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The Expression of Estrogen Receptors in Hepatocellular Carcinoma in Korean Patients

Ai Guo Wang,[1,#] Ki Young Lee,[2,#] Seong Yong Kim,[3] Jong Young Choi,[4] Kee Ho Lee,[5] Wook Hwan Kim,[6] Hee Jung Wang,[6] Jin Man Kim,[7] Moon Gi Park,[8] Young Il Yeom,[1] Nam Soon Kim,[1] Dae Yeul Yu,[1] and Dong Seok Lee[1,9*]

[1]Laboratory of Human Genomics, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Korea;
[2]Department of Anesthesiology and Pain Medicine, and Anesthesia and Pain Research Institute, Yonsei University College of Medicine, Seoul, Korea;
[3]Department of Biochemistry & Molecular Biology, College of Medicine, Yeungnam University, Daegu, Korea;
[4]Kangnam St. Mary's Hospital, Department of Internal Medicine, The Catholic University of Korea, Seoul, Korea;
[5]Laboratory of Molecular Oncology, Korea Institute of Radiological and Medical Sciences, Seoul, Korea;
[6]Department of Surgery, School of Medicine, Ajou University, Suwon, Korea;
[7]Department of Pathology, College of Medicine, Chungnam National University, Daejeon, Korea;
[8]Department of Surgery, SUN General Hospital, Daejeon, Korea;
[9]College of Animal Resource Sciences, Kangwon National University, Chunchon, Korea.

Expression of estrogen receptors (ER)-α and -β, as well as androgen receptor (AR), in hepatocellular carcinoma (HCC) is thought to be correlated with prognosis, survival, and male prevalence of HCC. These hypotheses are based on investigations of European patients; however the expression patterns of these receptors in Asian patients are largely unknown. In this study, we collected liver carcinoma and peritumor tissues from 32 patients (9 females and 23 males) in South Korea. The expression of ERs and ARs was studied using RT-PCR. Wild-type ER-α and AR were expressed in all of the samples investigated, and their expression was independent of the causal virus or patient sex. Expression of the ER-α variant was independent of sex (100% female vs. 91.3% male) and HCV and HBV status (91.3% vs. 100%). Wild-type ER-β was expressed more often in HCV patients than in HBV patients (95.7% vs. 44.4%; $p < 0.05$). In conclusion, the stronger ER-α variant expression in HCC tissues implies that this variant has an important role in HCC development. However, at least in Korean patients, expression of the ER-α variant (vER-α) is not related to male HCC prevalence. In addition, the predominant expression of ER-β in HCV patients suggests that it plays an important role in HCV-induced liver disease.

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#*These authors contributed equally to this work.*

Reprint address: requests to Dr. Dong Seok Lee, College of Animal Resource Sciences, Kangwon National University, Chunchon, Korea. Tel: 82-33-250-8612, Fax: 82-33-251-7719, E-mail: dslee@kangwon.ac.kr

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INTRODUCTION

The liver is a hormone-sensitive organ. Although sex hormones function in sexual organs, such as the breast,[1] they also affect many mammalian liver functions.[2,3] Both male and female livers contain androgen receptors,[4-6] as well as high-affinity, low-capacity estrogen receptors.[7,8] Several lines of evidence suggest that sex hormones and their receptors play a role in liver carcinogenesis.[9]

Clinical studies have shown that men are more prone to developing hepatocellular carcinoma (HCC) than women. Furthermore, studies have also shown that androgens might be involved in malignant liver transformation.[10] Some reports have shown that, in HCC, estrogen receptor level decreases, while androgen receptor level increases.[11] In addition, HCC tissues may take up testosterone actively,[12] Elevated serum testosterone levels increase the risk of HCC,[13] and male sex is one of the most prominent HCC risk factors.[9] Individuals with AR-negative tumors had

a 55% survival rate five years after surgery, while those with AR-positive tumors had a 0% survival rate.[14]

Conversely, estrogen also promotes hepatocarcinogenesis.[9] Specifically, Villa et al.[15] first reported the ER-α variant in human primary HCC tissues. The variant ER (vER)-α was also significantly associated with the male sex. Recently, Iavarone et al.[16] reported vER-β in liver diseases. In addition, ER-β was found to be significantly expressed in HCV-infected patients. This evidence implies that the expression of ERs and their variants plays a role in the progress of hepatocarcinogenesis and is associated with the male prevalence of HCC, as well as with specific viral infections. However, these studies were all conducted in European countries. Although, in Asia, HCC constitutes 30-40% of human tumors, the expression patterns of AR and ERs in Asian HCC patients are still largely unknown.

HCC is one of the most prevalent malignant tumors in Korea.[17] Therefore, we investigated the expression of AR and ERs in HCC patients from South Korea. In this study, we describe the different expression patterns of sex hormone receptors that are associated with HCC.

MATERIALS AND METHODS

Patients

We investigated 32 primary HCC tumors and adjacent peritumor tissues which were obtained from patients at the Catholic University of Korea (Dept. of Internal Medicine, Seoul, Korea), the Korea Institute of Radiological and Medical Sciences (Molecular Oncology laboratory, Seoul, Korea), the Ajou University School of Medicine (Dept. of Surgery, Suwon, Korea), the Chungnam National University College of Medicine (Dept. of Pathology, Daejeon, Korea), and the Yeungnam University College of Medicine (Dept. of Biochemistry & Molecular Biology, Daegu, Korea). The clinical features of the enrolled patients are described in Table 1. The tissue samples were snap-frozen and stored in liquid nitrogen until the RNA was extracted. Sixty-four samples were analyzed in total.

ER and AR assay

Primers were designed from published gene sequences (Table 2). When designing the primers, particular attention was paid to the high homology

Table 1. Epidemiological and Clinical Features of the 32 Patients Enrolled in the Study

	No. of patients	Sex (M : F)	Age (yrs)
HCV patients	23	16 : 7	65 ± 8
HBV patients	9	7 : 2	56 ± 8

No., number; yrs, years.

Table 2. The Primers and RT-PCR Product Size for the Genes Investigated

Gene name	Forward primers	Reverse primers	Wt	Vt
AR	5′-AAGGAACTCGATCGTATCATTGC-3′	5′-AATAGATGGGCTTGACTTTCCCA-3′	241 bp	
ER-α(1)	5′-GGAGACATGAGAGCTGCCAAC-3′	5′-CCAGCAGCATGTCGAAGATC-3′	439 bp	300 bp
ER-α(2)	5′-CACAAGCGCCAGAGAGATGA-3′	5′-CATCATGCGGAACCGAGATG-3′	516 bp	377 bp
ER-β(1)	5′-TCACTTCTGCGCTGTCTGCAGCG-3′	5′-CCTGGGTCGCTGTGACCAGA-3′	818 bp	679 bp
ER-β(2)	5′-GGCCAAGAGAAGTGGCGGCCACG-3′	5′-AAACCTTGAAGTAGTTGCCAGGAGC-3′	430 bp	291 bp
ER-β(3)	5′-GCTGGGCCAAGAAGATTCCCG-3′	5′-GTGACCAGAGGGTACATACTGG-3′	313 bp	174 bp
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′	230 bp	

Wt, wild type; Vt, variant type; AR, androgen receptor; ER, estrogen receptor.

between ER-α and ER-β. Specifically, the primer pair for ER-α was located in exons 4 and 6[18] in order to amplify a possible exon 5 deletion. The primer pair for ER-β was located in exons 2 and 7.[19] The GAPDH, AR, and additional ER-β primers were designed by our laboratory and checked on human cell lines to ensure that the products of the primers were the correct size and produced only a single band.

RT-PCR was performed to detect AR and ER expression in human HCC and related, adjacent peritumor tissues. The RT step was carried out using a Reverse Transcription kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The PCR step was performed. using AccPrime™ *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions. Four micrograms of total RNA were reverse transcribed into cDNA. Approximately 4-5 μL of cDNA were used for PCR amplification. The reaction mixture was heated at 95℃ for 4 min and then was subjected to a total of 35 cycles (1 min at 95℃, 1 min at 58℃ for ER-α or 62℃ for ER-β, and 1 min at 72℃). The reaction was terminated by a final incubation at 72℃ for 10 min, followed by cooling at 4℃. A total of 10 μL final PCR product was analyzed by 2% agarose gel electrophoresis, ethidium bromide gel staining, and examination under ultraviolet light.

All 64 tissue samples were tested for GAPDH expression and analyzed for AR and ER mRNA expression. All of the samples that tested negative for either ER-α or ER-β were re-tested. GAPDH was amplified as a control for RNA quality. As a positive control, total RNA from MCF-7 breast carcinoma cells was reverse-transcribed and amplified in parallel with the samples under investigation. As a negative control, reaction mixtures without RNA were processed. Deletion mutants were identified by their different electrophoretic mobilities and were confirmed by direct sequencing after purification (QIAquick Gel Extraction kit; Qiagen, Hilden, Germany).

Immunohistochemical staining

Paraffin sections of liver cancer tissues from patients were de-paraffinized with xylene and then rehydrated. Antigen retrieval was performed by submerging the samples in citrate buffer (pH 6.0), followed by heating the samples in the microwave. Next, the sections were treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity and incubated with 1% bovine serum albumin to block non-specific binding. A primary anti-androgen receptor antibody (1 : 50 dilution; Cell Signaling, Frankfurt, Germany) was incubated for 60 min at room temperature. After washing, the tissue sections were incubated first with biotinylated anti-rabbit secondary antibodies and then with streptavidin-horseradish-peroxidase complexes. The tissue sections were immersed in 3-amino-9-ethyl carbazol as a substrate, counterstained with 10% Mayer's hematoxylin (dehydrated), and mounted with a crystal mount. For the negative controls, the same isotype of the non-immune rabbit IgG or the dilution solution minus the antibody replaced the primary antibody.

Statistical analysis

The chi-square test was used to test differences among the groups.

RESULTS

AR expression in HCC patients

AR expression was determined in 64 human samples. To our surprise, AR was expressed in all of the examined samples, independent of sex, viral etiology, and tumor or peritumor tissues (Fig. 1A, B). To confirm whether AR expression differed between tumor and paired non-tumor tissue, the RT-PCR products were loaded together. No differences in the quantitative expression between the tumor and peritumor tissues were observed (Fig. 1C). Five patient blocks (four HBV patients and one HCV patient) were obtained in order to confirm the protein levels by immunohistochemistry. The results showed that the AR was expressed in the nuclei of both tumor and paired non-tumor tissues of HCV and HBV patients and that no obvious expression differences were found (Fig. 2). Moreover, of the 32 patients studied, seven survived longer than five years.

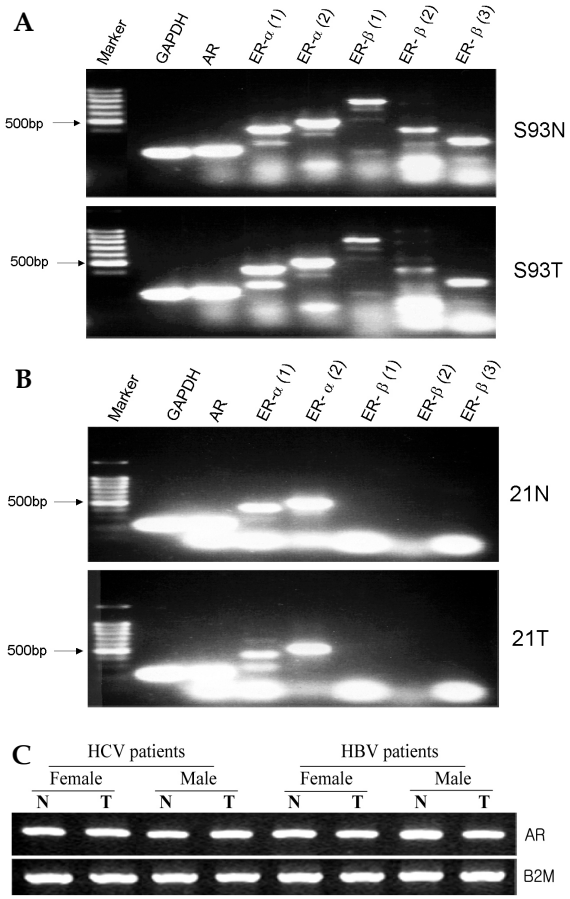

Fig. 1. The expression of AR and ER-α, -β in HCV and HBV patients as determined by RT-PCR analysis. (A) HCV patient. (B) HBV patient. ER-α (1), ER-α (2), ER-β (1), ER-β (2), and ER-β (3) are the ER-α, -β RT-PCR products using different primers (Table 1); S93 and 21 are patient identification numbers. The arrows show the vER-α bands. (C) AR expression in female and male HCV and HBV patients. N, peritumor tissues; T, tumor tissues.

ER expression in HCC patients

The expression of ER-α was observed in 32 patients (Table 3). Wild-type ER-α and vER-α expression were commonly detected in the samples and were independent of sex or viral etiology.

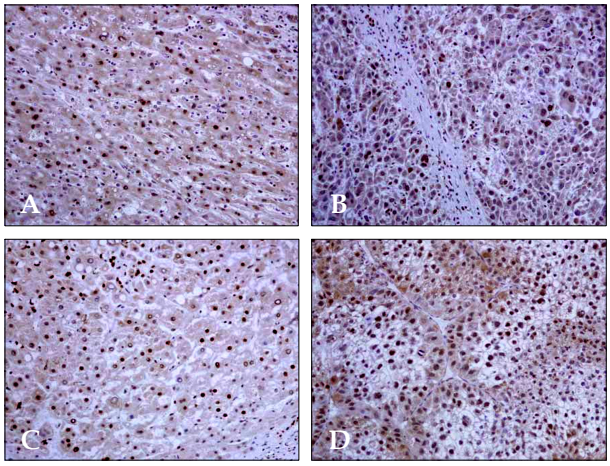

Fig. 2. Immunohistochemical staining of AR in hepatocellular carcinoma and paired peritumor tissues. AR protein was present in the nuclei of all normal hepatic and hepatocellular carcinoma cells. No difference was found in the expression of AR between tumor and non-tumor tissues or according to the viral agent. (A) non-tumor region of an HCV patient: (B) tumor region of a HCV patient; (C) non-tumor region of a HBV patient; (D) tumor region of a HBV patient. Magnifications: 200 ×.

However, the expression of vER-α was stronger in the tumor tissues than in the adjacent peritumor tissues (Fig. 1A, B). We detected significantly greater ER-β expression in HCV-infected patients than in HBV-infected patients (Table 4). Moreover, ER-β expression was not significantly related to sex (data not shown). We were unable to detect the expression of vER-β because the desired band was too weak to be confirmed by sequencing. We were unable to confirm this result even though we used the same RT-PCR primers and conditions that were used in a previously published paper, describing vER-β detection.[18,19]

DISCUSSION

The screening of gene expression profiles in

Table 3. Comparison of the Expression of ER-α Variant Type by Sex and Causal Agent

	Sex		Causal agents	
	Female	Male	HCV	HBV
Peri-tumoral tissues	9/9 (100%)	19/23 (82.6%)	20/23 (87.0%)	8/9 (88.9%)
HCC tissues	9/9 (100%)	21/23 (91.3%)	21/23 (91.3%)	9/9 (100%)

HCC, hepatocellular carcinoma; HCV, hepatitis C virus, HBV, hepatitis B virus.

Table 4. Comparison of the Expression of ER-α and ER-β in Patients by Causal Agent

	Patterns of ER expression			
	HCV patients		HBV patients	
	α+β+	α+β-	α+β+	α+β-
HCC tissues	22/23 (95.7%)*	1/23 (4.3%)	4/9 (44.4%)	5/9 (55.6%)

*Indicated a significant difference compared with other groups ($p < 0.05$).
No α- β- samples were detected.
HCC, hepatocellular carcinoma, HCV, hepatitis C virus, HBV, hepatitis C virus.

cancer provides useful information for further research on cancer mechanisms. Sex hormones affect many functions of the mammalian liver, and several lines of evidence suggest that sex hormone receptors may play an important role in liver tumorigenesis. Recently, investigations in Europe have examined the expression and activity of sex hormone receptors in the normal liver and liver cancer. Useful insights into liver cancer development have been obtained from these studies. Nevertheless, sex hormone receptor expression has not been investigated in Asian hepatocarcinoma patients, which account for 30-40% of all of the Asian cancer patients. Therefore, we performed this study to confirm that the results seen in European patients were also found in the Asian HCC patient population.

This study investigated 32 liver cancer patients. We could not find any obvious difference in the AR mRNA and protein expression profiles between male and female HCV and HBV patients. Interestingly, our results were very different from a previous report stating that AR-positive patients never survive longer than five years,[14] since seven of the AR-positive patients in our study group survived for more than five years. Our result implies that AR expression does not influence patient survival. At least in Koreans, AR expression is not significantly correlated with the five-year survival rate. Moreover, this result may explain the outcome of a clinical finding that anti-androgen receptor therapy was ineffective in treating HCC.[20] Combined, these results imply that the role of AR in hepatocarcinogenesis still needs to be elucidated in the Korean population.

For the ER-α expression profile, our results counter the study by Villa et al.,[15] which found that vER-α is almost always limited to male HCC patients and suggested that vER-α plays an important role in tumorigenesis, as well as in the male prevalence of HCC. We detected vER-α expression in both male and female patients. Nevertheless, vER-α may play a key role in hepatocarcinogenesis because vER-α had a stronger expression in tumors, compared to peritumor tissues. Therefore, at least in Koreans, vER-α is not a factor that is directly related to the male prevalence of HCC.

Our ER-β expression profile results are consistent with a previous report[17] of a significantly higher incidence of ER-β expression in HCV patients, compared to HBV patients. This finding implies that ER-β plays an important role in HCV-induced liver disease. However, in our study we could not confirm the extremely weak vER-β expression. Furthermore, vER-β function remains to be elucidated.

In addition, our study was predominantly performed at the mRNA level, using RT-PCR analyses of 32 patients. At the protein level, only AR expression was examined by the immunohistochemical staining of five patient samples. Although some of our data counter previous reports which focused on European patients, our results may have been influenced by the sensitivity of the RT-PCR system and the limited number of patients that we investigated. A larger, more detailed investigation of sex hormone receptor expression and activity in Asian patients should be performed.

In conclusion, we found different expression patterns of AR and ERs in liver cancer samples from Korean patients, compared to European patients. Our findings indicate that ER-β may have important functions related to HCV-induced liver disease. Furthermore, we found that vER-α is

not related to the male prevalence of HCC, but may function in hepatocarcinogenesis independently of sex. Overall, our results indicate that the AR and ER expression patterns differ according to race and that the functions of sex hormone receptors in HCC development must be further examined.

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