slow progression through all stages of HCV infection^[11,14]. Direct evidence of the therapeutic potential of estrogen has come from experimental studies demonstrating that administration of 17 β -estradiol inhibits HCC development in *in vitro* and *in vivo* animal models^[15,16]. However, clinical trials evaluating hormonal therapy using selective estrogen receptor modulators (SERMs) have shown inconsistent outcomes in HCC patients^[17-19]. These inconsistencies may be attributed to the differences in the expression of estrogen receptors (ERs) in the liver^[20].

Liver is a sexually dimorphic organ and expresses the ERs, ER α and ER β , making it responsive to the

actions of estrogen^[21,22]. These receptors classically function as transcription factors, shuttling between the cytoplasm and nucleus for regulating the expression of various genes involved in cell cycle, proliferation, apoptosis and inflammation^[23]. More recently, the non-classical role of ERs in cell signaling has become increasingly evident^[24,25]. The two subtypes, ER α and ER β share significant structural homology and ligand binding properties and yet function very differently, often antagonizing each other's actions^[26]. Thus, the relative expression of the two subtypes can have a significant impact on net cellular responses to estrogen.

In breast cancer, ER α :ER β expression ratio is thought to play a key role in estrogen-dependent tumor development^[27,28]. Like breast cancer, aberrant increase in ER gene expression has been reported in liver tumors when compared to normal or non-tumor parts of the liver in HCC patients^[29,30]. However, there is very limited information available on the relative expression of ER α and ER β subtypes and ER α :ER β expression ratio in livers of normals, HCV-related cirrhosis and HCV-related HCC.

We hypothesized that basal liver ER α and ER β expression differs in males vs females and this differential expression dictates host susceptibility to chronic HCV or its progression to hepatocellular carcinoma. In the present study, we evaluated the expression of ER α and ER β in the livers of normals, HCV-cirrhosis and HCV-related HCC at the mRNA and protein levels using different techniques. We further determined the correlation of ER subtype expression with the levels of inflammatory and oncogenic markers like NF- κ B and cyclin D1. Our findings show that the basal expression of ER subtypes is different between normal males and females. Furthermore, sub-cellular expression of both ER subtypes is altered in HCV cirrhosis and HCV-related HCC livers as compared to normals and correlates with the expression of inflammatory and oncogenic markers. This altered expression of ER subtypes in the liver may contribute to the progression of cirrhosis and cancer development during HCV-pathogenesis.

MATERIALS AND METHODS

Patients

Explant liver tissues from normal donors and HCV-related cirrhosis and HCV-related HCC patients (Tables 1-3) were obtained from the NIH Liver Tissue and Cell Distribution System (LTCDS) at the University of Minnesota. Liver explants were aseptically collected under the institutional review board (IRB) guidelines of University of Minnesota. The study was conducted at Oklahoma State University-Center for Health Sciences under IRB guidelines. In order to avoid interference of other co-factors, patients co-infected with human immunodeficiency virus (HIV) or hepatitis B virus (HBV)

or with a history of alcohol consumption or drug use were excluded from the study. Liver explants were snap frozen in liquid nitrogen and stored at -80 °C until further use.

Western blot analysis

Whole tissue lysates were prepared by homogenizing approximately 200 to 300 mg of liver tissues in liquid nitrogen followed by lysis in buffer comprising of 50 mmol/L Tris-HCl (pH 8.0), 400 mmol/L KCl, 10 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 μ g/mL aprotinin and 1 μ g/mL leupeptin. The homogenates were centrifuged at 30000 $\times g$ for 1 h at 4 °C. The supernatants were collected and stored at -80 °C until further use. Cytoplasmic and nuclear fractions were prepared from liver tissues using the NE-PER kit (Pierce Chemical Co., Rockland, IL, United States) as per the manufacturer's instructions. Protein concentrations were determined by Bicinchoninic acid assay (Pierce Chemical Co., Rockland, IL, United States). Thirty micrograms of protein from each lysate (whole tissue lysate, cytoplasmic or nuclear tissue lysates) was resolved on 4%-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, United States). The separated proteins were electrophoretically transblotted on to a 0.2 μ m nitrocellulose membrane. Purified recombinant ER α and ER β proteins were included as positive controls. The membrane was blocked in 5% non-fat dry milk made in TBS (20 mmol/L Tris-HCl, pH 7.6 and 136 mmol/L NaCl) containing 0.1% Tween-20 for 2 h and probed with antibodies specific for human ER α (rat monoclonal against human ER α , H222, Fitzgerald Industries International Inc., Concord, MA, United States), human ER β (mouse monoclonal against amino acids 1-153 of human ER β , 14C8, Affinity Bioreagents, Golden, CO, United States), phosphorylated I κ B-kinase (IKK) $\alpha\beta$ (rabbit polyclonal against a phosphopeptide corresponding to amino acids surrounding Ser176/180 of human IKK α , Cell signaling Technology, Danvers, MA, United States), IKK α (rabbit polyclonal against a peptide corresponding to 20 amino-terminal amino acids of human IKK α , Cell signaling Technology, Danvers, MA, United States), Cyclin D1 (rabbit polyclonal against amino acids 1-295 of human cyclin D1, H-295, Santa Cruz Biotechnology, Dallas, TX, United States), phosphorylated NF- κ B (mouse monoclonal against a phosphopeptide corresponding to amino acids surrounding Ser536 of human NF- κ B p65 subunit, Cell signaling Technology, Danvers, MA, United States), NF- κ B (rabbit monoclonal against a peptide corresponding to amino acids near the amino-terminus of human NF- κ B p65 subunit, Cell signaling Technology, Danvers, MA, United States) and β -actin (rabbit polyclonal against a peptide corresponding to amino acid 20-33 of actin, Sigma-Aldrich, St. Louis, MO, United States). After incubation with alkaline phosphatase conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, United States), the protein

bands were detected by enhanced chemiluminescence (Pierce Chemical Co., Rockland, IL, United States). To ensure the purity of cytoplasmic and nuclear fractions, the blots were also probed with anti-GAPDH (rabbit monoclonal against a peptide corresponding to a part of the carboxyl terminus of human GAPDH, Cell signaling Technology, Danvers, MA, United States) and anti-histone H1 (mouse monoclonal against nuclei from biopsies of myeloid leukemia cells, EMD Millipore, Billerica, MA, United States) antibodies respectively. Images of the protein bands were digitally captured with Alpha Innotech instrumentation (Alpha Innotech Corp, San Leandro, CA, United States) and quantified with Image analysis program, ImageJ (NIH, Bethesda, MD, United States). The phosphorylated forms were expressed as a ratio of the phosphorylated protein to total protein.

Immunohistochemistry

Immunohistochemistry was performed on normal and diseased explant liver tissue sections to confirm the protein expression of ER α , ER β and cyclin D1. Formalin-fixed liver tissues were embedded in paraffin and sectioned into 4- μ m thick sections. Non-enzymatic antigen retrieval was performed in citrate buffer (pH 6.0) using a microwave procedure. Slides were allowed to cool down for 20 min at room temperature and endogenous peroxidase was quenched with Dako EnVision system kit according to manufacturer's instructions (Dako, Carpinteria, CA, United States). The sections were washed and incubated for 30 min with 0.5% Triton X-100 in PBS followed by 5% rabbit serum. The tissue sections were incubated overnight with primary antibodies against ER α (rabbit polyclonal against a peptide corresponding to the carboxyl terminus of mouse ER α , MC-20, 1:50 dilution, Santa Cruz Biotechnology, Dallas, TX, United States), or ER β (mouse monoclonal against peptide corresponding to the carboxyl terminus of ER β , PPG5/10, 1:20 dilution, Dako, Carpinteria, CA, United States) at 4°C. Slides were washed with PBS containing 0.05% Tween and treated for one hour at room temperature with corresponding HRP labeled secondary antibodies (Dako, Carpinteria, CA, United States). The tissue sections were stained with 3,3'-diaminobenzidine and the nuclei were counterstained with Mayer's hematoxylin. A similar immunostaining procedure was used for cyclin D1 (rabbit polyclonal antibody against amino acids 1-295 of human cyclin D1, H-295, 1:150 dilution, Santa Cruz Biotechnology, Dallas, TX, United States). For negative controls, sections were incubated with PBS instead of the primary antibody. The stained sections were visualized using Nikon microscope (Nikon H600 L) and sections with staining of $\geq 10\%$ of the cells were defined as positive.

Real-time RT-PCR

Total RNA was isolated from liver tissues with Trizol

(Thermo Fisher Scientific, Waltham, MA, United States) followed by purification on RNeasy columns (Qiagen, Valencia, CA, United States) as per the manufacturer's instructions. Total RNA from each sample was treated with DNase (Thermo Fisher Scientific, Waltham, MA, United States) and then reverse-transcribed to synthesize cDNA using the High capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, United States). The expression of target genes was evaluated by quantitative real-time PCR using commercially available ER α and ER β specific Taqman primers and probe assay mixes (Applied Biosystems, Foster City, CA, United States) on an ABI StepOne Real-Time PCR System (Applied Biosystems Foster City, CA, United States). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) was included as the endogenous control. Relative differences in gene expression between groups were determined using the $2^{-\Delta\Delta CT}$ method using the normal group for normalization^[31].

Statistical analysis

The statistical methods of this study were performed and reviewed by Dr. Mark E. Payton from Department of Statistics, Oklahoma State University, Stillwater, United States. Data obtained from Western blotting and real time RT-PCR experiments were analyzed statistically using SAS software v9.4 (SAS Institute Inc., Cary, NC, United States). Statistical significance between appropriate groups was determined by non-parametric analyses that included the Kruskal-Wallis test and Mann Whitney *U* test. Post-hoc analyses were performed using Fisher's Protected Least Significant Difference comparisons using rank data test. Correlations of ER subtypes with other proteins were analyzed by using the Spearman's rank correlation test. $P \leq 0.05$ was considered statistically significant. All graphs were made and depicted using the GraphPad Prism v6 software (GraphPad Software Inc, La Jolla, CA, United States).

RESULTS

Basal expression of ER α is significantly higher in liver of normal males than females

Due to the well-established gender disparity in HCC prevalence, we initiated this study by investigating the expression of ER α and ER β in normal males and females. Whole tissue lysates of liver explants obtained from normal females and normal males (Table 1) were evaluated by Western blotting using validated ER α and ER β directed antibodies (Figure 1A). Densitometric analysis of protein bands showed comparable ER subtype expression in normal male and female tissues when the gender data was pooled (Figure 1B). However, when ER subtype expression data was segregated based on gender, we found that there was a significantly higher expression of ER α in males as

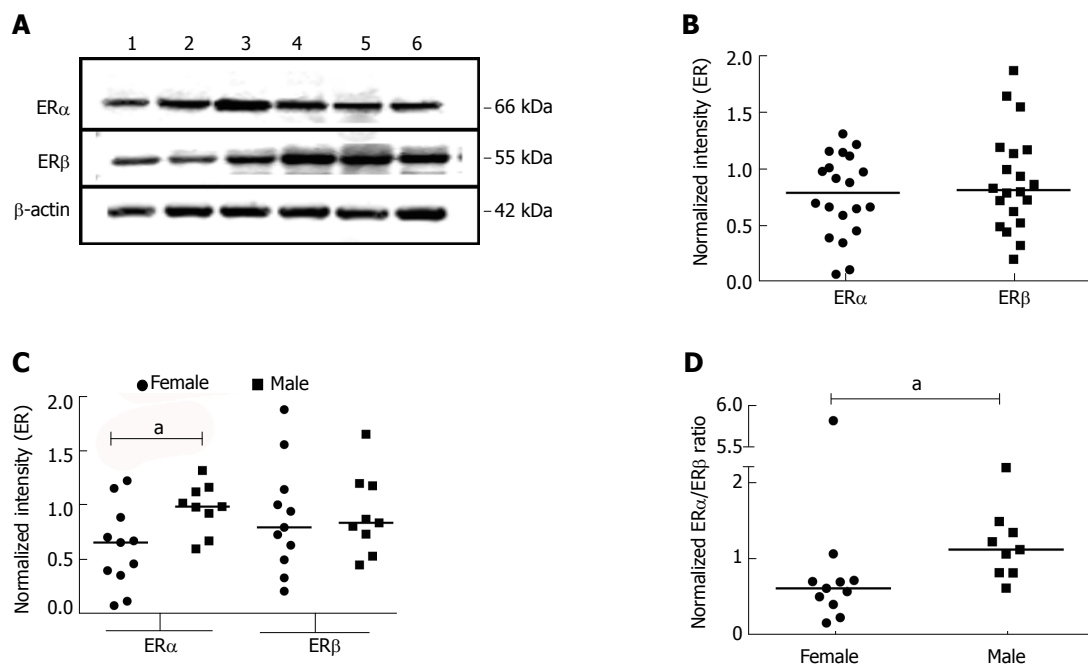


Figure 1 Expression of estrogen receptor subtypes in whole tissue lysates from normal male and female subjects. A: Whole tissue lysates from liver tissues of normal donors were subjected to Western blotting and probed with antibodies against ER α , ER β and β -actin. A representative blot is shown. B-D: The bands corresponding to ER α , ER β and β -actin were quantified by densitometric analyses using ImageJ. Each symbol represents one individual. Expression of the ER subtypes was normalized to the expression of β -actin and plotted (B). Gender-based ER subtype expression was evaluated by segregating the gender and plotted (C). The ER α :ER β expression ratio was also plotted for each gender group (D). ^a*P* < 0.05 was considered significant. ER: Estrogen receptor.

Table 1 Age ranges of normal and diseased subjects in whole tissue lysates

Group	No. of subjects	No. of females	Age range (yr)	No. of males	Age range (yr)
Normal	20	11	42-67	9	34-64
HCV	14	4	53-60	10	43-55
HCC	14	1	56	13	44-66

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 2 Age ranges of normal and diseased subjects in mRNA analysis

Group	No. of subjects	No. of females	Age range (yrs)	No. of males	Age range (yrs)
Normal	9	8	42-67	1	64
HCV	10	3	43-60	7	43-53
HCC	10	0	N/A	10	44-59

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

Table 3 Age ranges of normal and diseased subjects in nuclear and cytoplasmic tissue lysates

Group	No. of subjects	No. of females	Age range (yr)	No. of males	Age range (yr)
Normal	10	5	43-67	5	50-64
HCV	12	3	51-60	9	46-53
HCC	16	0	NA	16	44-66

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

compared to females (Figure 1C). No difference was found in the expression of ER β between males and females. Since physiological outcomes are determined by the expression of both, ER α and ER β , we compared the ER α :ER β expression ratio between male and females and found that males showed a significantly higher ER α :ER β expression ratio compared to females (Figure 1D).

Altered expression of ER α is observed in males with HCV-related cirrhosis and HCV-related HCC

The expression of ER subtypes was compared in liver explants of normals, HCV-related cirrhosis and HCV-related HCC (Tables 1 and 2) by real time RT-PCR and Western blotting (Figure 2). At the transcriptional level, there was an increase in the liver mRNA expression of ER α (ESR1) and ER β (ESR2) subtypes in chronic HCV and HCV-related HCC as compared to normals (Figure 2A and B). This change in expression significantly decreased the ER subtype ratio in the HCV-related cirrhosis group compared to normal (Figure 2C), suggesting that HCV infection may result in altering the expression of ER subtypes.

When we measured ER subtype protein levels by Western blotting, we could not detect any significant differences among the three groups when the data was evaluated without gender segregation (Figure 2 D-G). However, upon separating males and females,

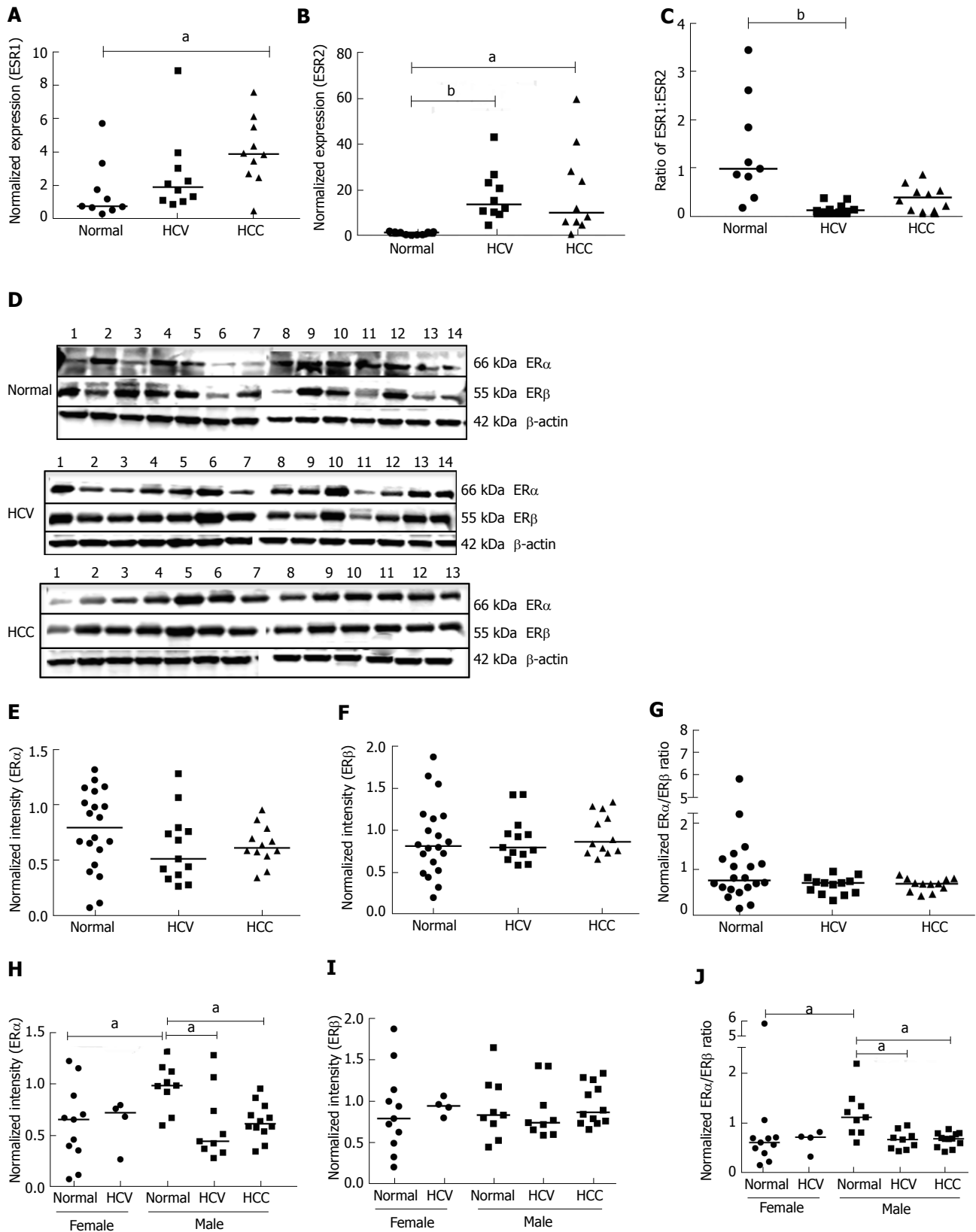


Figure 2 Expression of estrogen receptor subtypes from normal and diseased subjects. A-C: Total RNA extracted from normal, HCV-related cirrhosis and HCV-related HCC livers was subjected to RT-PCR. The abundance of ESR1 (A) and ESR2 (B) transcripts was determined by real time PCR and normalized to the expression of HPRT. ESR1:ESR2 expression ratio was also plotted for the three groups (C). ^a*P* < 0.05 or ^b*P* < 0.001 was considered significant. D: Whole tissue lysates from liver tissues of normal, HCV and HCC subjects were subjected to Western blotting and probed with antibodies against ERα, ERβ and β-actin. Representative blots for each group are depicted. E-J: The bands corresponding to ERα, ERβ and β-actin were quantified by densitometric analyses using ImageJ. Expression of the ERα and ERβ was normalized to the expression of β-actin and plotted (E and F). The ERα:ERβ expression ratio was also plotted for normal, HCV and HCC group (G). Expression of ER subtypes and ERα:ERβ expression ratio in the male population of normal, HCV and HCC groups was plotted separately (H-J). ^a*P* < 0.05 was considered significant. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

Table 4 Spearman correlations of ER α and ER β expression in whole tissue lysates with nuclear cyclin D1, nuclear phosphorylated NF- κ B and cytoplasmic phosphorylated IKK expression

	R-coefficient (P value)		
	Normal	HCV	HCC
Correlations with WTL ER α			
ER β	0.4154 (0.0692)	0.81944 (0.0006) ^b	0.70339 (0.0107) ^a
Nuclear cyclin D1	0.62189 (0.0549)	0.88842 (0.0075) ^a	-0.29785 (0.3737)
Nuclear pNF- κ B	0.02757 (0.9397)	0.84435 (0.0169) ^a	0.19351 (0.5686)
Cytoplasmic pIKK	-0.21577 (0.5494)	-0.09787 (0.8346)	-0.37679 (0.2534)
Correlations with WTL ER β			
Nuclear cyclin D1	-0.03251 (0.9290)	0.92648 (0.0027) ^a	-0.70135 (0.0162) ^a
Nuclear pNF- κ B	-0.18056 (0.6176)	0.71732 (0.0696)	0.41196 (0.2080)
Cytoplasmic pIKK	0.24608 (0.4931)	-0.21801 (0.6386)	-0.03438 (0.9201)

^aP < 0.05, ^bP < 0.001. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor; WTL: Whole tissue lysates.

we observed a significant decrease in the expression of ER α in diseased livers (HCV as well as HCC) compared to normal livers (Figure 2H) but no difference in the expression of ER β (Figure 2I) in males. This resulted in a significant decrease in ER α :ER β expression ratio in male diseased livers (HCV as well as HCC) compared to normal male livers (Figure 2J). Among the females, we could compare the expression of ER subtypes between normals and HCV-related cirrhosis only as there were no females in the HCV-related HCC category. We found that there were no significant differences in protein levels of ER α and ER β between normal livers and HCV-related cirrhotic livers in females (Figure 2H and I). We further observed a positive-correlation between the expression of ER α and ER β in HCV-related cirrhosis and HCV-related HCC group based on gender-pooled data (Table 4).

Thus the results from the mRNA and protein analyses performed in whole tissue extracts showed different trends. Since classical ERs shuttle between the nuclear and cytoplasmic compartments of the cells and mediate their regulatory functions by binding to estrogen response elements on DNA, we evaluated the expression of ER subtypes in nuclear and cytoplasmic extracts of liver tissues.

Increased nuclear translocation of ER subtypes is observed in HCV-related cirrhosis and HCV-related HCC

The distribution and expression of ER α and ER β in nuclear and cytoplasmic extracts of normal, HCV-related cirrhosis and HCV-related HCC group is depicted in Figure 3. We observed a higher expression

Table 5 Spearman correlations of ER α and ER β expression in nuclear tissue lysates with nuclear cyclin D1 and nuclear phosphorylated NF- κ B expression

	r-coefficient (P value)		
	Normal	HCV	HCC
Correlations with nuclear ER α			
Nuclear ER β	0.63880 (0.0468) ^a	0.63539 (0.0264) ^a	0.73192 (0.0008) ^b
Nuclear cyclin D1	0.12049 (0.7402)	0.75728 (0.0043) ^a	0.55714 (0.0202) ^a
Nuclear pNF- κ B	-0.62968(0.0511) ^c	0.207 (0.5186)	0.4793 (0.0516) ^c
Correlations with nuclear ER β			
Nuclear cyclin D1	0.44012 (0.2031)	0.64445 (0.0237) ^a	0.51223 (0.0355) ^a
Nuclear pNF- κ B	-0.52091 (0.1226)	0.10243 (0.7514)	0.25861 (0.3162)

^aP < 0.05, ^bP < 0.001, ^cP < 0.055. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

Table 6 Spearman correlations of ER α and ER β expression in cytoplasmic tissue lysates with cytoplasmic pIKK

	r-coefficient (P value)		
	Normal	HCV	HCC
Correlations with cytoplasmic ER α			
Cytoplasmic ER β	0.61233 (0.0599)	0.74376 (< 0.0001) ^a	0.79765 (0.0158) ^b
Cytoplasmic pIKK	-0.66837 (0.0346) ^a	-0.00698 (0.9828)	-0.56014 (0.0194) ^a
Correlations with cytoplasmic ER β			
Cytoplasmic ER α	0.61233 (0.0599)	0.91363 (< 0.0001) ^b	0.79765 (0.0001) ^b
Cytoplasmic pIKK	-0.60909 (0.0616)	-0.29717 (0.3482)	-0.60848 (0.0095) ^a

^aP < 0.05, ^bP < 0.001. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

of ER α in the nuclear extracts of normal males as compared to normal females but the increase was not statistically significant, probably due to the small sample size (Supplementary Figure 1). There was no change in the expression of nuclear ER β . These observations in normals for the expression of nuclear ER α and ER β were similar to the results observed in whole tissue extracts.

When we compared the expression of ER subtypes in nuclear and cytoplasmic extracts of normal and diseased livers, we could not perform statistically relevant gender analyses due to the small sample size and scarcity of female subjects in the diseased groups. Hence we have reported our results without gender segregation. We observed a significant increase in the expression of nuclear ER α and ER β in HCV-related HCC and HCV-related cirrhosis (Figure 3A, B and D) respectively when compared to normals. However, there was no significant difference in the expression of cytoplasmic ER α , while HCV-related HCC group showed a significantly higher expression of cytoplasmic ER β compared to HCV-related cirrhosis group (Figure 3C and E). An increase in the nuclear:

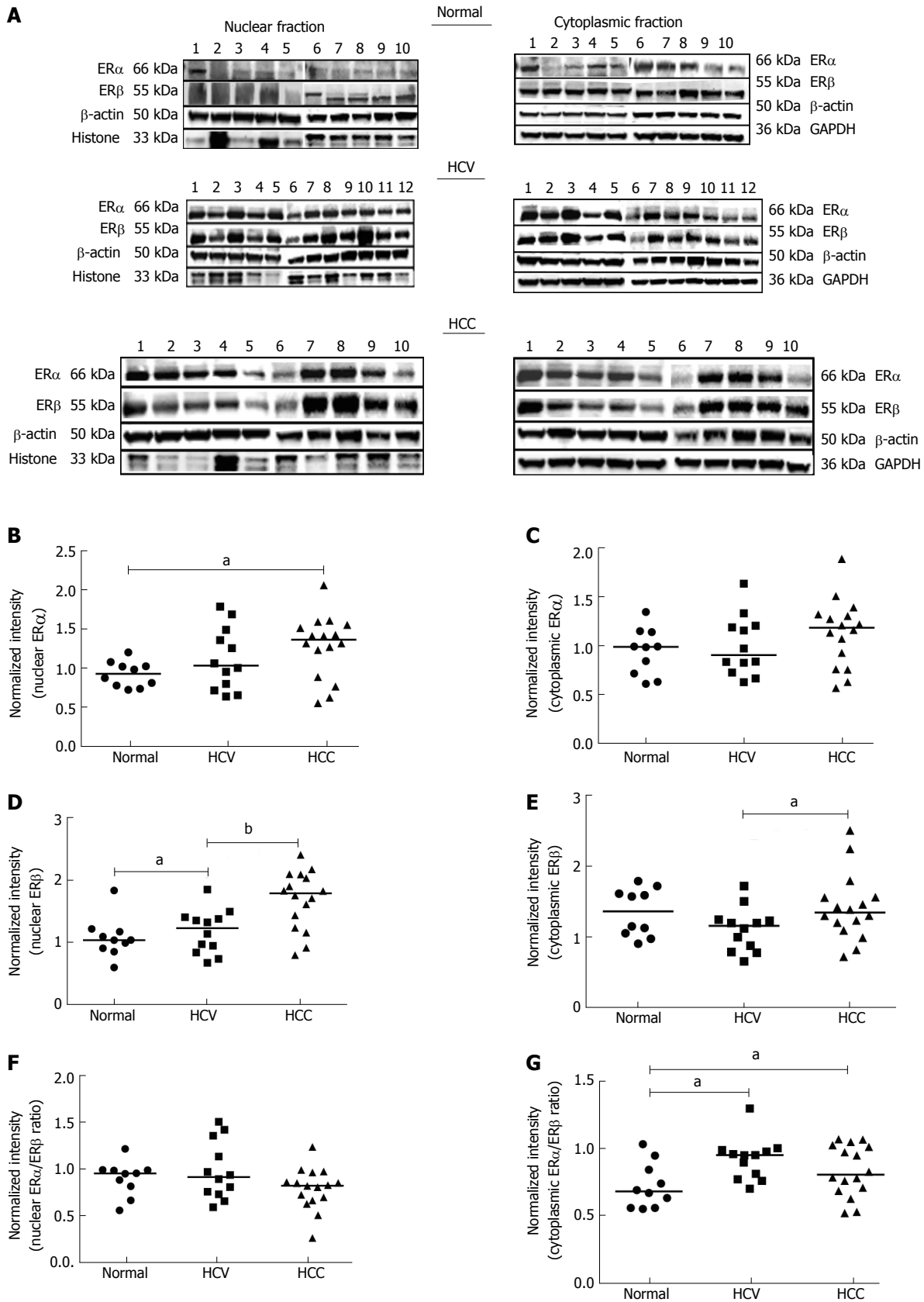


Figure 3 Expression of estrogen receptor subtypes in nuclear and cytoplasmic tissue lysates from normal and diseased subjects. A: Lysates prepared from nuclear and cytoplasmic fractions of liver tissues from normal, HCV and HCC subjects were subjected to Western blotting and probed with antibodies against ER α , ER β , β -actin, histone (nuclear lysates only) and GAPDH (cytoplasmic lysates only). Representative blots for each group are depicted. B-G: The bands corresponding to ER α , ER β and β -actin were quantified by densitometric analyses using ImageJ. Expression of the ER α and ER β was normalized to the expression of β -actin and plotted with (B and C). The ER α :ER β expression ratio was also plotted for normal, HCV and HCC group (D). Expression of ER subtypes and ER α :ER β expression ratio in the male population of normal, HCV and HCC groups was plotted separately (E-G). ^a*P* < 0.05 or ^b*P* < 0.001 was considered significant. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

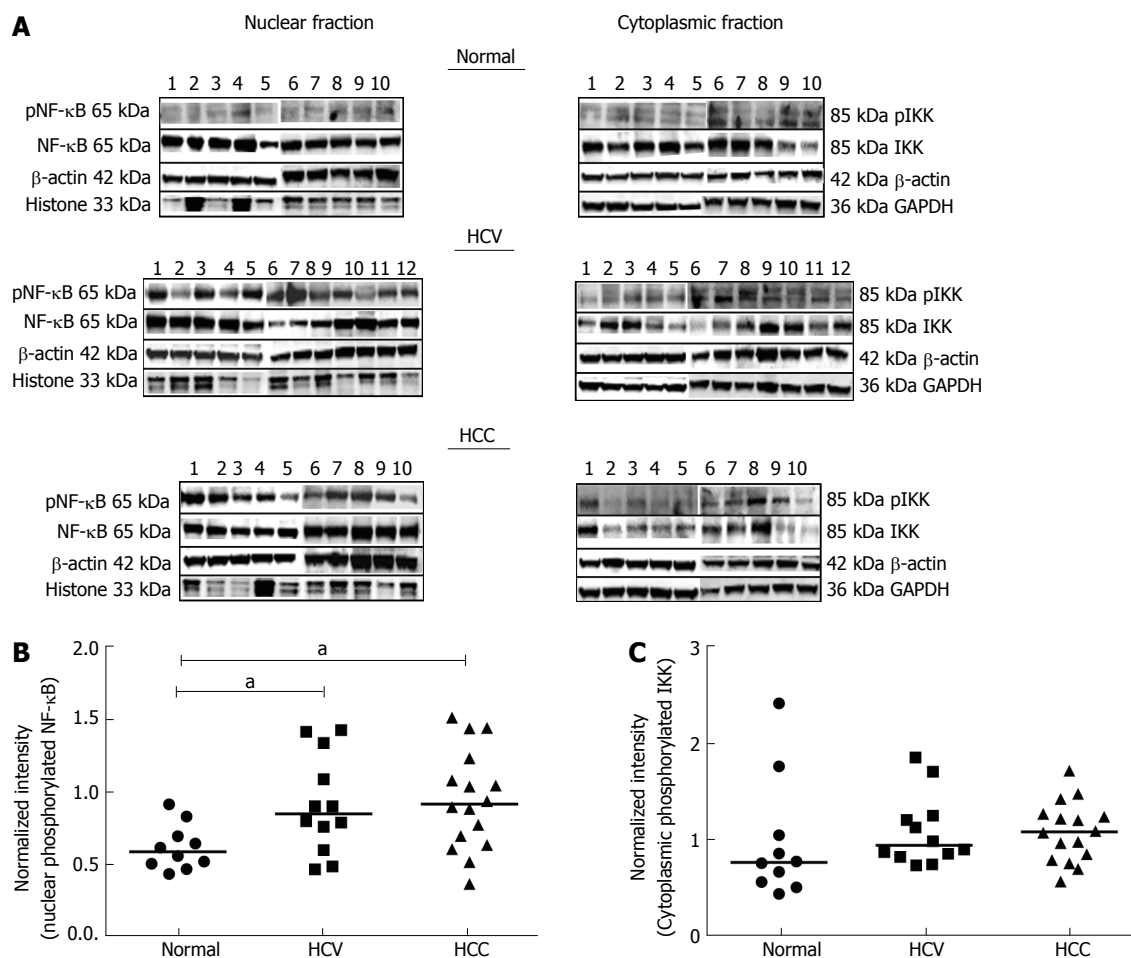


Figure 4 Expression of pNF-κB and pIKK in nuclear and cytoplasmic tissue lysates from normal and diseased subjects. A: Lysates prepared from nuclear and cytoplasmic fractions of liver tissues from normal, HCV and HCC subjects were subjected to Western blotting. Nuclear tissue lysates were probed with antibodies against pNF-κB, NF-κB, β-actin, and histones while cytoplasmic tissue lysates were probed with antibodies against pIKK, IKK, β-actin and GAPDH. Representative blots for each group are depicted. B and C: The bands corresponding to pNF-κB, NF-κB, pIKK, IKK and β-actin were quantified by densitometric analyses using ImageJ. The ratio of pNF-κB:NF-κB was normalized to the expression of β-actin and plotted (B). The ratio of pIKK:IKK was normalized to the expression of β-actin and plotted with gender pooled (C) subjects. ^a*P* < 0.05 was considered significant. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ER: Estrogen receptor.

cytoplasmic ratio of both, ER α and ER β , in the diseased groups were observed compared to the normals (Supplementary Figure 1) suggesting that there is increased transcriptional activation of ER subtypes in the progression to HCV-related cirrhosis to HCV-related HCC. Furthermore, there was a significant increase in the ratio ER α :ER β in the cytoplasmic compartment of the HCV-related cirrhosis and HCV-related HCC groups compared to normals (Figure 3G) but there was no significant difference in the ratio of the nuclear ER subtypes (Figure 3F).

HCV-related cirrhosis and HCV-related HCC group show higher activation of NF-κB

HCV infection is known to trigger inflammatory pathways and livers displaying chronic inflammation are more at risk of developing hepatocellular carcinoma^[32,33]. Most inflammatory pathways involve NF-κB, hence we evaluated the activation of the NF-κB signaling pathway by detecting the phosphorylation of the p65 subunit and IKK; the kinase that phosphorylates IκB, resulting in its dissociation from

NF-κB and degradation by the proteasome. Livers chronically infected with HCV and HCV-related HCC tissues showed significantly higher expression of phosphorylated NF-κB (Figure 4A and B) compared to normals. There was an increase in the expression of pIKK from normal livers to HCV cirrhosis and HCV-related HCC, but the increase was not statistically significant (Figure 4C). We also observed a weak negative correlation between nuclear ER α and pNF-κB in normal liver tissues (*r* = -0.62968, *P* = 0.0511; Table 5). On the contrary, HCV-related HCC group demonstrated a weak positive correlation between nuclear ER α and pNF-κB (*r* = 0.4793, *P* = 0.0516; Table 5). In addition, expression levels of pIKK and the cytoplasmic ER subtypes showed a strong negative correlation in the HCC group (ER α : *r* = -0.56014, *P* = 0.0194; ER β : *r* = -0.60848, *P* = 0.0095; Table 6).

ER α levels correlate with expression of cyclin D1 in both HCV cirrhosis and HCV-related HCC

In order to study the correlation of ERs with oncogenic markers in diseased livers, we determined the

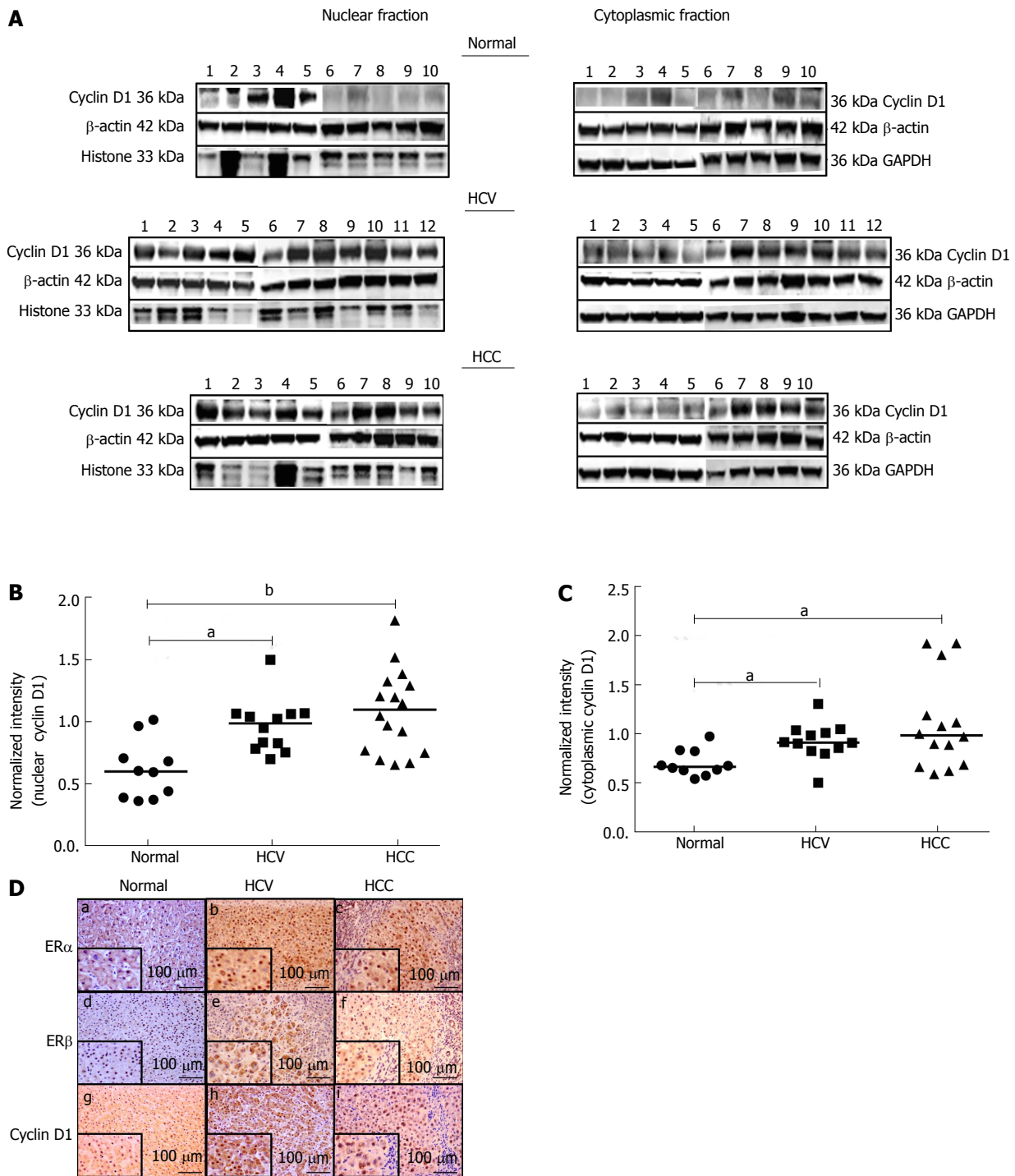


Figure 5 Expression of cyclin D1 in nuclear and cytoplasmic tissue lysates from normal and diseased subjects. A: Lysates prepared from nuclear and cytoplasmic fractions of liver tissues from normal, HCV and HCC subjects were subjected to Western blotting and probed with antibodies against cyclin D1, β -actin, histones (nuclear lysates only) and GAPDH (cytoplasmic lysates only). Representative blots for each group are depicted. B and C: The bands corresponding to cyclin D1 and β -actin were quantified by densitometric analyses using ImageJ. Expression of cyclin D1 was normalized to the expression of β -actin in nuclear (B) and cytoplasmic (C) lysates and plotted. ^a $P < 0.05$ or ^b $P < 0.001$ was considered significant. D: Immunohistochemical staining of ER α , ER β and cyclin D1 in normal (a, d, g), HCV-related cirrhosis (b, e, h) and HCV-related HCC (c, f, i) is depicted. A magnified region of the stained tissue section is depicted as an inset in each corresponding image. Scale bar = 100 μ m. HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

expression of cyclin D1 in the nuclear and cytoplasmic liver tissue extracts of normals, HCV-related cirrhosis and HCV-related HCC group by Western blotting (Table 3). As shown in Figure 5, there was a significant increase in the expression of cyclin D1 in both, the nuclear and cytoplasmic fractions of diseased

livers, suggesting that chronic HCV infection primes hepatocytes to proliferate. A positive-correlation between the expression of nuclear ER α and ER β with nuclear cyclin D1 was observed in both HCV-related cirrhosis (ER α : $r = 0.75728$, $P = 0.0043$; ER β : $r = 0.64445$, $P = 0.0237$; Table 5) and HCV-related HCC

(ER α : $r = 0.55714$, $P = 0.0202$; ER β : $r = 0.51223$, $P = 0.0355$; Table 5) groups but not in normals, signifying the importance of ER subtypes in cyclin D1 activation (Table 5). We further determined the expression of ER subtypes and cyclin D1 by immunohistochemistry (IHC). As observed with Western blotting, IHC showed over-expression of ER α and cyclin D1 in HCV-related cirrhosis and HCV-related HCC groups (Figure 5D, Supplementary Tables 1-3).

DISCUSSION

Various epidemiological studies around the globe have recognized the role of gender bias in the progression of HCV infection to chronic liver disease and cirrhosis due to poor therapeutic responses or further development of HCV-related HCC in these patients^[34]. Remarkable differences have been observed in the incidence, progression of HCV-related chronic liver disease and outcome of interferon therapy in males, postmenopausal females and premenopausal females^[13,35]. This has generated a significant interest in the scientific community to investigate the role of estrogen as the key host factor in HCV-related pathogenesis. Recent studies in postmenopausal women with HCV-related fibrosis clearly show clinical benefits from estrogen replacement therapy in conjunction with standard HCV treatment^[12]. One of the most compelling evidence is the inhibition of HCV life cycle by 17 β -estradiol in an ER α dependent manner in human liver cell lines^[36,37]. Raloxifene, a SERM, treatment has been shown to improve the efficacy of antiviral treatment in HCV-infected postmenopausal women^[38]. These findings suggest a protective role of estrogen affecting not only viral replication but also HCV pathogenesis in the host.

Despite the recognition of estrogen as a potentially significant host factor in HCV pathogenesis, little progress has been made to understand the underlying mechanisms on how ER subtypes may be involved in progression to HCV chronic disease in some patients and rapid clearance of HCV in others. Perhaps this gap in understanding is one of the major factors contributing to the current debatable potential of estrogen therapy^[17,19]. In addition, most of the clinical studies did not monitor ER α and ER β expression in the liver of the enrolled patients or the hormonal status of females at the time of recruitment or during treatment^[20]. Some studies evaluated ER subtype expression by measuring mRNA levels in the liver or performing less specific ligand binding assays^[39, 40].

One of the most important premises of the current study was to evaluate the gender-based (males vs females) ER subtype protein expression in the liver of normals (basal expression) and further evaluate the changes in expression of these subtypes in HCV-related cirrhosis or HCV-related HCC patients. Further, we employed subcellular fractionation (nuclear versus cytoplasmic) protein studies to examine the relative

expression of ER subtypes and correlate their presence with activation of NF- κ B or cyclin D1 in these fractions. Our findings in whole tissue extracts of normal livers indicated that males express significantly higher levels of ER α protein compared to females but comparable levels of ER β . We also found a significant increase in the ER α :ER β expression ratio in males as compared to females. To our knowledge, this is the first report demonstrating gender-based differences in ER α at the basal level thereby affecting the ratio of ER subtypes in the liver. Whether these differences in ER subtype expression at basal levels predispose males to severe and fast disease progression of HCV infection needs further investigation.

We were able to detect ER α and ER β transcripts in normal, HCV-related cirrhosis and HCV-related HCC groups. Similar observations were made in a Korean population study where they observed ER α and ER β mRNA transcripts in more than 95% of HCV-related HCC liver tissues^[40]. There was an increase of ER α and ER β transcripts in diseased livers compared to normals but there were no changes in the protein levels of ERs among the groups in whole tissue lysates. However, gender analyses of ER expression in whole tissue lysates revealed that there was a significant decrease in ER α but not ER β expression only in diseased male patients suggesting its disease specific role in these patients. These findings are in agreement with Villa *et al* who showed that there was reduction or even loss of wild-type ER α in male HCV-related HCC livers but an increase in the expression of variant ER α , which we have not evaluated^[41,42].

Further, ER expression in the nuclear and cytoplasmic fractions from gender-pooled diseased livers differed when compared to normals. In the nuclear fraction, we found a higher expression of ER α and ER β subtypes in HCV-mediated HCC and HCV-mediated cirrhosis tissues respectively compared to normal liver tissues, which is in contrast to the findings in the whole tissue extracts. HCV-mediated HCC tissues also showed significantly higher expression of cytoplasmic ER β only when compared to HCV-cirrhosis tissues. We found a significant increase in the ER α to ER β ratio in the cytoplasmic fraction than in the nuclear fraction of both HCV-related cirrhosis and HCV-related HCC livers as compared to normal livers. The clinical relevance of these changes in ER subtype expression in the subcellular compartments needs to be further investigated. ER protein levels have been shown to vary under physiological states during breast tumor progression and hormonal therapy^[43]. A recent study in the MCF7 cell-line has shown that estrogen stimulation involves dynamic changes in protein subcellular spatial distribution rather than changes in total protein abundance and propose that cells may be transformed by perturbations in nucleocytoplasmic shuttling of nuclear hormone receptors and other proteins^[44,45]. In addition to breast cancer, ERs have also been implicated to play important roles

in gastrointestinal cancers like gastric, colon and colorectal cancers^[46-48]. Since ERs primarily function as nuclear receptors and act as transcription factors for gene regulation, we propose that evaluating the expression of these proteins in cellular fractions as opposed to the whole tissue lysates would be a more accurate representation of their biological activity. Furthermore, our immunohistochemistry analysis also supported the increase in nuclear and cytoplasmic expression of ER α in both groups of diseased livers compared to normal livers (Supplementary Table 1). The expression of cytoplasmic ER β was comparable among all the three groups by immunostaining. However, we observed a loss of nuclear ER β in some HCC tissues (Supplementary Table 2). Loss of ER β was also reported by Iavarone *et al.*^[39], who evaluated the expression of ER subtype mRNA in liver cancer tissues. Additionally, in breast, colon and prostate cancers, there are reports that indicate that presence of ER β leads to better prognosis^[49-52]. These findings about differential expression of nuclear or cytoplasmic ER β expression in HCV cirrhosis or HCC can be substantiated only by increasing the sample size in the study.

Inflammation has been associated with the initiation and progression of hepatocarcinogenesis^[53]. Since HCV infection triggers inflammation, and chronic inflammation promotes carcinogenesis, NF- κ B has been viewed as an important player in inflammation-induced cancer^[54,55]. In the current study, we found a significant increase in activated NF- κ B in HCV-related cirrhosis and in HCV-related HCC liver tissues. Significant increase in ERs in the cytoplasmic fraction of diseased livers may modulate the activity of pIKK thereby influencing the activation of NF- κ B and inflammation outcome. ER α and NF- κ B have been shown to directly interact in the nuclei of U2-OS cells treated with 17 β -estradiol and aberrant ER α and NF- κ B expression correlated with invasion and metastasis in HCC^[56,57]. 17 β -estradiol, *via* ER α , can also inhibit the transport of p65 to the nucleus and downregulate inflammation^[58]. Detailed mechanistic studies are warranted to explore the cross talk between ER α and NF- κ B to unfold the nature of their interaction in a HCV infection setting.

Unlike mammary carcinoma, the target genes and mechanism for ER regulation in liver are not well studied. There is a correlation between dysregulation in ER subtype with the overexpression of cyclin D1^[59]. In our study, we observed increased expression of cyclin D1 in HCV-related cirrhosis and HCV-related HCC compared to normal livers and the expression of nuclear cyclin D1 correlated with the expression of nuclear ER subtypes in diseased livers. These findings along with other studies suggest that ERs may play a role in dysregulation of cellular proliferation by participating in increased transcription of cyclin D1 that aids in the progression of HCV infection to cirrhosis and eventually to hepatocellular carcinoma^[60].

Cyclin D1 expression can also be regulated by ER α *via* non-genomic signaling. Treatment of HepG2 cells with 17 β -estradiol activates the MAPK/ERK pathway by membrane-localized ERs resulting in increased expression of the cyclin D1 gene^[61]. Apart from its role in cell cycle progression, cyclin D1 has recently been recognized to play a role in influencing sex steroid metabolism by increasing estrogen levels and decreasing androgen levels^[62]. This is the first study that correlates the expression of ER subtypes with cyclin D1 in HCV-related cirrhosis and HCV-related HCC.

Increased expression of different isoforms or variants of ER α in diseased livers has been reported by mRNA studies and their involvement to activate oncogenic signaling pathways in cell lines has been reported^[63,64]. The variants, ER α 36 and ER α 46 have been shown to modulate signaling pathways linked to proliferation and apoptosis^[65,66]. Differential expression of ER α 36 has been observed in various cancers like breast, gastric, and endometrial cancer^[67] and its expression correlates with a worse prognosis in breast cancer^[68] but its role in HCV-mediated HCC has not been thoroughly investigated. Various ER β variants have also been detected in different tissues but their role in carcinogenesis has not been evaluated as extensively as the ER α variants^[69]. The present study focused to fill the gap in knowledge for the expression of wild type ER α and ER β in normal and diseased livers. However, the expression of variant ER receptors also needs to be investigated to understand their role, if any, in HCV-related carcinogenesis. In addition to ER subtypes, the expression of androgen receptors should also be evaluated as HCV infection enhances androgen receptor-mediated signaling in an *in vitro* model^[70] but it is not clear if androgen receptors play a role in carcinogenesis due to existing conflicting reports^[71,72].

Finally, in the current study, we have demonstrated for the first time that the basal expression of wild-type ER α in the liver is higher in males than females. Furthermore, the increased sub-cellular expression of ER subtypes observed in diseased livers correlates with the expression of inflammatory and oncogenic markers. This altered expression of ER subtypes in the liver may contribute to the progression of cirrhosis and cancer development during HCV-pathogenesis. These observations have been made in end stage HCV-related cirrhosis and HCC. In future, early disease or stage specific prospective longitudinal studies that include a larger sample size (in both males and females category) can be conducted to further clarify the role of nuclear or cytoplasmic ER expression at early stages of HCV-related cirrhosis or HCV-related HCC. Impact of gender associated ER subtype ratio distribution in the liver can also be studied relative to oncogenic markers and that may help us to monitor host therapeutic responses, viral clearance or improved prognosis during the carcinogenesis process. In conclusion, we expect that by evaluating the expression of sex

hormone receptors and understanding the signaling pathways they affect, gender and stage-related personalized therapies can be designed to treat and manage chronic HCV infections and HCV-related HCC.

ACKNOWLEDGMENTS

We thank Dr. Senait Assefa and Zachary Lovato for their technical assistance and Ayantika Sen for reviewing this manuscript. Liver tissues were obtained from the NIH Liver Tissue and Cell Distribution System (LTCDS; Contract No. HHSN276201200017C).

COMMENTS

Background

Hepatitis C virus (HCV) infection-related fibrosis and cirrhosis lead to the development of chronic liver disease that can ultimately result in hepatocellular carcinoma (HCC). According to epidemiological and clinical studies, chronic HCV infection mediated cirrhosis is more prevalent in males and progresses to cancer development faster as compared to premenopausal females who show better therapeutic responses and clear the infection. However, females with menopausal estrogen deficiency may have reactivation of the HCV infection resulting in accelerated progression to fibrosis and HCC development, confirming estrogen related etiology. Previous hormonal therapies in HCC have been inconclusive perhaps due to them poor understanding of estrogen receptor (ER) subtype expression in the normal livers from both males and females and also how changes in the expression of ERs may contribute to HCV-related disease progression. In the current study, they evaluated the basal liver ER α and ER β expression in males versus females and have studied the changes in liver ER subtype expression and their correlation with inflammatory and proliferative markers in HCV-related cirrhosis and HCV-related HCC

Research frontiers

Gender disparity in immunity to chronic infectious disease, autoimmunity and cancer development is increasingly being recognized in various clinical and experimental studies. Systemic estrogens in females play an important role in host innate immunity, however, the contributions of estrogen receptors are poorly recognized. In addition to hormonal cancers like breast, prostate and uterus, ER subtypes are being increasingly implicated in non-hormonal tissue cancers as well that include liver, colon, pancreas and stomach.

Innovations and breakthroughs

This is the first report demonstrating gender-based differences in the basal expression of ER α in the liver that also results in change in ER subtype ratio. In addition to ER subtype expression in the whole liver tissue extracts, subcellular fractionation of liver extracts for expression of ER subtypes, inflammatory and proliferative markers in nucleus and cytoplasm of both, normal and diseased livers, were also evaluated. To our knowledge, this is the first study that correlates the expression of ER subtypes with cyclin D1 in livers of HCV-related cirrhosis and HCV-related HCC.

Applications

Investigating gender associated changes in ER subtype ratio in the liver during the course of HCV-related disease progression along with oncogenic marker expression may help us to monitor gender specific host therapeutic responses, viral clearance and cancer development more effectively. Furthermore, these ER subtype expression studies will further complement existing treatments and pave the way to develop gender and stage-related personalized therapies that are highly needed to treat and manage chronic HCV infections and HCV-related HCC.

Terminology

Cirrhosis of the liver is damage caused by chronic Hepatitis C viral infection. Cirrhosis involves replacement of normal liver tissue with scar tissue that affects

liver function and leads to end-stage disease.

Peer-review

Authors investigated ER expression in liver hepatocytes from normal subjects and patients with hepatitis C virus-related cirrhosis and Hepatocellular Carcinoma. The expression of ER subtypes, ER α and ER β , were evaluated for the first time in relation to gender.

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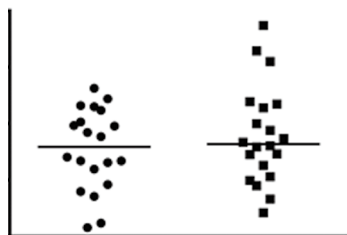
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P- Reviewer: Sukocheva OA S- Editor: Gong ZM L- Editor: A E- Editor: Ma YJ









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ISSN 1007-9327

