Possible involvement of androgen receptor alterations in hepatocarcinogenesis

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1. Introduction

It is well documented that the prevalence of hepatocellular carcinoma is higher in males than females both in humans and experimental animals [1–5]. Considerable experimental and clinical evidence suggests a pivotal role for androgens in hepatocarcinogenesis [6–9].

Recently, the role of male sex steroid hormones in the promotion of hepatocarcinogenesis has been confirmed by the demonstration that activation of the androgen receptor (AR) is amplified by the presence of the HBx protein, a fact that could explain the higher prevalence of HCC in HBV as compared to HCV male cirrhotic patients [17,18]. However, studies conducted on diethylnitroamine-treated mice demonstrate that in this animal model, the lower incidence of HCC in females compared to males is due to a protective role of estrogens [19]. Finally, using the HBV transgenic mouse model, that spontaneously develops HCC, we have recently found that ovariectomy-induced estrogen depletion does not reduce the number of HCC nodules but only their growth [20]. All these data suggest that the gender disparity in HCC development may not be simply due to the levels of estradiol or testosterone taken singularly but could depend on the testosterone/estrogen ratio [20,21].

All these data suggest a role for androgens in the pathogenesis of this common malignancy but the precise mechanism mediating the oncogenic effect of androgens remains to be elucidated.

Studies on prostate cancer tissue and human prostate tumour cell lines indicate that AR mutations involving the ligand-binding domain region would make ARs susceptible to activation also by other ligands (progestins, estrogens, adrenal androgens and antian-drogens). This alteration would increase the receptor’s function as a steroid hormone-activated transcription factor and consequently promote cell growth [22,23].

In the present study, starting from this hypothesis, we evaluated whether AR mutations involving the hormone binding domain could increase AR function and promote carcinogenesis, as suggested for prostate cancer.

Aims: Herein, we evaluated qualitative (point mutations involving the hormone binding domain) and quantitative AR alterations and their possible correlation with cell proliferation and tumour grading.

Materials: Carcinomatous and non-cancerous surrounding liver tissue was collected from 14 Caucasian patients with hepatocarcinoma. They were all affected by cirrhosis with different aetiologies.

Methods: AR missense mutations, AR mRNA and protein levels, AR distribution in the liver, liver cell proliferation, and tumour staging were evaluated by DNA sequencing, quantitative real-time PCR, Western blot analysis, immunofluorescence, PCNA immunostaining, and conventional histological techniques, respectively.

Results: AR gene regions encoding the hormone binding domain did not contain any missense mutation. AR mRNA and protein levels were increased in hepatocarcinoma compared to non-cancerous surrounding tissue. Cell proliferation was significantly increased in the tumour compared to non-cancerous surrounding tissue.

Conclusions: Mutations of the AR regions studied were not involved in hepatocarcinogenesis. Elevated AR levels in transformed cells could have a tumour promoting effect by stimulating cell growth.
gene exons encoding for the ligand-binding domain region in both tumoural and peritumoural tissue from HCC cirrhotic patients. In addition, AR mRNA and protein levels, as well as tumour grading and cell proliferation, were determined in the same tissues in order to establish any possible correlation between these biological parameters and AR molecular (quantitative and qualitative) alterations.

2. Materials and methods

2.1. Patients

From January 1989 to April 1990, 25 male patients underwent hepatic resection for single or multiple hepatic tumours in the Department of Surgery at Bari University Hospital. Since then, as the more frequent application of locoregional therapies has limited the number of cases treated with surgical resection, interrupting this research program.

Before enrolment in the study, informed consent was obtained from all patients. In 20 patients, neoplastic and perilesional neo-plastic tissue was collected, immediately frozen and stored in a tissue bank. The diagnosis of hepatocarcinoma on cirrhosis was confirmed by histopathological evaluation in 14 of these patients. All our patients were affected by virus (HBV, HCV) or non-virus confirmed by histopathological evaluation in 14 of these patients.

Normal hepatic tissue (control) was obtained from 5 patients undergoing liver resection for non-neoplastic hepatic diseases (echinococcosis, liver trauma). Informed consent was also obtained from these patients.

This study was approved by the local Ethics Committee. All the patients with hepatocellular carcinoma included in the present study are now deceased.

2.2. Molecular biology studies

2.2.1. RNA preparation and first-strand cDNA synthesis

Total RNA was extracted from human liver tissue using the RNeasy Mini Kit (Qiagen GmbH, Germany) according to the manufacturer’s instructions. Aliquots of total RNA (1 μg) were reverse transcribed using random hexamers from the TaqMan Reverse Transcription Reagents and RT Reaction Mix using 3.12 U/μl of MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, California, USA).

2.2.2. Quantitative real-time PCR for AR

Quantitative real-time PCR (qPCR) was performed on an ABI Prism 7900 PCR cycler (Applied Biosystems, Foster City, CA) as previously described [24]. The following PCR primers and TaqMan MGB probes (FAM labelled) were used: human androgen receptor (AR) (Assay ID: 4331182_Hs00171172_m1) and human glyceralde-

2.2.3. DNA sequencing

Gene sequencing was performed to characterise point mutations, using the method described by Sanger et al. [26], on high resolution separation of DNA strands that differ by one single nucleotide.

AR gene mutations were analysed with reference to the AR mRNA sequence reported in GENEBANK (Homo Sapiens NM_000044). We focused our attention on exons V, VI and VIII, considering that in these regions there are the numerous point mutations that have been correlated with prostate carcinoma, as reported by the Androgen Receptor Gene Mutation Database (ARDB).

Denatured cDNA (2 μl) was amplified in 50 μl of 10× PCR buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl), dNTPs (0.1 mM), specific sense and antisense primers (50 μM) (Table 1). MgCl2 2 mM and 2.5 U of AmpliTaq polymerase (PerkinElmer, Elmer Cetus Norwalk, CT, USA); A test tube without RNA was considered as negative control. The cDNA fragments were amplified by 40 cycles (94 °C, 56–60 °C × 1′, 56–60 °C × 1′ for different primer sets, 72 °C × 1′) and visualised by gel electrophoresis (agarose 2%) with ethidium bromide. All PCR products were purified using the Wizard PCR prep kits (Promega, Madison, WI, USA). Then they were submitted to the DNA sequencing reaction (performed with the same primers for PCR) described by Sanger et al. [26], using a Dye Terminator 3.1 Ready Reaction Kit, as indicated by the manufacturer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed on the two strands of each PCR product with the automated DNA sequencer ABI Prism 377 (Applied Biosystems, Foster City, California, USA) and the resulting nucleotide sequence was aligned using the Sequence Navigator software package (Applied Biosystems, Foster City, California, USA).

The sequence of primers used for PCR-sequencing is described as follows:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
<th>No. of EXON</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR F1 5′: ACT CTG GCA GCC CGG AAC CT</td>
<td>2987–3005</td>
<td>V</td>
<td>249 bp</td>
</tr>
<tr>
<td>AR F1 5′: ACT CTG GCA GCC CGG TAG AG</td>
<td>3236–3215</td>
<td>VI</td>
<td>153 bp</td>
</tr>
<tr>
<td>AR R1 5′: CCA GTT CAT TGA GCC TAG AG</td>
<td>3415–3436</td>
<td>VI</td>
<td>249 bp</td>
</tr>
<tr>
<td>AR F2 5′: TCT GGT TTT CAA TCA GTT GCC C</td>
<td>3568–3548</td>
<td>V</td>
<td>153 bp</td>
</tr>
<tr>
<td>AR R2 5′: CCA GTT CAA TAA TCG TGA AGA G</td>
<td>3708–3728</td>
<td>VIII</td>
<td>103 bp</td>
</tr>
</tbody>
</table>

2.3. Protein studies

2.3.1. Cytosol extraction

Liver tissue samples (about 150 mg) were initially homogenised in a solution (1:3, w/v) containing Tris–HCl 10 mM, EDTA 1.5 mM, Na molybdate 5 mM, aprotinin 5 μM, PMSF 0.1 μM and β-mercaptoethanol 1 mM, pH 7.4, on ice. The homogenate was...
centrifuged at 18 × 10^3 g for 30 min at 4 °C and an aliquot of the supernatant was used to evaluate the protein concentration, as described by Lowry et al. [27]. The remaining supernatant (cytosol) was stored at −20 °C until used for immunoblot assays.

2.3.2. Western blotting

Aliquots containing 50 μg of proteins, obtained from normal, tumoural and non-tumoural surrounding liver tissue or from a prostate cell line with elevated AR expression (LNCaP cells) [28], were first denatured by the addition of β-mercaptoethanol (5%) and exposure at 95 °C for 5 min, and then used to perform SDS-PAGE electrophoresis. Proteins were immediately transferred to a nitrocellulose membrane (Trans-Blot Transfer 0.2 g/ml, BIORAD), at 4 °C. After transfer, molecular weights markers were stained with Red Ponceau S (Sigma St. Louis, MI, USA), while the part of the membrane containing our samples was incubated in 3% non-fat milk in TTBS (Tris Base 20 mM, NaCl 0.5 mM, Tween 20 0.005%, pH 7.5) for 3 h, in order to block non-specific immunoblotting sites. The AR protein was detected by incubating the membrane overnight, at 4 °C, with a mouse monoclonal antibody anti-human AR (DAKO-Carpinteria, CA, USA) (1:250) in TTBS with 1% non-fat dry milk.

After washing with TTBS, a goat anti-mouse immunoglobulin peroxidase conjugate (1:5000) was added for 2 h, at room temperature. Then, detection of immune complexes was performed by chemiluminescence (ECL Western Blotting detection, Amersham Biosciences) and densitometric analysis of the bands was made with the Optilab System 2.6.1 (BIORAD).

2.4. Histological and immunohistochemical studies

Formalin-fixed, paraffin-embedded tissues were used to prepare 2–4 μm sections utilised for hematoxylin/eosin (H-E) staining, histochemical, and immunohistochemical determinations. With the exception of H-E preparations, silane-coated slides were used for immunohistochemical studies.

2.4.1. Histological evaluation of tumour grading and proliferation

Histological evaluation of tumour grading was performed using the Edmondson–Steiner staging system [29].

The proliferative activity of liver cells was estimated by immunostaining detection of proliferating cell nuclear antigen (PCNA) using a mouse monoclonal antibody anti-PCNA (Dako, Glostrup, Denmark), as previously reported [30]. The percentage of immunolabelled cells over the total cells counted, i.e. the labelling index (LI) for PCNA (PCNA LI) was semiquantitatively evaluated in a blinded protocol by two-independent observers who counted the percentage of immunoreactive nuclei in at least 10 randomly selected high power fields (400×). The PCNA-LI was separately assessed in the non-tumoural tissue and carcinomatous areas. Only cells that exhibited strong nuclear immunostaining were considered PCNA positive cells.

2.5. Evaluation of AR by confocal laser scanning microscopy

AR expression and localisation were evaluated by immunofluorescence and confocal microscopy analysis using specific antibodies.

Frozen sections of 7 μm, obtained from liver samples tissue enclosed on a tissue freezing medium, were fixed with ice-cold acetone for 20 min, and washed in phosphate-buffered saline (PBS). Incubation with permeability solution (Triton 0.25% in PBS) and with blocking solution buffer (5% bovine serum albumin, 1% fetal calf serum PBS) was carried out, and then the sections were incubated with rat monoclonal anti-human androgen receptor antibody (4 μg/ml) (Abcam Ltd., Cambridge, UK) overnight at 4 °C. The immune complex was identified by incubating the section for 2 h with the secondary antibodies Alexa Fluor 488 goat anti-rat IgG–FITC conjugate (1:200) (Molecular Probes, Inc., Eugene, OR), and then the nuclei were counterstained with TO-PRO-3 (Invitrogen-Molecular Probes, The Netherlands) diluted 1:10,000 in PBS.

Slides were then mounted in Gel/Mount (Biomedica Foster City, CA) and sealed. Sections were analysed by confocal laser scanning microscopy using the Leica TCS SP2 (Leica, Germany), and adopting a sequential scan procedure during acquisition of the two double immunolabelling fluorophores. Confocal images were taken at 430-nm intervals through the z-axis of the section, covering a total of 7 μm in depth. Images from individual optical planes and multiple serial optical sections were analysed, digitally recorded and stored as TIFF files in Adobe Photoshop software (Adobe Systems, California, USA).

2.6. Statistical analysis

Statistical evaluation of the results was performed by Student’s t-test. Pearson’s test was used to correlate AR expression to cell proliferation. In all cases, a value of p < 0.05 was considered statistically significant.

3. Results

Table 1 shows the characteristics of the patients enrolled in this study. All patients were males and most were HBV-infected. The stage of malignancy was equally distributed in groups II–IV, according to the Edmondson–Steiner classification.

Fig. 1 shows AR cell localisation in control tissue (testicle; Fig. 1A) and in tumoural and peritumoural cirrhotic tissue (Fig. 1B and D, respectively), using confocal laser scanning microscopy. Our results clearly demonstrate a uniform distribution of AR positivity in the nuclei of most of the cells examined (Fig. 1A–C). The AR positivity is more evident in the tumoural tissue (Fig. 2C) as compared to peritumoural tissue (Fig. 2B).

The evaluation of possible genomic mutations involving the AR ligand-binding domain (AR qualitative alterations) was performed both in HCC tissue and non-cancerous surrounding liver tissue.
Quantitative analysis of AR mRNA levels in control, peritumoural and tumoural tissues. AR gene expression was calculated by qPCR as reported in Section 2. All values represent the mean ± S.D.

using the DNA sequencing technique described in Section 2. AR gene mutations were analysed with reference to the AR mRNA sequence reported in GENEBANK, focusing our attention on exons V, VI and VIII, since these regions contain numerous point mutations that have been correlated with prostate carcinoma. Our findings demonstrated that the DNA sequences encoding the ligand-binding domain region of the AR receptor, and in particular exons V, VI and VIII (Table 2), do not contain any mutation.

In order to evaluate possible modifications of AR mRNA levels (AR quantitative alterations) in HCC as compared to non-cancerous surrounding tissue, we performed qPCR. Our results (Fig. 2) demonstrate a significantly higher AR mRNA level in tumoural tissues as compared to peritumoural cirrhotic tissues (p < 0.04). This increase was also significantly higher when compared to the value obtained in normal liver tissues (p < 0.05) (Fig. 2). No significant difference in AR mRNA levels was found between peritumoural and control tissue (Fig. 2).

**Table 2**

<table>
<thead>
<tr>
<th>Samples</th>
<th>Exons identification by PCR</th>
<th>Sequencing for identification of mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>Samples 1–5</td>
<td>Positive</td>
</tr>
<tr>
<td>Tumoural tissue</td>
<td>Samples 1–14</td>
<td>Positive</td>
</tr>
<tr>
<td>Peritumoural tissue</td>
<td>Samples 1–14</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Fig. 3**

Semiquantitative evaluation of AR protein levels in controls, peritumoural and tumoural tissues. Western blotting of androgen receptor (AR). LNCap cells extract (line 1), normal liver (line 2), peritumoural (lines 3–5) and tumoural liver tissue (lines 6–8). The blots shown are representative of three runs obtained using three different tissue samples and show 50 µg protein/lane.

**Fig. 4**

Proliferative activity in tumoural and surrounding non-cancerous tissue expressed by immunohistochemical detection of PCNA. PCNA positive cells in (A) non-cancerous surrounding tissue and (B) tumoural tissue (200×). (C) Quantitative evaluation of PCNA positive cells. Data represent the mean ± S.D. *p < 0.0001.
4. Discussion

The androgen receptor gene is more than 90 kb long and encodes for a protein that has three major functional domains: the N-terminal domain, with modulatory functions (transactivation domain), the DNA-binding domain, and the androgen-binding domain.

Upon binding to the hormone ligand, the receptor dissociates from accessory proteins, translocates into the nucleus, dimerizes, and then stimulates the transcription of androgen responsive genes, some of which are involved in the regulation of immunity and mitotic activities. The N-terminal domain of the protein, encoded in exon 1, contains 2 polymorphic trinucleotide repeat segments [(CAG)n and (GGC)n].

Essentially, two types of mutations can be observed in the AR gene: the shortening of CAG repeat segments (normally containing 11–38 GAG repeats) and nucleotide changes (point mutations). The former is associated with an intrinsically higher transactivation capability of ARs (Fig. 3) and seems to play a role in the transactivation of ARs in tumoural tissue as compared to non-tumoural surrounding tissue, a finding in agreement with previous data in literatures.

Interestingly, in our tumoural tissue, the increased AR levels were associated to an enhanced proliferative activity, a result that assumes an important biological significance if we consider that in the tumoural tissue there was a fourfold increase of mitoses as compared to non-tumoural surrounding tissue (Fig. 4). These results seem to be in agreement with those reported by Nagase et al., demonstrating an inverse correlation between AR expression in tumoural tissue and prognosis after surgical resection. However, probably due to the limited number of examined cases, we did not find any correlation between AR protein levels and proliferative activity in the tumour (data not shown).

In conclusion, our data seem to exclude a possible role of an altered ligand-binding capacity of ARs in the pathogenesis of HCC. However, the biological significance of the quantitative increase of ARs in tumoural tissue as compared to non-tumoural surrounding tissue and normal hepatic tissue remains to be clarified.

Conflict of interest statement
None declared.

References

[6] Nagasue N, Takei K, Ichihara M, Takeda T, Moriyama T, Arai H, et al. A further confirmation of the consistency of our AR mRNA evaluation was gained by the analysis of AR protein levels (Fig. 3), demonstrating the presence of higher AR levels in tumoural tissues as compared to surrounding non-tumoural tissue, a finding in agreement with previous data in literatures [12,13].

In conclusion, our data seem to exclude a possible role of an altered ligand-binding capacity of ARs in the carcinogenic process of this organ; in addition, we hypothesised that, as suggested by Villa et al. for estrogens receptors, also for ARs there could be an association between an alteration of the ligand-binding domain and HCC. Finally, we extended our study to the possible correlations of these mutations with proliferative activity and tumour stage.

Once the presence of quantitative AR alterations had been excluded we evaluated the possible correlations of AR mRNA and protein levels with proliferative activity and tumour stage, i.e. the possibility that the carcinogenic process could be associated to quantitative variations of AR levels. The data illustrated in Fig. 2, obtained by qPCR, clearly demonstrate an increased AR mRNA expression in tumoural tissues as compared to peritumoural cirrhotic tissue and controls. Tavian et al. [39] found higher AR mRNA levels in tumoural than in the corresponding peritumoural tissue in a relatively small percentage (16%) of HCC samples, while Wang et al. [40] report similar AR levels in tumoural and peritumoural tissues in a Korean population with HCC. These conflicting results could be partly explained by a different patient selection and degree of tumour differentiation, as previously suggested [39], and partly by the method used for AR mRNA quantitation (we used qPCR that is a more sensitive and accurate method than comparative RT-PCR, used in previous studies).

Additional information

A further confirmation of the consistency of our AR mRNA evaluation was gained by the analysis of AR protein levels (Fig. 3), demonstrating the presence of higher AR levels in tumoural tissues as compared to surrounding non-tumoural tissue, a finding in agreement with previous data in literatures [12,13].

Interestingly, in our tumoural tissue, the increased AR levels were associated to an enhanced proliferative activity, a result that assumes an important biological significance if we consider that in the tumoural tissue there was a fourfold increase of mitoses as compared to non-tumoural surrounding tissue (Fig. 4). These results seem to be in agreement with those reported by Nagase et al. [14], demonstrating an inverse correlation between AR expression in tumoural tissue and prognosis after surgical resection. However, probably due to the limited number of examined cases, we did not find any correlation between AR protein levels and proliferative activity in the tumour (data not shown). In this scenario, the elevated AR levels in HCC could represent the tissue counterpart of the elevated levels of circulating testosterone observed in male cirrhotic patients with an increased risk of developing HCC [15,16,41]. However, the elevated aromatase activity of human HCC tissues leading to a higher transformation of testosterone to estrogen makes this interpretation questionable. At this point it should be noted that ARs show a different biological behaviour as compared to ERα that reduces its quantitative expression [43] and undergoes mutations that negatively influence the clinical course of HCC [44].

In conclusion, our data seem to exclude a possible role of an altered ligand-binding capacity of ARs in the pathogenesis of HCC. However, the biological significance of the quantitative increase of ARs in tumoural tissue as compared to non-tumoural surrounding tissue and normal hepatic tissue remains to be clarified.

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