Histol Histopathol (2002) 17: 1113-1119

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Androgen receptor mRNA under-expression in poorly differentiated human hepatocellular carcinoma

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Summary. Many studies suggest that hepatocellular carcinoma (HCC) is an androgen-dependent tumor with an incidence five times higher in males, but few data are available on the androgen receptor (AR) mRNA levels in different physiological classes of human liver specimens.

In this study 108 human hepatic samples have been analyzed for AR mRNA expression by a comparative RT-PCR assay. These consisted of 35 non-tumoral hepatic samples (3 normal parenchymas, 4 steatosis, 10 hepatitis, 18 cirrhosis), 38 tumoral specimens derived from uninodular and multinodular HCCs and 35 peritumoral hepatic tissues.

Normalized AR mRNA levels in tumoral and peritumoral liver tissues spanned from 0 to 146% and from 7 to 125% respectively. Only in a relatively small percentage of HCCs, the levels of expression of AR mRNA were higher than in the corresponding peritumoral tissues (16% of total HCCs). Although extremely variable, the AR mRNA levels were related to histological tumoral differentiation and proved to be lower in the highly dedifferentiated HCCs as compared to the well differentiated ones.

Therefore, the evaluation of AR expression in HCC patients might be relevant for the planning of clinical studies on anti-androgen therapies, which might be useful only in the cases in which a high level of AR mRNA is detected, considering the high heterogeneity of AR mRNA levels which characterizes HCC samples. It is likely that the HCCs, expressing low or undetectable levels of AR mRNA, would not benefit by the anti-androgen therapy.

Key words: Hepatocellular carcinoma, Androgen receptor, mRNA expression, Tumoral differentiation, Anti-androgen therapies

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Introduction

In the last years the number of patients with early stage resectable HCC has rapidly increased, since the adoption of ultrasound scan (US) for the screening of high risk patients. However a 5-year survival is between 25 and 45 % according to different authors (Colleoni et al., 1998; Portolani et al., 1999).

Human HCC is more prevalent in men than in women and there are indications suggesting that androgen steroids are implicated in its development (Goldfarb, 1976; Okuda, 2000). This is substantiated by the observations that therapy with substituted androgenic steroids, in patients with Fanconi' s anemia and anaplastic anemia, has been associated with the development of HCC (Meadows et al., 1974; Mulvihll et al., 1975) and that the expression of androgen receptor protein in the tumors is significantly correlated with a shorter recurrence-free survival (Nagasue et al., 1989). Finally, a large retrospective study, analysing serum samples from a cohort of 9691 male adults, showed that high serum testosterone levels represent a risk factor for the development of HCC in humans (Yu and Chen, 1993).

There are no data on AR mRNA expression levels in different physio-pathological classes of human liver specimens, with the exception of the study of Negro et al. (1994) which underlined the importance of AR mRNA evaluation in HCCs by non radio-isotopic in situ hybridization assay (NISH), before starting an antiandrogen therapy. Moreover Nakagama et al. (1991) performed, a qualitative evaluation of AR mRNA expression by RT-PCR and Northern blot analysis in eight cases of peritumoral and tumoral liver samples showing that variable levels of AR mRNA expression could be identified in these tissues. Several groups have observed the expression of AR protein in HCC tissues by hormone binding assays, but controversial results have been obtained (Iqbal et al., 1983; Nagasue et al., 1985; Ohnishi et al., 1986; Eagon et al., 1991; Boix et al., 1993).

The aim of the study was to perform a comparative

evaluation of AR mRNA expression among different classes of hepatic tissues in order to verify whether AR gene transcription is oncodevelopmentally regulated (or is associated to molecular mechanisms of hepatocarcinogenesis) and to verify, in HCC patients, whether there are possible benefits for an antiandrogen therapy. The results obtained showed that: 1) the tumor samples, in particular the HCCs G1/2, display a high variability in AR mRNA and most of them are characterized by high levels of expression; 2) in the highly dedifferentiated HCCs (G3/4), significantly lower levels of AR mRNA expression were found, as compared to the well differentiated HCCs (G1 and G2), the control liver samples and the correspondent peritumoral tissue; and 3) no differences in AR mRNA expression were evidenced between peritumoral or tumoral tissues of male and female HCC patients.

Materials and methods

Chemicals

Earle's minimum essential medium (MEM), Dulbecco's medium and gentamicin were from Gibco (Paisley, Scotland), fetal calf serum (FCS) from Seromed (Berlin, FRG), penicillin and streptomycin from Squibb (Rome, Italy); PBS from Oxoid (Basingstoke, UK); dNTPs from Pharmacia (Milwaukee, Wisconsin); the hexamer primer mixture, Bst XI restriction enzyme, DNA molecular weight marker V and III from Boeringher Mannheim (FRG); Moloney murine leukemia virus reverse transcriptase (MMLV-RT), EcoRI restriction enzyme and Trizol™ from Gibco BRL (Gaithersburg, MD); RNasin and Thermus aquaticus Taq DNA polymerase were from Promega (Madison, USA); and AR-1F and AR-2R, FN-1R and FN-2R primers were supplied from Life Technologies.

Human cell line and tissues

Human hepatocarcinoma cells (SK-Hep-1: ATCC HTB 52) were grown in Earle's MEM with 10% FCS, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

The 108 specimens of human hepatic tissues examined were as follows: 35 non-tumorous livers (3 normal parenchymas, 4 steatosis, 10 livers with hepatitis, 18 cirrhotic livers); 38 tumoral specimens derived from multinodular and uninodular HCCs, and 35 peritumoral livers, resected 1-2 cm from the tumor (11 chronic hepatitis with HBV or HCV etiology, 22 cirrhosis and 2 normal parenchymas). For the two multinodular HCCs (with three and two malignant lesions), only one PT specimen was analysed. All the samples from human HCCs and the surrounding non-tumorous ones were obtained from the surgically resected specimens and from fine-needle cytoaspirates for pathological examination and evaluation of tumoral grading (grades I to IV in Edmondson's scale); as shown in Table 1 the evaluation of tumoral grading was not available for 6 HCC tissue specimens. Some liver tissues (with normal parenchyma or steatosis) were surgically resected from patients with a primitive carcinoma in a distal organ (colon, rectum, pancreas, stomach), others (with non-specific reactive hepatitis, chronic hepatitis, steatosis) were derived from patients undergoing non complicated cholecystectomy and considered non-tumorous after histological examination. Informed consent was obtained from each patient. After resection, the biopsy fragments (100-300 mg) were treated as previously described (Tavian et al., 1994). Fine-needle cytoaspirates were rapidly extracted by Trizol.

RNA extraction and comparative RT-PCR analysis of AR mRNA expression levels in human liver biopsies

Total RNA purification, analysis and retro-transcription was performed as already reported (De Petro et al., 1998).

We used AR-F (forward) and AR-R (reverse), FN-F (forward) and FN-R (reverse) primers which (according to the AR and FN sequences deposited at the EMBL data bank) were as follows:
AR-F 5'-CATCCTGCTCAAGACGCTTC-3' (2705-2724)
AR-R 5'-CACAGAGATGATCTCTGCCA-3' (2859-2840)
FN-F 5'-GCCTGGTACAGAATATG-3' (3908-3929);
FN-R 5'-ATCCCAGCTGATCAGTAGGCTGGTG-3' (4327-4303);

Primers were selected from two exons separated by at least one intronic sequence, allowing possible identification and exclusion of RNA samples eventually contaminated by traces of genomic DNA. The sizes of the amplified cDNA fragments were 155 bp for AR and 419 bp for FN.

PCR products were analyzed on 2% agarose gel electrophoresis and the specificity of the reaction was tested by appropriate restriction site analysis with Bst XI (for AR) and BamHI (for FN) (not shown).

For each AR RT-PCR experiment 29 rounds of amplification were performed on a 9600 Applied Biosystem DNA-RNA amplifier with the following cycling profile: denaturation at 95 °C for 1 min, annealing at 51 °C for 30 s and extension at 72 °C for 20 s for the first round; denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 20 s for 27 cycles; and denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and terminal extension at 72°C for 3 min for the last cycle.

For each FN RT-PCR experiment 26 rounds of amplification were performed as previously described (Tavian et al., 1994; De Petro et al., 1998). Primer concentration was 25 pmoles for AR and for FN PCR amplification with 1.6 mM dNTPs.

The Magiscan Image Analysis System (M.I.A.S.) was used to scan the RT-PCR cDNA products directly from the images of the agarose gel (De Petro et al.,

1998; Tavian et al., 2000). For each band, a relative value of integrated density (I.D.) was expressed in pixels. Each I.D. value was normalized versus the reference value, considered as 100%, corresponding to the III-9 FN and AR RT-PCR product obtained in the reference sample, SK-Hep-1 cells. In order to allow a comparative evaluation among different experiments, the cDNA from SK-Hep-1 sample was included in all the experiments (Fig. 1).

The III-9 region of FN mRNA was expressed at comparable levels in all cDNA preparations analyzed (De Petro et al., 1998; Tavian et al., 2000).

The comparative evaluation of AR mRNA in the different specimens implied: 1) the accuracy in the control of quality and amount of RNA (1 μg) used for reverse transcription; 2) the constant amount of cDNA used for the amplification reactions with FN-F/FN-R and for AR-F/AR-R primers (100 ng); 3) the inclusion of the same reference sample, SK-Hep-1, in all the experiments of amplification for FN and for AR gene; 4) the use of a limited number of PCR cycles in a range far from plateau values (FN: 26 cycles; AR: 29 cycles); 5) the measurement of the amounts of RT-PCR products by a digital system; 6) the normalization of the III-9 FN RT-PCR product obtained in each hepatic sample versus that expressed in the reference sample (SK-Hep-1), considered as 100%; 7) the normalization of the AR RT-PCR product obtained in each hepatic sample versus the RT-PCR amount expressed in the same reference sample (SK-Hep-1), considered as 100%; and 8) the normalization of the value corresponding to the amount of amplification product of the AR gene product versus the III-9 FN mRNA expressed in the same sample (Fig. 1).

DNA extraction and PCR analysis of AR gene in HCC tissues

Genomic DNA from 10 peritumoral and tumoral hepatic tissues has been extracted using the LiCl method (Gemmell and Akiyama, 1996). 1 μg of genomic DNA was used to perform AR gene amplification with AR-F/AR-R primers. PCR cycling profile was as follows: denaturation at 95 °C for 1 min, annealing at 49°C for 30 s, extension at 72 °C for 90 s for the first round; denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s and extension at 72 °C for 90 s, for 28 cycles; and denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s and terminal extension at 72 °C for 3 min for the last cycle. The size of the amplified fragment was of 830 bp (spanning a portion of exon 7, intron 7 and a portion of exon 8) and was analyzed on a 1% agarose gel electrophoresis.

Statistical analysis

The data obtained from AR comparative RT-PCR assay were analyzed using the statistical program Primit and the analysis of variance (Bonferroni's t test) as well as non parametric analysis (Mann-Whitney's V-test) were employed to assess statistical significances. The data were considered to be significant when $p \leq 0.05$.

Results

AR expression in non-tumoral liver biopsies

The expression level of AR mRNA was determined by comparative RT-PCR analysis in 7 control liver biopsies (3 normal livers and 4 steatotic livers), 10 livers with hepatitis and 18 with cirrhosis, from patients without HCC.

The control tissues tested showed AR RT-PCR products with normalized I.D. values spanning from 19 to 140% (X = 72), the hepatitic livers from 4 to 112% (X = 81) and the cirrhotic livers from 4 to 132% (X = 76) (Fig. 2).

The AR mRNA expression level of livers with hepatitis and with cirrhosis was not significantly different from that of control tissues (HEP: p=0.6; CIR: P=0.8).

AR expression in human HCCs and in the surrounding tissues

AR mRNA was evaluated in 38 HCCs lesions (derived from uninodular and multinodular HCC) and in their surrounding tissues (for details see Materials and methods). The HCC relative values spanned from 0 to 146% (X=57) and for peritumoral tissues from 7 to

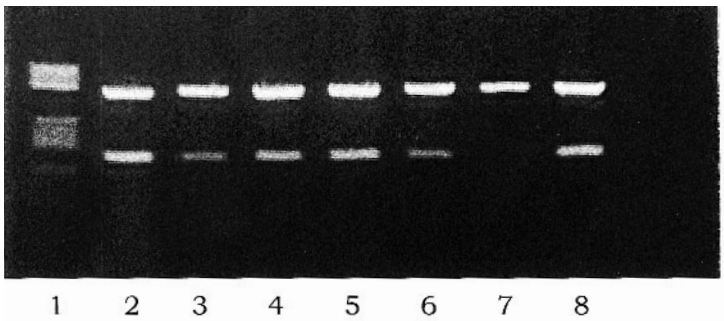

Fig. 1. RT-PCR detection of AR and III-9 cDNA products (155 and 419 bp respectively) by single PCR assays in three HCCs (lanes 3, 5 and 7), in the correspondent peritumoral tissues (lanes 2, 4 and 6) and in the positive control, SK-Hep-1 cells (lane 8). Lanes 3 and 7 refer to highly dedifferentiated HCCs (G4); lane 5 represents a well differentiated HCC (G1). Lane 1 refers to the DNA molecular marker V.

125% (X=83) (Table 1). AR mRNA expression in peritumoral and tumoral tissues was characterized by a marked variability, which was also present in non tumoral hepatic samples (Fig. 2). The relative AR mRNA levels in the HCC and in the correspondent surrounding tissue were not significantly different from those of control (p=0.4; p=0.3) Table 1). There was no significant difference between female and male samples either in the peritumoral or in the tumoral samples (p=0.485; p= 0.923) (Table 1).

The expression of AR mRNAs was generally lower in HCC than in the correspondent peritumoral tissue, showing a significant p value of 0.004 (X_{PT} =83; XHCC=57). In fact, 21 out of 38 HCCs were characterized by the underexpression of AR gene, while 11 out of 38 HCCs showed an equal expression and only 6 out of 38 HCCs showed an overexpression of AR mRNA in comparison with the correspondent perilesional tissues (Table 1).

Considering the tumoral grading available for 32 HCC lesions, it has been found that the most undifferentiated HCCs (G3/4) were characterized by very low or undetectable levels of AR mRNA expression, contrary to the G1 and G2 HCCs (Figs. 1-3). As a control, in all the HCC samples in which AR mRNA could not be found, genomic DNA was amplified with AR primers and a specific band was identified, thus indicating that the gene was present in these HCC samples (data not shown). The difference in the level of AR mRNA expression between G1 and G2 and G3/4 HCCs was highly significant (p<0.001), as well as that between G3/4 HCC samples and control hepatic tissues (p<0.001). On the contrary, no significant difference was found between the AR mRNA expression level of G1 and G2 HCCs and control liver samples (p=0.98).

Table 1. Clinicopathological characteristics of HCCs and AR mRNA expression in malignant and peritumoral tissues.

PATIENTS		UNDERLYING LIVER	TUMOR SIZE (cm)	DIFFERENTIATION DEGREE	AR mRNA EXPRESSION		
Age	Sex				HCC	PT	HCC/PT
61	F	Cirrhosis	<5	1	87	90	0.97
n.d.	F	Cirrhosis	<5	1	52	92	0.56
65	F	Cirrhosis	<5	1	81	84	0.96
48	M	Cirrhosis	<5	1	132	117	1.13
71	M	Cirrhosis	<5	1	100	97	1.03
60	M	Hepatitis	<5	1	120	95	1.26
71	M	Hepatitis	>5	1	44	59	0.75
52	M	Cirrhosis	<5	1	20	58	0.35
54	M	Cirrhosis	<5	1	76	91	0.83
65	M	Cirrhosis	<5	1	10	67	0.15
69	M	Normal	>5	1	93	101	0.92
55	F	Cirrhosis	>5	2	120	101	1.20
55	F	Cirrhosis	>5	2	98	n.d.	/
55	F	Cirrhosis	>5	2	105	n.d.	/
n.d.	F	Normal	n.d.	2	52	69	0.75
37	F	Hepatitis	>5	2	1	10	0.10
62	F	Hepatisis	<5	2	19	105	0.18
79	F	Cirrhosis	<5	2	63	103	0.61
62	M	Hepatitis	<5	2	32	125	0.26
71	M	Cirrhosis	<5	2	61	58	1.05
71	M	Cirrhosis	<5	2	60	n.d.	/
67	M	Hepatitis	>5	2	90	110	0.82
63	M	Cirrhosis	n.d.	2	62	48	1.29
59	M	Cirrhosis	>5	2	146	75	1.95
63	M	Cirrhosis	>5	3	0	85	0
77	M	Cirrhosis	<5	3	4	124	0.03
56	M	Hepatitis	>5	3	0	115	0
57	M	Cirrhosis	>5	3	15	92	0.16
61	M	Cirrhosis	<5	3	1	49	0.02
n.d.	M	Hepatitis	<5	3	2	65	0.03
69	F	Hepatitis	<5	4	39	114	0.34
n.d.	F	Hepatitis	n.d.	4	0	76	0
n.d.	F	Cirrhosis	<5	n.d.	98	98	1
62	F	Cirrhosis	<5	n.d.	80	65	1.23
n.d.	M	Cirrhosis	<5	n.d.	47	70	0.67
66	M	Hepatitis	<5	n.d.	104	115	0.90
55	M	Cirrhosis	<5	n.d.	67	84	0.80
n.d.	M	Cirrhosis	<5	n.d.	0	7	0

F: female; M: male; n.d.: not detected

In the G3/4 HCCs, AR mRNA expression was always lower than in peritumoral tissues, while in the G1/2 HCCs the expression was variable. In one subgroup of the G1/2 HCCs, the AR mRNA was increased in comparison with the correspondent perilesional tissues and these AR levels were higher than control liver samples showing almost a significant p value (p = 0.054).

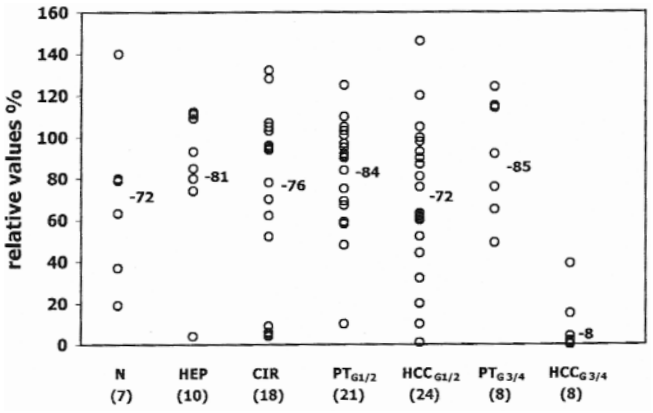

Fig. 2. Evaluation of AR RT-PCR expression in human liver biopsies: the relative percentage values obtained, as described in Materials and Methods, are shown on the Y axis. The number of the cases tested for each group of examined tissues is reported on the X axis. N: control livers (with normal parenchyma or steatosis); HEP: livers with hepatitis but without tumors; CIR: cirrhotic livers without tumors. All these tissues, derived from patients without HCC, were considered non tumorous after histological examination (see Materials and Methods). PTG1/2: peritumoral liver tissues of G1/2 hepatocellular carcinomas; HCCG1/2: hepatocellular carcinoma of G1/2 grading; PTG3/4: peritumoral liver tissue of G3/4 hepatocellular carcinomas; HCCG3/4: hepatocellular carcinoma of G3/4 grading. In the panel, the average relative value for each group of examined tissues is reported.

Discussion

The role of androgens in liver carcinogenesis has been studied mainly in animal models giving support for a biological basis of the antiandrogen treatment of hepatocellular carcinoma (HCC) in man (Nagasue et al., 1996; Yu et al., 1996; Pignata et al., 1998).

Regarding human liver, it is known that normal parenchyma expresses the androgen receptor (AR) which belongs to a superfamily of nuclear receptors (Bannister et al., 1985). In certain HCCs the level of AR is significantly higher in the tumor as compared to the surrounding liver tissue, suggesting the possible involvement of androgens in liver carcinogenesis (Nagasue et al., 1985).

These data prompted us to carry out the evaluation of AR expression in different classes of human hepatic specimens, in order to verify the possible relationships between AR expression and the clinicopathological characteristics of HCC in view of the possible use of anti-androgen therapies.

There are few studies on AR mRNA expression in normal liver parenchyma, steatosis, hepatitis and cirrhosis, as well as between these non tumorous liver conditions and the tumorous ones. Our data show that in control liver tissues, hepatitis and cirrhosis, the AR is always detectable and its level of expression does not show any statistical difference among the different physiopathological conditions. Bannister et al. (1985) measured the androgen receptor protein in 11 needle-byopsies of human cirrhotic liver and also in two cases of normal liver parenchyma demonstrating that AR concentration was reduced in cirrhosis. In this study, as well as in most of the published works on AR protein, a receptor binding assay has been used for the detection of the cytosolic fraction, thus excluding the nuclear fraction of androgen receptor protein. This might be the main reason for the discrepancy from our results. Indeed our data are in agreement with the AR protein

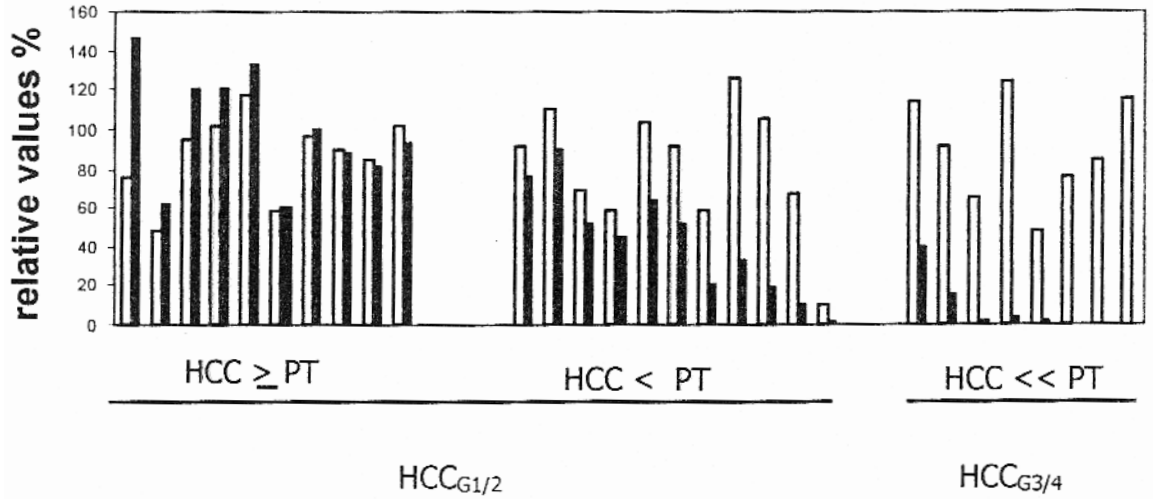

Fig. 3. Evaluation of AR RT-PCR expression in G1/2 and G3/4 hepatocellular carcinomas and in the correspondent peritumoral tissues. The black bars represent tumor samples, while the white ones represent the peritumoral tissues.

determinations reported by Eagon et al. (1991) implying both the cytosolic and the nuclear fractions of the hepatic tissue tested.

Concerning AR mRNA expression in human HCC, our data show that this level is generally lower in the malignant tissues, as compared to the corresponding peritumoral tissues and that tumoral grading is strongly correlated with AR expression pattern. The highly dedifferentiated HCCs were characterized by low levels of AR mRNA expression and in some cases, AR expression was below the detection limit; in these samples the AR gene was also present, as verified by PCR analysis. In all the G3/4 HCCs the level of AR mRNA was lower than that of the corresponding peritumoral tissues. These results seem to indicate that the level of AR mRNA expression is inversely correlated with the dedifferentiation of tumoral hepatocytes. Indeed, it has been shown that the only cells producing AR mRNA in HCC and peritumoral tissues are the hepatocytes and not the other cell types (Kupffer's, stromal and vascular endothelial cells) (Negro et al., 1994).

These results are in line with those obtained in rat liver regeneration, in which a parallel decline of serum testosterone levels and AR content in the liver was observed, suggesting that both G3/4 HCC hepatocytes and regenerating liver cells do not require AR for their growth (Thiel et al., 1991).

For the possible use of anti-androgen therapies, the few clinical data reported, mainly on the treatment of patients with unresectable HCC, indicate that only certain kinds of anti-androgenic compounds might be of benefit only in subgroups of unresectable HCC. As summarized by Pignata et al. (1998), the nitulamide (a pure antiandrogen compound) was effective only in 1.6% patients (4/244), and the cyproterone acetate (a steroidal antiandrogenic compound with progestogenic activity) showed activity in 12-19% of patients (3/25, 3/16). It might therefore be of interest to design clinical studies on anti-androgenic therapies of resectable HCC by different compounds taking into account the evaluation of AR mRNA for the selection of the most responsive patients. This might be a general consideration for all HCC patients and particularly for those with G1 and G2 HCCs, who show a more marked heterogeneity in AR mRNA expression in our study. In the G1 and G2 HCC population we found that AR mRNA expression levels were significantly higher than those identified in G3/4 HCC, and that the G1 and G2 HCC subpopulations, which overexpresses AR mRNA in comparison with its peritumoral tissue, is characterized by very high levels of mRNA. This HCC fraction (corresponding to 20% of G1/2 HCC and to 16% of the total HCC population) corresponds to the percentage of HCC patients, responding to cyproterone acetate treatment (15%-22%) (Forbes et al., 1987; Pignata et al., 1998) in those studies in which patients with unresectable HCC were treated, but not evaluated for the expression of AR in the tumor.

Concerning male and female HCC patients, we did not find any significant difference in AR mRNA expression level identified in peritumoral and tumoral biopsies; we cannot exclude that gender, via the androgen receptor system, is not responsible for the unbalanced HCC male-female incidence, because we have not studied androgen levels circulating in our patients, which were presumibly different according to sex.

In conclusion, the results obtained from our study raise several issues on the transcriptional regulatory mechanisms of AR in human HCC, about its variable expression and also its under-expression observed in the G3/G4 HCC samples. In this context we are aware of the need to extend this study to a larger number of cases; nevertheless, the present data indicate that the patients affected by highly undifferentiated HCC (G3/4) will not have any advantage with antiandrogen therapies. Among the other HCC patients, antiandrogen therapy might help patients with differentiated HCC, showing a derepression of AR mRNA in the tumoral as compared to the peritumoral tissues. This possibility should be clinically tested following evaluation of AR mRNA expression in HCC liver biopsies.

Acknowledgements. The authors thank Dr. Alessandro Salvi for analyzing the AR gene in some HCC samples and Mrs. Bruna Arici for excellent technical assistance. The authors also thank Dr. Anna Benetti for evaluation of HCC grading (Chair of Pathological Anatomy, University of Brescia), Dr. Giovanni Parinello (Division of Statistics, University of Brescia) for invaluable suggestions for the statistical analysis. This work was supported by grants awarded by the Ministero dell' Università e Ricerca Scientifica eTecnologica, Centro Eccellenza IDET and by CIB.

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Accepted July 8, 2002