The clinical and pathogenetic significance of estrogen receptor-? expression in chronic liver diseases and liver carcinoma

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The Clinical and Pathogenetic Significance of Estrogen Receptor-β Expression in Chronic Liver Diseases and Liver Carcinoma

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BACKGROUND. Estrogen receptor-α (ERα) is variably expressed in hepatocellular carcinoma (HCC) and is believed to be correlated with prognosis and survival. Recently, another estrogen receptor (ERβ) has been identified, but its relevance in liver diseases is unknown.

METHODS. The expression of ERβ in the liver of 42 patients with HCC (10 with paired extratumoral tissues) and 26 with chronic liver disease without HCC was studied by a reverse transcriptase-polymerase chain reaction method, and correlated with the expression of ERα and severity of the liver disease.

RESULTS. Both ERβ and wild-type ERα were found to be expressed more often in patients with chronic liver disease compared with those with HCC (69% vs. 45% [$P = 0.046$] and 46% vs. 10% [$P = 0.0008$], respectively). ERs were similarly expressed in HCC and in the paired extratumoral tissue. Wild-type receptors, either alone or together with the deleted mutants ERδ5, were more often coexpressed in chronic liver disease (58%) than in HCC (29%); in 13 tumors (31%), either ERδ5 or no receptors at all were detected ($P = 0.006$). Hepatitis B virus (HBV)-related tumors either did not appear to express ERs or expressed ERδ5 more often than hepatitis C virus (HCV)-related tumors (67% vs. 15%; $P = 0.007$). The same was true for multinodular compared with single nodular tumors (50% vs. 19%; $P = 0.04$).

CONCLUSIONS. Both receptors were expressed in chronic liver disease and neoplastic livers demonstrating different patterns in relation to the etiology and clinical presentation of the tumor. These differences might underscore different pathogenetic mechanisms in HBV-related and HCV-related HCC and a different evolutionary course for the tumor. ***Cancer* 2003;98:529–34.**

KEYWORDS: estrogen receptor (ER)-α, estrogen receptor-β, hepatocellular carcinoma (HCC), chronic hepatitis, cirrhosis, hepatitis B virus (HBV), hepatitis C virus (HCV).

Several lines of evidence suggest that sex hormones and their receptors may play a role in liver carcinogenesis.[1] Estrogen receptor-α (ERα) is expressed in the liver of both healthy individuals and patients with chronic hepatitis and hepatocellular carcinoma (HCC), apparently with no differences in the pattern of expression.[2,3] Conversely, the mutant form with the entire exon 5 deleted (ER$\alpha\delta$5) is preferentially expressed in patients with HCC compared with patients with normal livers or chronic hepatitis.[4,5] Because ER$\alpha\delta$5 lacks the hormone-binding domain, HCC patients expressing mutated receptors might form a subset of patients who respond less favorably to antiestrogen drugs.[5]

With the identification of ERβ,[6,7] the cellular network of ERs

TABLE 1
Epidemiologic and Clinical Features of the 68 Patients Enrolled into the Study

	Chronic liver disease without HCC	HCC
No. of patients	26	42
Males, no.	18	35
Age (yrs)[a]	55 ± 12	65 ± 9
HBsAg	2	9
Anti-HCV	23	26
Alcohol consumption	1	2
Mixed	0	2
Cryptogenic	0	3

HCC: hepatocellular carcinoma; HBsAg: hepatitis B surface antigen; HCV: hepatitis C virus.
[a] Mean ± the standard deviation.

appears to be more complex than previously thought, thus partly explaining the different patterns of clinical response to anticancer hormones reported to date.[6] Despite the high degree of sequence homology between ERα and ERβ,[8] animal studies have revealed distinct patterns of expression for each receptor, with ERα being expressed preferentially in female reproductive organs and ERβ being predominant in nonreproductive organs and in the male reproductive tract.[6–9] The ERα:ERβ expression ratio apparently changes during carcinogenesis and is believed to play a role in tumor development.[10] In tissues of the breast, prostate, and colon, ERβ appears to be underexpressed during malignant transformation and tumor progression.[10–14]

Coexpression of ERα and ERβ may further contribute to the range of clinical responses to hormone therapy because it increases the chances of producing heterodimer receptors, whose transcriptional profiles are different from those given by homodimer receptors.[8]

To assess whether the pattern of ERs plays a role in human liver carcinogenesis, we studied the expression of ER subtypes in the liver in a well defined group of patients with chronic hepatitis and HCC and correlated the results with disease etiology and severity.

MATERIALS AND METHODS

Patients

The current study included 68 patients who were attending the Liver Center in the Division of Hepatology at Maggiore Hospital in Milan (Table 1). There were 26 patients with chronic liver disease without HCC (9 with chronic viral hepatitis with a mean necroinflammatory activity score of 9 ± 2 as assessed by the Ishak score and 17 with compensated cirrhosis). Forty-two patients had a histologic diagnosis of HCC (40 patients had underlying cirrhosis and 26 had a single-nodular tumor measuring 38 mm, on average). The grade of tumor differentiation was assessed according to the Edmondson-Steiner score and was high (Grade 1) in 2 patients (5%), moderate (Grade 2) in 16 patients (38%), poor (Grades 3-4) in 19 patients (45%), and not evaluable in 5 patients (12%). Ten patients with HCC also had paired tissues from extratumoral liver. None of the patients had received antiviral or antitumoral treatment at the time of the study. All liver biopsies were performed with a 16-gauge Tru-Cut® needle (TSK, Tokyo, Japan) under ultrasound guidance. Approximately two-thirds of each tissue sample was formalin fixed and embedded in paraffin for routine histopathology, whereas the remainder of the tissue sample was snap-frozen at − 80 °C until RNA extraction. Overall, 78 liver tissue samples were analyzed.

ER Assay

Total RNA was extracted from frozen tissue by RNAzol B (TEL-TEXT, Inc., Friendswood, TX), and the acid guanidium-thyocianate-phenol-clorophorm method. Primers were designed from published gene sequences, with particular attention paid to the high homology between ERα and ERβ. In particular, the primer couples for ERα were located in exons 4 and 6 (ER1: 5′-GGAGACATGAGAGCTGCCAAC-3′, ER2: 5′-CCAGCAGCATGTCGAAGATC-3′, ER3: 5′-CACAAGCGCCAGAGAGATGA-3′, and ER4: 5′-CATCATGCGGAACCGAGATG-3′), according to Fuqua et al.,[15] to allow the amplification of a possible exon 5 deletion. With regard to primer couples for ERβ, these were located in exons 2 and 7 (ER-β1: 5′-TCACTTCTGCGCTGTCTGCAGCG-3′, ER-β2: 5′-CCTGGGTCGCTGTGACCAGA-3′, ER-β3: 5′-GGCCAAGAGAAGTGGCGGCCACG-3′, and ER-β4: 5′-AAACCTTGAAGTAGTTGCCAGGAGC-3′), according to the method of Vladusic et al.[16]

Total RNA was reverse transcribed to cDNA. A total of 10 μL of RNA were added to a volume of 20 μL containing 4 μL of polymerase chain reaction (PCR) buffer 5×, 2.5 μL of dNTPs (2.5 mM), 5 μL of specific outer antisense primer (ER4 for ERα and ERβ2 for ERβ), 15 units (U) of reverse transcriptase-avian myeloblastosis virus (RT-AMV) (Thermoscript™ [RNase H$^-$ Reverse Transcriptase], Life Technologies, Gaithersburg, MD), 40 U of RNase inhibitor (RNase OUT; Invitrogen, Groningen, the Netherlands), and 1 μL of DTT (0.1 mM, Life Technologies). Complementary DNA (cDNA) was synthesized by incubation at 42 °C for 60 minutes, cooled rapidly at 4 °C, and stored at − 20 °C until first PCR.

Approximately 10 μL of cDNA were added to a final volume of 50 μL containing 5 μL of PCR buffer 10×, 3 μL of dNTPs (2.5 mM), 2 μL each primer

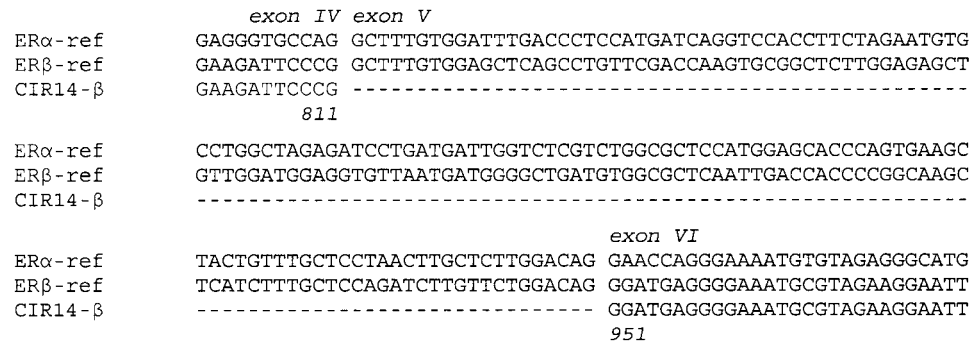

FIGURE 1. Example of a deleted sequence of estrogen receptor-β (ERβ) in a patient with cirrhosis (CIR14-β). Reference sequences (ERα-ref and ERβ-ref) were found in GenBank (National Institutes of Health, Bethesda, MD), access numbers NM_000125 and NM_001437, respectively.

couples (ER3/ER4 primers for ERα and ERβ1/ERβ2 for ERβ), and 2.5 U of Taq Gold DNA polymerase (AmpliTaq Gold®; Applied Biosystems, Foster City, CA). The reaction mixture was heated at 95 °C for 4 minutes and then was subjected to a total of 40 cycles (1 minute at 95 °C, 1 minute at 58 °C for ERα and 62 °C for ERβ, and 1 minute at 72 °C). The reaction cycle was terminated by heating at 72 °C for 10 minutes and cooling at 4 °C. Second PCR steps were performed with 5 μl of the product of the first step of PCR, using the inner primer couples (ER1/ER2 primers for ERα and ERβ3/ERβ4 for ERβ), with the same profile as the first PCR. A total of 5 μL of the final PCR product was analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide, and detected by ultraviolet lights.

The expected size was 438 base pairs (bp) for ERα and 430 bp for ERβ. Deletion mutants were identified by the different electrophoretic mobility and confirmed by direct sequencing, after purification (QIAquick PCR Purification Kit; Qiagen, Milan, Italy).

All samples that tested negative for either ERα or ERβ were retested and β-actin was amplified as a control of RNA quality. As a positive control, total RNA from MCF-7 breast carcinoma cells was reverse-transcribed and amplified in parallel with samples under investigation. As a negative control, reaction mixtures without RNA were processed.

Histopathologic Studies

Liver tissue was fixed in 10% formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin. The histologic diagnoses of chronic hepatitis, cirrhosis, and HCC were based on internationally accepted criteria. Each liver biopsy was reviewed by a single pathologist (M. F. D.) and scored for histologic activity[17] or grade of tumor cell differentiation.[18]

Serum Assays

Hepatitis B surface antigen (HBsAg) and antibody to HBsAg were detected by a microparticle enzyme immunoassay (AXSYM; Abbott Laboratories, North Chicago, IL). Antibody to hepatitis C virus (HCV) was measured by a second-generation test system (Ortho DS, Raritan, NJ). Serum α-fetoprotein (AFP) was measured using a standard assay (immunoradiometric assay [IRMA]; Abbott Laboratories).

Statistical Analysis

The Fisher exact test, chi-square test, and Student *t* test were used to test the differences between groups.

RESULTS

All 78 tissue samples reacted for β-actin and therefore were analyzed for ER mRNA.

Chronic Liver Disease without HCC

ERβ mRNA was detected in 18 patients with chronic liver disease (69%). Fifteen patients had wild-type ERβ alone, 1 patient coexpressed wild-type ERβ and a deletion variant, and 2 patients were found to have the deletion variants alone. Sequence analysis of the ERβ deletion variant PCR product revealed the absence of 139 bp within the hormone-binding domain (i.e., the presence of the mutant ER$\beta\delta$5) (Fig. 1). ERα mRNA was detected in all tissues from patients with chronic liver disease. Twelve patients had wild-type ERα, 13 coexpressed wild-type ERα and ER$\alpha\delta$5, and 1 patient had ER$\alpha\delta$5. Combining the ERα and ERβ results, we obtained four different patterns. Fifteen tissue samples (58%) coexpressed ERα and ERβ (either the wild-type alone or together with the deleted mutants), 1 tissue sample (4%) expressed ER$\alpha\delta$5 together with wild-type ERβ, and 10 tissue samples (38%) expressed wild-type ERα alone or in association with ERδ5, together with ER$\beta\delta$5 or without ERβ expression. All tissue samples demonstrated some form of ER expression (Table 2).

Hepatocellular Carcinoma

ERβ mRNA was detected in 19 HCC tumor samples (45%). Fifteen samples expressed wild-type ERβ alone, 1 sample coexpressed wild-type ERβ and ER$\beta\delta$5, and 3

TABLE 2
Expression of ERα and ERβ mRNA in 26 Chronic Liver Disease Tissues and 42 HCC Specimens

	Patterns of ER mRNA expression			
Liver tissue	α+β+	α−β+	α+β−	α−β−
Chronic liver disease without HCC	15 (58%)	1 (4%)	10 (38%)	0
HCC	12 (29%)	4 (9%)	13 (31%)	13 (31%)

$P = 0.006$, chi-square test.

ER: estrogen receptor; HCC: hepatocellular carcinoma; α+: either ERαwt alone or both ERαwt and ERαΔ5; α−: ERαΔ5 or no expression of ERα at all; β+: either ERβwt or both ERβwt and ERβΔ5; β−: ERβΔ5 or no expression of ERβ at all.

TABLE 3
Clinical and Histologic Characteristics of 42 HCC Specimens According to ER status

	Patterns of ER mRNA expression			
	α+β+	α−β+	α+β−	α−β−
Patients	12	4	13	13
Males	9	3	11	12
Age (yrs)[a]	64 ± 6	65 ± 9	64 ± 6	65 ± 10
HBsAg	1	1	1	6
Anti-HCV	8	3	11	4
Alcohol consumption	0	0	1	1
Multifocal HCC[b]	4	1	3	8
Portal invasion[b]	2	0	1	1
Serum AFP, 7 IU/mL	9	3	7	10
Tumor grade[c] 1–2	4	2	7	5
3–4	6	2	3	8

HCC: hepatocellular carcinoma; ER: estrogen receptor; α+: either ERαwt alone or both ERαwt and ERαΔ5; α−: ERαΔ5 or no expression of ERα at all; β+: either ERβwt or both ERβwt and ERβΔ5; β−: ERβΔ5 or no expression of ERβ at all; HBsAg: hepatitis B surface antigen; HCV: hepatitis C virus; AFP: α-fetoprotein.

[a] Mean ± the standard deviation.

[b] Detected by abdominal ultrasound.

[c] According to Edmondson–Steiner score.

samples expressed ERβδ5 only. ERα mRNA was detected in 41 HCC samples (98%). Four patients had wild-type ERα, 21 samples coexpressed wild-type ERα and ERαδ5, and 16 samples were found to express ERαδ5. Combining the results of ERα and ERβ expression provided four different patterns. Twelve samples (29%) co-expressed ERα and ERβ, either wild-type alone or associated with deleted forms; 4 samples (9%) expressed ERαδ5 together with wild-type ERβ; 13 samples (31%) expressed wild-type ERα alone or associated with ERαδ5 together, with ERβδ5 or without ERβ expression; and 13 samples (31%) expressed either deleted forms or no ER mRNA. The four patterns of ER expression did not appear to be influenced by age, gender, alcohol consumption, portal invasion by the tumor, serum level of AFP, or tumor grading (Table 3). However, more hepatitis B virus (HBV)-related tumors either did not express ERs or expressed only deleted forms compared with HCV tumors (67% vs. 15%; $P = 0.007$). Either negative or deleted ER was more common among multinodular tumors compared with single-nodulular tumors (50% vs. 19%; $P = 0.04$).

ERβ was found to be expressed less frequently in HCC compared with in chronic hepatitis or cirrhotic tissues (45% vs. 69%; $P = 0.046$), whereas ERα was similarly expressed in both. However, wild-type ERα was expressed significantly more often in chronic hepatitis cases (5 of 9 cases) compared with cirrhosis cases (7 of 17 cases) and HCC cases (4 of 42 cases) (chi-square test, $P = 0.0017$). The four patterns of expression of ER mRNA significantly differed for chronic liver disease and HCC tissues (Table 2). Benign tissues co-expressed wild-type forms of both ERα and ERβ (58%) alone or together with the deleted forms more often than malignant tissues (29%), whereas 31% of tumors were found to have either deleted forms of both receptors or no receptors at all, a pattern that was not present in benign liver disease cases (overall, $P = 0.006$).

Among the 10 extratumoral cirrhotic livers, 7 cases had no or deleted ERβ. Seven extratumoral samples expressed ERαδ5 alone. The analysis of paired biopsies demonstrated no differences in ER expression between tumoral and extratumoral samples.

DISCUSSION

Carcinogenesis is a complex mechanism that involves constitutive cell proliferation, with the expression of ER believed to play an important role in this process.[1] Estrogens, in fact, are main regulators of those cell functions associated with the growth and development of well defined tumors such as those of the uterus and breast.[19] The fact that the effects of estrogen and its antagonist on various target tissues are markedly different and can be largely influenced by alterations in the structure of ER itself[19] makes the study of ER expression of potential interest in liver carcinogenesis as well. A potential role for ER in liver carcinogenesis has been suggested by the striking prevalence of HCC observed in males, the increased expression of the variant transcript of ERα (ERαδ5) in the peritumoral cirrhotic tissue of patients with HCC, and the correlation between ERα and increased proliferation rates in HCC cases.[1] Recently, Villa et al.[5] demonstrated high rates of ERαδ5 expression in men at high-risk for HCC development.

To our knowledge, this is the first study to report the expression of ERβ and its variants in the liver of patients with chronic liver diseases and liver carci-

noma. Previously, ERβ was found to be expressed in breast, prostate, and gastrointestinal tissues of healthy individuals or patients with a variety of clinical conditions including cancer.[10–14] We found that ERβ was underexpressed compared with ERα in patients with chronic hepatitis or cirrhosis and those with HCC, with a tendency toward loss of ERβ expression in HCC patients compared with those with chronic inflammatory liver diseases. Because a similar behavior of ERβ already has been demonstrated during neoplastic transformation in breast, prostate, and gastrointestinal tissues,[10–14] one could hypothesize that the neoplastic transformation of several epithelial lineages, including the liver, is associated with a reduction in expression of ERβ.

In the current study, all but one tissue sample expressed ERα. In greater than half the cases, ERα was expressed as the deletion mutant ERαδ5, with the tissue expression of this receptor being higher in patients with HCC than in patients with chronic hepatitis or cirrhosis, as already demonstrated by Villa et al.[5] The higher prevalence of ERαδ5 in neoplastic livers and paired extratumoral samples in the current study is in keeping with the hypothesis that sex hormones may play a role in HCC.[5] The presence of mutated ERα may be biologically significant because the growth rate of HCC in patients with this variant was found to be significantly higher than in tumors expressing wild-type ERα only.[20] Expression of ERαδ5 also may be a useful clinical predictor because in one study[21] patients with this variant were found to have shorter survivals than those expressing wild-type ERα. Moreover, ERαδ5-positive tumors are more resistant to palliative treatment with antiestrogen therapy, whereas they respond better to progestinic drugs in terms of patient survival at 1 year.[22]

To our knowledge, the current study was the first to assess the clinical significance of ERα and ERβ coexpression in liver tissue, and demonstrated higher coexpression of ERα and ERβ in patients with chronic liver disease compared with those with HCC (58% vs. 29%) and excess ERαδ5 associated with wild-type ERβ among patients with severe liver disease and/or HCC. These other observations suggest that a lack of coexpression could have pathogenetic implications in HCC patients. A previous work reported less expression of wild-type ERα among patients with cirrhosis and HCC with respect to patients with mild liver disease. In that study, diminution of ERα paralleled the increased expression of ERαδ5. An in vitro study[23] demonstrated that ERαδ5 could inhibit wild-type ERβ, thus confirming the interaction between ERs as well as the potential importance of ER coexpression in a patient's response to hormone therapy. Our finding that the deletion mutant of ERβ mRNA lacked a domain that exactly corresponds to exon 5 of ERα, parallels the observations in MCF-7 breast carcinoma cells and in the estrogen-unresponsive breast carcinoma cell line MDA-MB-231[16] and stresses further the importance of assessing ER expression in the liver prior to the initiation of hormone therapy in patients with HCC.

Isolated expression of wild-type ERα was demonstrated in six patients with chronic hepatitis or cirrhosis and in two patients with a well differentiated single-nodular HCC. Expression of ERα only in patients with liver disease is unprecedented because previous studies have lacked concurrent analyses for ERβ.

It is interesting to note that we demonstrated lower ERβ expression associated with ERαδ5 in HCC and in paired extratumoral cirrhotic tissue. In vitro studies have demonstrated a correlation between ERα and ERβ, often in forms of heterodimer-activated receptors, a condition in which the transcriptional profiles are different from those generated by homodimer-activated receptors.[8] Coexpression of ERα and ERβ may have a significant clinical impact because epithelial tumors that coexpress ERα and ERβ are reported to be less invasive and dedifferentiated than tumors expressing a single ER.[10–14] Studies of chemical carcinogenesis suggested that ER may modulate HCC risk by inhibiting the malignant transformation of preneoplastic liver cells.[24,25] It also is interesting to note that we found that wild-type forms of ER were more often coexpressed in the less severe, single-nodular tumors compared with multinodular HCCs.

In the current study, ERα and ERβ were found to be underexpressed in HBV-related tumors in terms of either an excess of deletion mutants for both receptors or no ER expression. These data are in keeping with recent studies based on polymorphic microsatellite markers that suggested the existence of two distinct chromosome patterns underlying distinct pathways of hepatocarcinogenesis, viral etiology, and clinical severity.[26] HBV-related tumors appear to have greater chromosome instability, poorer differentiation, and poorer prognosis compared with HBV-negative tumors. The number and size of HCC nodules, that are clinically significant surrogates for liver cell dedifferentiation and vascular invasiveness, also were found to be the two stronger predictors of survival after radical treatment of HCC.[27]

The results of the current study demonstrated expression of ERβ in both chronic liver disease and liver carcinoma, and a change in the coexpression pattern of ERα and ERβ in patients with HCC. The differences in ER expression may point to different pathogenetic mechanisms in HBV-related and HCV-related HCC and a different evolutionary tumor course.

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