Variant Estrogen Receptor Messenger RNA Species Detected in Human Primary Hepatocellular Carcinoma

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Abstract

The development of hepatocellular carcinoma (HCC) in addition to cirrhosis affects males in a significantly higher proportion than females. Liver estrogen receptors increase when HCC develops in males; however, these tumors usually respond poorly to antiestrogens. We have, therefore, hypothesized that, similar to breast cancer, estrogen receptors in males with HCC may be mutated.

Variant estrogen receptor transcripts (lacking exon 5 of the hormone binding domain) were investigated by reverse transcription-PCR in 14 patients (7 males and 7 females) with HCC. While females mostly displayed the wild-type transcript (both in peritumoral and in tumor liver tissue), males showed both transcripts in the cirrhotic tissue and almost only the variant in the tumor. As the variant ER transcripts when translated could give rise to truncated receptors still able to constitutively activate transcription, they may be key factors in favoring deregulated proliferation in the male liver.

Introduction

HCC is one of the leading causes of death worldwide. A common characteristic in the different geographical areas is the striking male prevalence; the male:female ratio varies, in fact, from 4:1 to 7:1 in the different series reported (1). Both male and female human livers have been shown to contain ERs (2, 3). During the progression of chronic liver disease, especially when this is due to alcohol (4) or when hepatocellular carcinoma develops (5, 6), ER levels in males increase. The possible involvement of ERs in the pathogenesis of HCC has suggested that antiestrogens, could have a role in the treatment of patients with inoperable HCC; however, results of two trials with tamoxifen were poor (7, 8). This suggests that the relationship between the presence of ERs and the effect of tamoxifen could not be as straightforward as initially thought. In breast cancer (a tumor which is often dependent upon estrogen for growth), the progression from hormone dependence to hormone independence and the contemporary development of a more aggressive phenotype has been associated with the onset of variant liver ERs (9–13). Of special interest is one of these variants, which appears to be missing the hormone-binding domain of the receptor but which maintains a constitutive transcriptional activity (14).

We have hypothesized that similar mutational events could also occur during hepatocarcinogenesis; we have, therefore, investigated ER transcripts in individual primary HCC, especially looking for ER variants in the hormone-binding domain because the maintenance of the constitutive transcriptional activity could be especially relevant when investigating carcinogenic events.

Materials and Methods

Clinical Data

Liver tissue was obtained from 14 patients (7 females, mean age of 57 ± 8; 7 males, mean age of 55 ± 9) undergoing surgical resection of primary HCC associated with cirrhosis. All patients were anti-hepatitic C virus-positive. Normal liver tissue was obtained from five male patients undergoing elective cholecystectomy. Written informed consent was obtained from each patient. In HCC patients, several paired samples of 2 cm³ were obtained; they were cut into halves, and one-half was used for RNA and DNA extraction, while the other was paraffin embedded for histopathology. In controls, a 1 cm³ sample was similarly processed.

Nucleic Acids Extraction

The HCC and peritumoral tissue were separated immediately after operation. The contamination of HCC samples with noncancerous liver cells was less than 10% as determined by histopathology.

DNA Extraction. After homogenization in lysis buffer, samples were digested overnight with proteinase K and extracted with phenol-chloroform (15).

RNA Extraction. RNA was extracted by the acid guanidinium-thiocyanate-phenol-chloroform method after pulverization of the frozen tissue (16). Residual DNA was always removed by DNase treatment. RNA integrity was checked by electrophoresis; the concentration was determined spectrophotometrically before further use.

Southern and Northern Hybridization Analysis

For Southern analysis, 10 μg of DNA were digested with EcoRI and PvuII (15 units/μg DNA), fractionated by electrophoresis on 0.8% agarose gel, and transferred onto Zetaprobe filters. Northern analysis was performed on total cellular RNA using standard methods (15). Hybridizations were done at 60°C, and stringent posthybridization washings were done at 55°C. The ER probe used was pOR8, a 2.1-kilobase cDNA kindly provided by Dr. Pierre Chambon (Institute de Chemie Biologique, Strasbourg, France). A human β-actin cDNA probe was also used to check the integrity of both RNA and DNA. Probes were labeled using [α-32P]dCTP and a multiprime labeling kit (Amersham).

Reverse Transcription-PCR Amplification

Reverse transcription reaction (obtained with 1 μg of total RNA, spectrophotometrically evaluated) and subsequent PCR amplification were carried out using primers located in exons 4 and 6, according to Fuqua et al. (17). An amplified fragment corresponding to the wild-type ER was 438 base pairs, while the variant ER was 296 bp.

PCR products were either electrophoresed on 6% nondenaturing polyacrylamide gel or applied to a 4% NuSieve (FMC Bioproducts) gel, electrophoresed, and transferred to Zetaprobe membranes (Bio-Rad). Hybridization was carried out with an oligonucleotide (terminally labeled with [γ-32P]dATP)
VARIANT ER TRANSCRIPTS IN HCC

internal to the amplified fragment and corresponding to the antisense strand of the ER cDNA sequence 1039–1067 (17).

Ligand Binding Assay

This was performed as already described (3) except that [3H]Moxestrol was used in place of [3H]estradiol. Briefly, cytosol was incubated with various concentrations (0.35 to 11 nmol/liter) of [3H]Moxestrol in the absence (total binding) or presence (nonspecific binding) of a 200-fold excess of unlabeled Moxestrol for 16 h at 4°C. Data were analyzed using Scatchard plots to determine dissociation constant and receptor levels. These were expressed as fmol/mg cytosol protein (Bio-Rad protein dye reagent). ER concentrations >10 fmol/mg cytosolic protein were considered positive.

Statistical Analysis

Data were analyzed with Fisher’s exact two-tailed test. P < 0.05 was considered significant.

Results

Southern and Northern Hybridization Analysis. The PvuII and EcoRI RFLP patterns did not show any modification in HCC patients in comparison with normal liver. There was no allele loss demonstrable in HCC-derived DNA. In Northern analysis, all samples, both from patients with HCC (tumors and peritumoral tissue) and with normal liver, contained the 6.5-kb mRNA; five tumors (from patients 5 and 8–11) contained a smaller sized mRNA of 3.8 kb; no smaller-sized ER mRNA. These data suggest that the variant ER transcript most probably arises as result of a posttranscriptional event, although the occurrence of an alternatively spliced transcript due to subtle gene mutations cannot be excluded.

Discussion

Different variant ER transcripts have been reported in breast cancer (11–15, and references therein). Although not all authors agree on their possible significance in determining resistance to anti-hormonal therapy (18), their presence is considered a relevant event in the natural history of breast cancer. The variant ER transcript which we have demonstrated in the liver of patients with primary HCC appears to have the same characteristics of those reported in breast cancer; the ER gene, as it appears from RFLP analysis, does not seem to be grossly deleted or rearranged. The variant-sized ER mRNA, at Northern analysis, has never been detected in the absence of the 6.5-kb mRNA. These data suggest that the variant ER transcript most probably arises as result of a posttranscriptional event, although the occurrence of an alternatively spliced transcript due to subtle gene mutations cannot be excluded.

Although the liver is not classically a sex hormone target, it contains high affinity, low capacity ERs (2, 3) and responds to estrogen by increasing the rate of synthesis of many proteins (19). Therefore, the demonstration, in a substantial proportion of patients with HCC, of a unique variant ER transcript altered in the sequence corresponding to the hormone binding domain suggests, in the first instance, that tumors bearing this variant ER would probably be hormone resistant and, consequently, also unresponsive to tamoxifen, in case this was used for palliative therapy. This could be one of the reasons for the discordant results of the published trials of tamoxifen in HCC patients (8, 9). The evaluation of the type of liver ER in HCC only. The wild-type form was always conserved in females, while in males, the wild-type transcript was present in only one patient (P < 0.01; Table 1; Fig. 2).

The presence of wild-type ER positively related with the measured levels of ER by ligand binding assay, both in the normal liver and in the cirrhotic, peritumoral liver tissue. ER levels ranged between 13 ± 4 fmol/mg cytosol protein (normal male liver), 15 ± 5 fmol/mg cytosol protein (nontumor liver tissue from HCC patients), and 37 ± 9 fmol/mg cytosol protein (tumor liver tissue from female HCC). No specific binding was obtained in male HCC liver tissue.

Table 1 Distribution of wild-type (438-bp) and variant (296-bp) ER transcripts in the peritumoral and tumoral tissue of 14 patients with HCC and 5 controls

<table>
<thead>
<tr>
<th>Sex</th>
<th>Peritumoral tissue</th>
<th>Tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>438 bp</td>
<td>296 bp</td>
</tr>
<tr>
<td>H. H.</td>
<td>F</td>
<td>+</td>
</tr>
<tr>
<td>S. G.</td>
<td>F</td>
<td>++</td>
</tr>
<tr>
<td>D. C. L.</td>
<td>F</td>
<td>+</td>
</tr>
<tr>
<td>S. A. T.</td>
<td>F</td>
<td>+</td>
</tr>
<tr>
<td>C. A.</td>
<td>F</td>
<td>+</td>
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<tr>
<td>D. F.</td>
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</tr>
<tr>
<td>L. T.</td>
<td>F</td>
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<td>S. G.</td>
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<tr>
<td>E. C.</td>
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</tr>
<tr>
<td>C. P.</td>
<td>M</td>
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<tr>
<td>Z. L.</td>
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<tr>
<td>P. G.</td>
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</tr>
<tr>
<td>R. E.</td>
<td>M</td>
<td>±</td>
</tr>
<tr>
<td>A. R.</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>Controls</td>
<td>Normal liver</td>
<td></td>
</tr>
<tr>
<td>D. B. C.</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>F. R.</td>
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<td>M. D.</td>
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<tr>
<td>G. G.</td>
<td>M</td>
<td>+</td>
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<tr>
<td>Z. G.</td>
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</table>

* Northern analysis in these patients revealed smaller-sized mRNA (3.8 kb), together with the normal 6.5-kb mRNA.
patients would be necessary for the interpretation of the tamoxifen results and could address the use of endocrine therapies not acting through the ER (14).

The marked difference between males and females (the occurrence of the variant transcript being limited almost exclusively to males) suggests that this molecular event could be relevant in the preferential development of HCC in males. It is difficult to determine whether the appearance of the variant ER in HCC patients is an early or a late event during the carcinogenic process. However, the finding, in the peritumoral cirrhotic tissue of these patients, especially of males, of a greatly increased (although lower than in tumor tissue) expression of the variant ER transcript in comparison with normal liver tissue suggests that a gradual transformation may take place during the progression of the cirrhotic process. As these variant ER transcripts would give rise to truncated receptors modified in the hormone-binding domain but still able to constitutively activate transcription, they might favor deregulated proliferation in the male liver. It has been hypothesized for breast cancer that the presence of ERs in neoplastic cells, apart from being a marker of differentiation, would confer a survival advantage to the neoplastic cell (20). This would be even more so for these ERs altered in the hormone-binding domain, because normal dependence from hormonal control would be totally lost, therefore offering a potential mechanism for the escape of the tumor from hormone control (14). In human HCC, this mechanism would be mainly working in males, because tumors in females retain, in most instances, the wild-type ER, therefore remaining under a normal hormonal constraint.

References
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