Detection and Identification of Tumor-Associated Protein Variants in Human Hepatocellular Carcinomas

Evelyn Zeindl-Eberhart,[1] Sibylle Haraida,[1] Sibylle Liebmann,[1] Peter Roman Jungblut,[2] Stephanie Lamer,[2] Doris Mayer,[3] Gundula Jäger,[4] Stephen Chung,[5] and Hartmut Manfred Rabes[1]

The proteomic approach is a valuable tool to detect and identify proteins that are associated with cancer. In previous investigations on experimentally induced rat hepatomas, we detected aldose reductase-like protein (ARLP) as a highly significant marker protein. Our present study was intended to look for the presence of similar tumor-associated marker proteins on human hepatocellular carcinomas (HCC). We found several novel tumor-associated protein variants that represent members of the aldo-keto reductase (AKR) superfamily. Human aldose reductase-like protein-1 (hARLP-1) was the most prominent tumor-associated AKR member detected in HCC by 2-dimensional electrophoresis (2-DE) and identified by mass spectrometric fingerprinting. The enzyme was found in 4 distinct forms (hARLP-1, 36/7.4 (kd/pI); hARLP-2, 36/7.2; hARLP-3, 36/6.4; and hARLP-4, 33/7.35). In addition, a human aldose reductase-like protein (hARLP-5, 36/7.6) was identified that differed from hARLP-1 by 1 amino acid (D313N), indicating 2 allelic forms of the human aldose reductase-like gene. A novel antibody directed against common parts of the hARLPs revealed hARLP reactivity in human HCC by immunohistochemistry. Furthermore, aldose reductase (AR) was identified and characterized as a tumor-associated variant. In conclusion, in all investigated human HCCs at least one of the various types of the described tumor-associated proteins of the AKR superfamily was clearly present. Of these HCC samples, 95% were positive for hARLPs as proven by 2-DE analysis and/or by use of the antibody directed against hARLP. Thus, hARLP is a strong candidate for use as an immunohistochemical diagnostic marker of human HCC. (HEPATOLOGY 2004;39:540–549.)

Human hepatocellular carcinoma (HCC) ranks fifth in worldwide cancer incidence and is an important component of public health. Many HCC risk factors are known, including hepatitis B or C (HBV or HCV) infection, ingestion of aflatoxin-contaminated food, and alcohol.[1,2] The development of HCC is associated with multiple changes at the messenger RNA (mRNA) and/or protein level, some of them serving as tumor markers, *e.g.*, α-fetoprotein,[3] or, less specifically, cyclin D1 or the proliferating cell nuclear antigen.[4]

Misprogramming of genetic information in cancer is reflected by quantitative and/or qualitative protein alterations. These protein alterations might represent tumor markers that are useful in the diagnosis of human tumors and may also help the understanding of mechanisms of tumor induction and development. Proteome analysis of liver proteins and HCC were predominantly performed using either chemically induced hepatomas in animals (predominantly the rat[5–10]) or human HCC cell lines, such as HepG2 and Huh7 cells,[11] BEL-7404 cells,[12] or HCC-M cells.[2,13,14] Numerous so-called tumor-associated or cancer-related proteins were identified; these provide valuable information for the establishment of HCC protein databases.[2,11–14] Comparative analysis of liver tissue and hepatocellular carcinomas might give additional insights into the induction or repression of tumor-associ-

Abbreviations: ARLP, aldose reductase-like protein; HCC, hepatocellular carcinoma; AKR, aldo-keto reductase; hARLP, human aldose reductase-like protein; 2-DE, 2-dimensional electrophoresis; HBV, hepatitis B virus; HCV, hepatitis C virus; MS, mass spectrometry; ARL-1, aldose reductase-like-1; PMF, peptide mass fingerprinting; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; AR, aldose reductase; acc, accession number; rARLP, rat aldose reductase-like protein; TST, thiosulfate sulfurtransferase.

From the [1]Institute of Pathology, Ludwig Maximilians University of Munich, Munich; the [2]Max Planck Institute for Infection Biology, Berlin; [3]Research Group in Hormones and Signal Transduction, German Cancer Research Center, Heidelberg; the [4]Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Munich, Germany; and the [5]Institute of Molecular Biology, University of Hong Kong, Hong Kong, China.

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Address reprint requests to: Evelyn Zeindl-Eberhart, Institute of Pathology, University of Munich, Thalkirchnerstrasse 36, D-80337 Munich, Germany. E-mail: Evelyn.Eberhart@lrz.uni-muenchen.de; fax: +49-89-5160-4083.

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ated protein variants within an intact organism. Clinical material was used for proteome analysis comparing normal liver tissue and HCC,[15–17] or normal liver, cirrhotic liver, and HCC[18] or sera from HCC patients for the identification of tumor autoantibodies.[19] Different protein variants were proposed as tumor-marker candidates. An integrated proteome database for the study of human HCC has been constructed.[20] As a result of worldwide interest in liver diseases, in particular human HCC, initiatives of the human proteome project (HUPO)[21] led to the initiation of the human liver proteome project (HLPP).

In previous investigations on chemically induced rat hepatomas, we detected a number of tumor-associated protein variants by 2-dimensional electrophoresis (2-DE), predominantly in an area of 30–40 kd/pI 6.2–7.6, and identified them by mass spectrometry (MS) as members of the aldo-keto reductase (AKR) superfamily.[5,6] Their induction/inhibition was dependent on the type of initiating carcinogen.

The AKR superfamily includes several main enzyme families. The largest family (AKR1) contains the aldehyde reductases (AKR1A), the aldose reductases (AKR1B), and the hydroxysteroid dehydrogenases (AKR1C); nomenclature is from Jez et al.[22] Members of the AKR superfamily are monomeric cytoplasmic proteins; they share identical sequences and possess similar physical and chemical properties. Most of them catalyze the NADPH-dependent reduction of a large variety of xenobiotics and endogenous aldehydes and ketones and metabolize a wide range of substrates; they are all involved in detoxification processes.[22,23] Members of the AKR superfamily are described as being involved in carcinogenic processes. These are 4 aldose reductase-like proteins (ARLPs) predominantly detected in nitroso-compound-induced rat hepatomas.[5] In human HCC, an aldose reductase-like-1 (ARL-1) mRNA was expressed.[24,25] Two aflatoxin B1-inducible aldehyde reductases were discovered in aflatoxin-B1-induced rat hepatomas[26] and in human colonic carcinomas.[27]

These results led us to perform a proteome analysis of human HCC. New patterns of tumor-associated protein variants were observed in 2-DE gels of human HCC, predominantly in a 2-DE area of ≈30–40 kd/pI 6.2–7.8. We identified these tumor-associated protein variants as members of the AKR superfamily by peptide mass fingerprinting (PMF) obtained by matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) and showed that they might be valuable as diagnostic markers of human HCC in immunohistochemistry.

Patients and Methods

Tissue Specimens. Human HCC samples from two different groups, Caucasians (group I) and Asians (group II), were investigated. Ten different tissue samples of human HCC (group I) from liver explants collected between 1994 and 1998 were provided by P. Bannasch, Division of Cytopathology, German Cancer Research Center, Heidelberg, Germany. The HCC tissue collections were obtained with informed consent and were approved by the ethics committee of Heidelberg University. These tumor samples were designated 237/98, 126/98, 261/98, 321/97, 14/98, 193/98, and 8/98 (males), and 260/98, 91/97, and 107/94(females). Of the patients in group I, 40% were positive for HBV infection and 50% for HCV infection. One patient was positive for HCV with an alcohol anamnesis; another was alcoholic. All tumor samples were histologically verified; classification of differentiation was performed according to the WHO grading system. The samples were stored at −80°C until use. Control liver samples from 2 healthy males,1 female (age adjusted), and a fetal liver (5th month) were received from the Institute of Forensic Medicine, Munich, and designated 51HLN and 29HLN (males), 30HLN (female), and FET (fetal liver), respectively. In addition, we used 5 other histologically examined human HCC samples together with the corresponding normal liver tissue from the Institute of Molecular Biology, University of Hong Kong. These tissue samples (group II) were designated identically to those published by Cao et al.[24]: (T, tumor; N, normal tissue) T2;N2, T12;N12, T16;N16, T19;N19, and T23. HBV/HCV status was determined in these HCC samples by polymerase chain reaction for HBV DNA and HCV RNA. All patients were positive for HBV infection; additional clinical data were not available.

Preparation of Protein Samples. Soluble protein fractions were extracted from all samples. The group II HCC samples underwent a lyophilization step prior to the extraction of the soluble protein fraction.

For lyophilization liver samples frozen at −196°C were weighed and transferred to a mortar cooled at −196°C. Two ml of bidistilled water were added in pellet form to each sample. Frozen components were ground to powder in liquid nitrogen. Five ml of bidistilled water (4°C) were added to each powdered sample, the suspension was quickly transferred into a lyophilization flask, and the samples were freeze-dried, weighed, and sent to our laboratory. The original mass of the samples was restored by adding bidistilled water. The extraction method of the soluble protein fraction was the same for all samples following the method of Klose[28] as previously described.[5]

Two-Dimensional Electrophoresis. Soluble proteins were separated by high-resolution 2-DE according to Klose,[29] using Iso-Dalt –equipment (Hoefer Scientific Instruments, San Francisco, CA).[30,31] Isoelectric focusing was performed with 4% carrier ampholytes (4 parts Servalyte, pH 5-7, Serva, Heidelberg, Germany; 1 part Pharmalyte, pH 3-10 and 1 part Ampholine, pH 3.5-9.5, both Amersham Pharmacia Biotech, Uppsala, Sweden). The second dimension was run on gels ($16 \times 16 \times 0.15$ cm) with a 10%-to-16% polyacrylamide gradient in a Dalt tank as previously described in detail.[32] Four hundred μg of soluble protein (Lowry protein assay kit, Sigma-Aldrich, Deisenhofen, Germany) were separated for Coomassie Brilliant Blue staining (CBB R-250, Merck, Darmstadt, Germany), and 100 μg of soluble protein were used for silver staining.[33]

The soluble protein fraction of each sample (n = 24) was applied for 2-DE separation at least twice. Because of the variable levels of albumin in the different human samples (first run) that are included in the protein determination, the applied protein amount was adjusted to identical actin amounts as an internal standard (second run) for every sample. In group I, 2-DE gels of 10 different HCC samples were compared with 3 different normal liver tissues; in group II, 5 different HCC samples were compared with their corresponding normal liver tissues. The selected area in 2-DE gels, ≈30–40 kd/pI ≈6.2–7.8, according to the rat model,[5] was evaluated visually at first according to several criteria. All protein spots that were changed in quantity and/or quality in at least 80% of all HCCs investigated (n = 15) were defined as candidates for tumor-associated protein variants. In addition, spots that previously had been defined as being tumor-associated by others were included as candidates for tumor-associated proteins. Also included were newly detected proteins that represent highly related forms of proven tumor-associated variants and protein variants predominantly detected in 1 of the 2 groups investigated.

The relative spot intensity of different candidates of tumor-associated protein variants was determined in silver-stained 2-DE gels from normal liver tissue and HCC samples of Caucasian and Asian patients by TopSpot evaluation program (Algorithmus, Berlin, Germany). Spot intensities of all candidates of tumor-associated variants were normalized against the actin spot intensity, which is in the linear range of the intensity curve and does not vary between HCC and controls. In order to define significant tumor-associated protein variants, Student's t test was performed. Protein spots that fulfilled the criterion $P \leq .025$ were defined as tumor-associated variants.

For exclusion of possible unspecific protein changes in 2-DE gels due to the lyophilization procedure (performed for group II samples only), we tested both preparation methods using a rat hepatoma and a human liver sample and analyzed the 2-DE pattern run in parallel with and without lyophilization. Samples that underwent both preparation methods showed identical protein amounts in their 2-DE gels. An influence of lyophilization on the 2-DE protein pattern could be excluded.

Protein Identification. For identification, several protein spots per spot of interest were excised from CBB-stained gels. PMF after tryptic in-gel digestion was used[34] as described.[5] Identification was performed by searching the National Center for Biotechnology Information (NCBI) and/or Swissprot databases with MS-Fit or PeptIdent, respectively. Sequence database was limited to human/rodent proteins. A mass tolerance of 0.1 Da and 2 incomplete cleavages were allowed. Identifications were accepted when they covered at least 30% of the whole sequence and when comparable molecular mass/P values were obtained from the databases.

Immunoblotting of Human Aldose Reductase-like Protein (hARLP). After 2-DE separation of the soluble protein fraction of HCC tissue (400 μg protein/sample), 2-DE gel areas (≈30–40 kd/pI ≈6.2–7.8) containing the hARLP region were excised from 2-DE gels and blotted onto hydrophobic polyvinylidene difluoride membranes (0.2 μm Bio-Rad, Hercules, CA) under semidry conditions for 3 hours at 1 mA/cm^2 membrane at room temperature with 50mM borate buffer, pH 9.0, 20% methanol. For immunodetection of hARLP the polyclonal antibody directed against the last 17 amino acids of the C-terminus of the hARLP, accession number (acc) O60218,[24] was used (dilution 1:1,000) according to the method described earlier.[35] The hARLP antibody was produced for our laboratory by Bio-Genes (Berlin, Germany).

Immunohistochemical Detection of hARLP in Liver Sections. Formaldehyde-fixed, paraffin-embedded tumor samples from 10 additional Caucasian patients, different from those used for proteome analysis, were used: HCC (n = 6); hepatocellular adenoma (n = 1); liver metastasis of a colonic adenocarcinoma (n = 1); focal nodular hyperplasia (n = 1); and fibrolamellar carcinoma (n = 1). Human ARLP expression was shown on serial 4 μm sections using the polyclonal antibody described above and the Vectastain –ABC Kit (rabbit immunoglobulin G; Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma) as described.[8] After unmasking in diluted Target Retrieval Solution (Dako Diagnostica, Hamburg, Germany) for 30 minutes in an autoclave at 1 atm, endogenous peroxidase of the sections was inhibited by 3% H_2O_2. Human ARLP antibody was used in a dilution of 1:80 in antibody diluent (Dako). Antibody di-

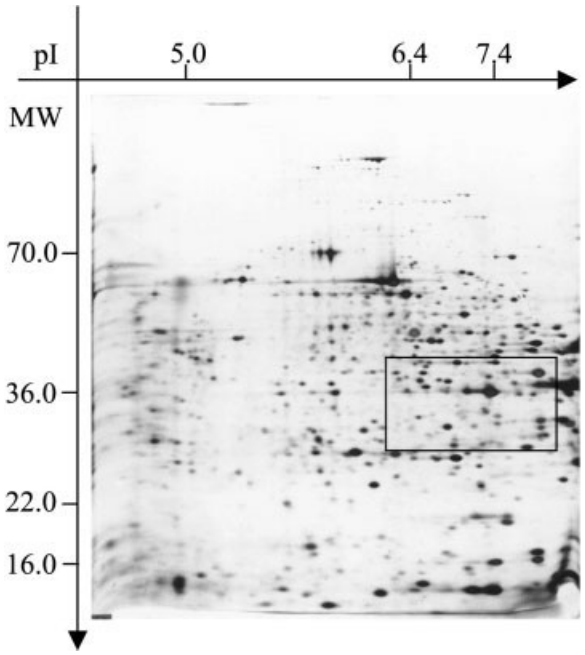


Fig. 1. Two-dimensional gel of soluble proteins from human HCC. One hundred μg of soluble proteins (sample T23) were separated in the first dimension by isoelectric focusing using 4% carrier ampholytes (pH 3-10). Separation in the second dimension was performed using an acrylamide gradient (10%-16%) followed by silver staining. M_r axis was calibrated by standard proteins (Serva). Evaluated area (**framed**): ≈30–40 kd/pI ≈6.2–7.8.

luent without antibody served as negative control. In addition, the APAAP (alkaline phosphatase–anti-alkaline phosphatase) method was performed. After unmasking as described above, hARLP antibody was used in a dilution of 1:80 in antibody diluent for 17 hours at 4°C. The following steps were performed according to the manufacturer's instructions (Dako): (1) Fast Red TR/Naphthol AS-MX was used as a detection system for alkaline phosphatase according to the product information (Sigma); (2) slides were counterstained with Mayer's hematoxilin for 2 minutes, blued for 15 minutes in running water, and cover-slipped with Kaiser's glycerol gelatin (Merck).

Results

High-resolution 2-DE gels performed with soluble protein fractions of human HCC from Caucasian (group I, n = 10) and Asian (group II, n = 5) male and female patients, compared with controls (n = 8), showed a reproducible protein pattern with some interindividual variation. A representative 2-DE gel is shown in Fig. 1. The area ≈30–40 kd/pI ≈6.2–7.8 was selected for the evaluation of tumor-associated protein variants. In this 2-DE area, approximately 90 different spots were registered. Thirteen were classified as tumor-associated variants according to the criteria outlined above. Each represents a well-defined spot with regard to MW and pI. Seven protein spots represent known proteins of different protein groups (publication in preparation). Five spots of the selected area were identified by PMF-MS (Table 1); 1 was characterized by immunoblotting. All 6 spots are members of the AKR superfamily and are subjects of this report.

Three spots with the same M_r but different pI (36 kd/pI 7.4; 7.2; 6.4, respectively) were identified by MS as different forms of hARLP. An additional spot (33 kd/pI 7.35) reacted with the hARLP antibody, obviously representing an additional hARLP. We designated these related proteins hARLP-1, hARLP-2, hARLP-3 and hARLP-4, respectively. These proteins are not found in normal liver tissue; but present exclusively in human HCC (Fig. 2A and B, Table 2) and were defined as tumor-associated variants.

We identified an additional related form of hARLP in HCC; it was designated hARLP-5 (Fig. 2C). This protein was first detected by immunoblotting in HCC samples from 2 female patients (260/98 and 91/97). MS analysis confirmed this result. However, compared to hARLP-1 (36/7.4), the hARLP-5 (36/7.6) was different in 1 specific mass peak. The mass peak 2,272.03/2,271.98 (user mass/matching mass) found in hARLP-1 characterized a peptide of the amino acid positions 298 to 316

Table 1. Tumor-Associated Protein Spots in 2-DE Gels Determined by PMF MALDI-MS

2-DE		MS					
kd/pI of Tumor-Associated Spots	Tissue Examined	Peptide Matches	*Identified Protein	Sequence Coverage (%)	Amino Acids	Calculated kd/pI	†Accession
36.0/7.4	HCC	16/30	hARLP-1	64.9	316	36.0/7.12	060218
36.0/7.2	HCC	17/35	hARLP-2	67.4	316	36.0/7.12	060218
36.0/6.4	HCC	13/36	hARLP-3	48.7	316	36.0/7.12	060218
36.0/7.6	HCC	15/25	hARLP-5	54.4	316	36.0/7.7	075890
36.0/6.8	HCC	11/21	AR	36.0	316	35.8/6.5	178489

Abbreviations: HCC, hepatocellular carcinoma; hARLP, human aldose reductase-like protein-1, -2, -3, and -5; AR, aldose reductase.

*The identified proteins originated either from HCC or from normal human liver tissue.

†NCBI and/or Swissprot were used to evaluate MS data.

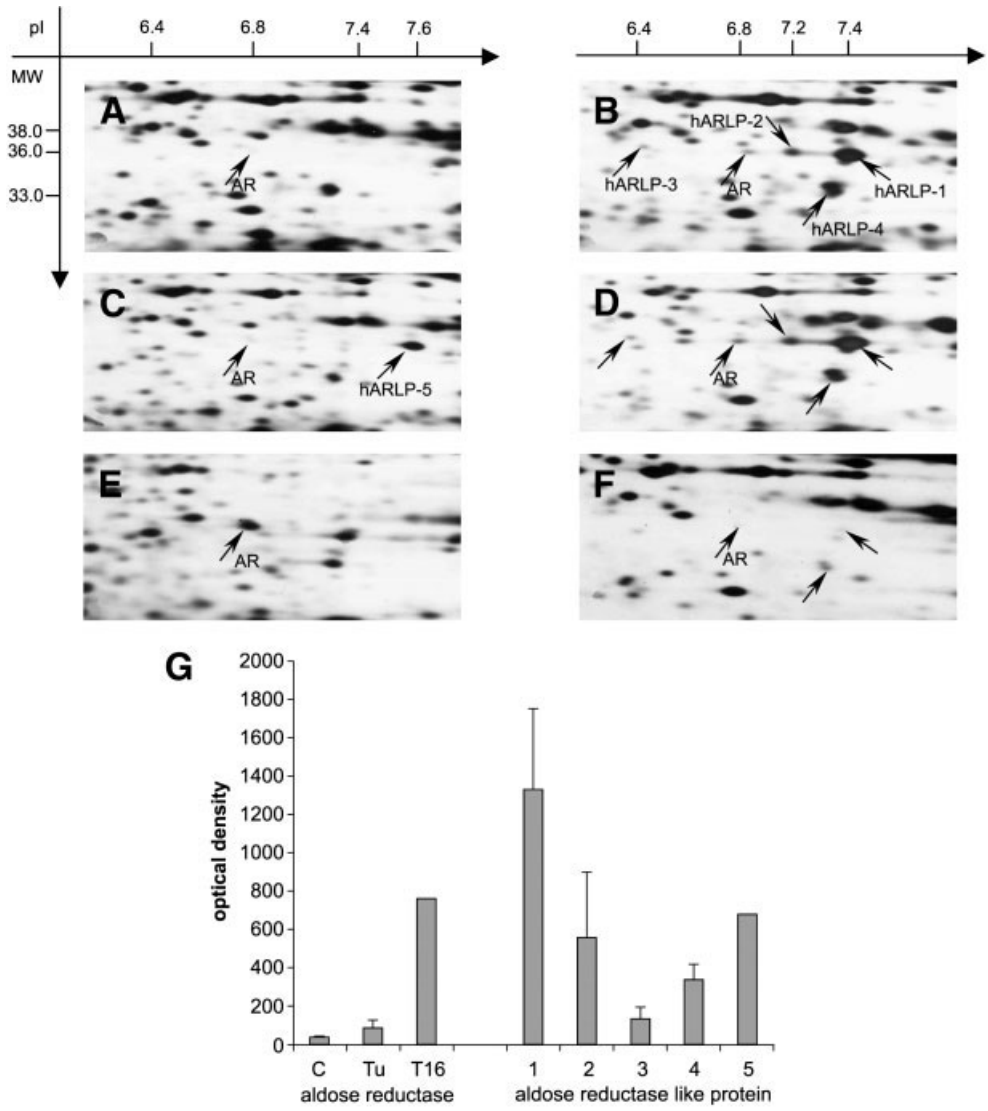


Fig. 2. Members of the AKR superfamily represent tumor-associated protein variants in human HCCs. The area of 2-DE gels ≈30–40 kd/pI ≈ 6.2–7.8 was evaluated for tumor-associated protein variants. Members of the AKR family (**arrows**)—hARLPs and AR—were changed in amount in HCCs compared to normal liver. (A) Normal liver tissue (sample 51HLN). (B) Human HCC; hARLP-1, hARLP-2, hARLP-3, and hARLP-4 (Caucasian; 193/98). (C) Human HCC; hARLP-5 (260/98). (D) Human HCC; hARLP-1, hARLP-2, hARLP-3, and hARLP-4 (Asian; T2). (E) Human HCC; AR (T16). (F) Fetal liver; hARLP-1 and hARLP-4. (G) Quantitation of tumor-associated protein variants analyzed by TopSpot evaluation program. Optical density (mean ± SD) of AR levels in control liver (C; n = 5), in human HCC (T; n = 8), and in sample T16; hARLP-1 to hARLP-4 in HCC (n = 8) and hARLP-5 (n = 1). Areas in 2-DE gels of normal liver corresponding to the site of hARLP –spots; the staining intensity was in the range of the background intensity (11 ± 2).

(ACNVLQSSHLEDYPFDAEY; acc O60218). The corresponding peptide of hARLP-5 was slightly different: 2,271.01/2,271.002 (user mass/matching mass). In amino acid position 313, the aspartic acid (D) present in hARLP-1 was substituted by an asparagine (N) (acc O75890) in hARLP-5. A detail of these MALDI mass spectra is shown in Fig. 3.

Human aldose reductase (AR) was identified by PMF-MS located at 36 kd/pI 6.8 in 2-DE gels obtained from the HCC sample T16 (group II). T16 shows the highest amount of AR of all HCC investigated (Fig. 2E). In normal liver tissue, AR could be detected in only minimal amounts but was found slightly increased in 8 additional HCC samples as exemplified in Fig. 2A and B and in Table 2. AR ($P = .0014$) was defined as a tumor-associated variant.

Interestingly, most of the HCC investigated showed hARLP-1 to hARLP- 4 (12 out of 15 samples). In 3 cases (260/98 and 91/97 for hARLP-5; T16 for AR), hARLP-5 and AR were present as 1 distinct spot in 2-DE gels, but hARLP-1 to hARLP-4 were absent in these samples (Fig. 2C and E). In 8 HCC samples, AR was detected to be slightly enhanced in amount but was regularly accompanied by hARLP-1 to hARLP-4, as shown in Fig. 2B and D and Table 2. In 2-DE gels of fetal liver, small spots of hARLP-1 and hARLP-4 were observed (Fig. 2F).The relative spot intensity of hARLP-1 to hARLP–5 and of AR was quantified in 2-DE gels from normal liver tissue and in HCC material of both groups using TopSpot as software (Fig. 2G).

Immunoblots using the polyclonal antibody directed against hARLP showed that the antibody detected all forms of hARLP but not AR. Six different tumor samples and 3 control liver samples were tested. In 4 of the tumor samples (193/98, 14/98, 321/97, and T23), the presence of hARLP-1 to hARLP-4 and the absence of hARLP-5

Table 2. Characteristics of Tumor-Associated Protein Spots in 2-DE Gels Determined by PMF MALDI-MS and Identified as Members of the Aldo-Keto Reductase Superfamily

									Human Aldose Reductase-like Protein				
Case No.	Gender	Age (yr)	HBV/HCV	Chronic Active Hepatitis	Alcoholism	Cirrhosis	Tumor Grade[1]	AR	1	2	3	4	5
Group I													
51HLN	M	52	*	*	*	*	*	+	–	–	–	–	–
29HLN	M	45	*	*	*	*	*	+	–	–	–	–	–
30HLN	F	36	*	*	*	*	*	+	–	–	–	–	–
FET	F	0.42	*	*	*	*	*	–	+	–		+	–
126/98	M	57	neg.$_2$	pos.	pos.	pos.	II-III	↑	+++	+	+	+	–
261/98	M	51	C	severe	neg.	pos.	II	+	++	++	+	+	–
321/97	M	54	C	severe	neg.	pos.	I-II	↑	+++	++	+	++	–
14/98	M	51	C	pos.	neg.	pos.	II	↑	+++	++	++	++	–
193/98	M	45	C	severe	pos.	pos.	III	↑	+++	++	++	++	–
237/98	M	55	B	mild	neg.	pos.	II-III	↑	+++	++	+	+	–
8/98	M	54	B	pos.	neg.	pos.	II-III	+	+++	+	+	+	–
107/94	F	63	C	mild	neg.	pos.$_3$	III	↑	+	+	+	+	–
260/98	F	49	B	severe	neg.	pos.	II-III	+	–	–	–	–	++
91/97	F	51	B	severe	neg.	pos.	II-III	+	–	–	–	–	++
Group II													
N2	*	*	B	*	*	*	*	+	–	–	–	–	–
N12	*	*	B	*	*	*	*	+	–	–	–	–	–
N16	*	*	B	*	*	*	*	+	–	–	–	–	–
N19	*	*	B	*	*	*	*	–	–	–	–	–	–
T2	*	*	B	*	*	*	*	↑	+++	++	+	+	–
T12	*	*	B	*	*	*	*	+	+	+	+	+	–
T16	*	*	B	*	*	*	*	↑ ↑ ↑	–	–	–	–	–
T19	*	*	B	*	*	*	*	–	++	+	+	+	–
T23	*	*	B	*	*	*	*	↑	+++	+	++	++	–

NOTE. Tumor-associated protein variants detected in human HCC (group I, Caucasians; n = 10; group II, Asians; n = 5) were identified by MS. Amount of tumor-associated proteins was compared with normal liver tissue (HLN, Caucasians) and (N, Asians) and with fetal liver (FET).

Abbreviations: AR, aldose reductase; hARLP-1 to -5, human aldose reductase-like protein-1, -2, -3, -4, and -5; B, HBV-positive; C, HCV-positive; neg.$_2$, absence of HBV and HCV markers; pos.$_3$, cirrhosis incomplete; *, "no data available". Degree of protein amount: +, low; ++, medium; +++, high; –, absent; ↓, reduced; ↑, increased; ↑ ↑ ↑, highly increased.

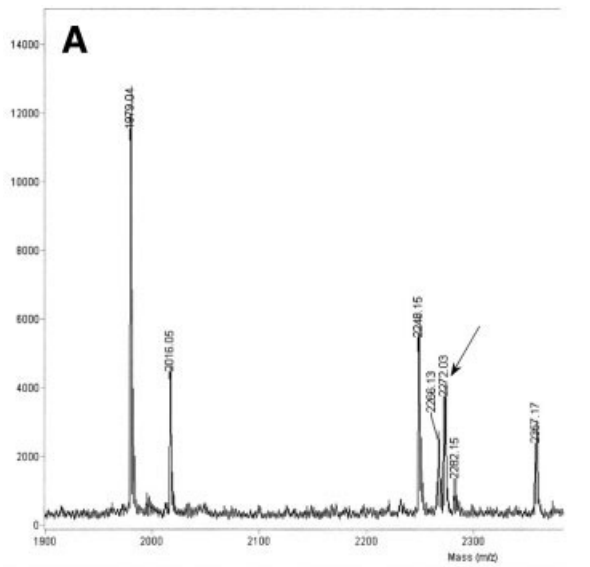
[1]Grading according to WHO. Caucasian patients have been transplanted because of HCC, serum levels of AFP, GOT GPT are not available.

were clearly confirmed. In sample 260/98, only hARLP-5 was detected, not the other hARLP forms. T16 showed no hARLP reactivity. In normal liver tissue (51HLN, 29HLN, and 30HLN), hARLP was below the limit of detection. As shown in Fig. 2B and D, hARLP-4 was slightly overlayed/neighbored by an additional spot. This neighbored spot was identified by MS as thiosulfate sulfurtransferase (TST; acc Q16762), an enzyme present in each control liver tissue as in most of the HCC samples investigated. Human ARLP-4 closely positioned to TST was assigned by immunoblottings. An immunoblot of HCC (sample T23) and of normal liver tissue (sample 29HNL) with hARLP antibody is demonstrated in Fig. 4A.

All HCC from an additional 6 Caucasian patients reacted positively (10% to 75% of HCC cells) with the hARLP antibody in histological sections. Human ARLP reactivity ranging from faint- to strong-staining was mainly observed in the cytoplasm of HCC cells. Cirrhotic liver parenchyma, focally with large liver cell dysplasia adjacent to the HCC, and inflammatory cells, were negative except for a few scattered, faintly positive cells. Furthermore, some of the positively reacting cells in the HCC—as in the adjacent cirrhotic and noncirrhotic reference liver—were identified as apoptotic cells. Hepatocellular adenoma, focal nodular hyperplasia, fibrolamellar carcinoma, and liver metastasis of a colonic adenocarcinoma failed to stain positively (Fig. 4B).

Discussion

In our attempt to identify and characterize tumor-associated protein variants in HCC from Caucasian and Asian patients, we analyzed 6 variant proteins by 2-DE and PMF-MS. These proteins all represent members of the AKR superfamily. Besides an elevated amount of AR in several HCCs, 5 different forms of hARLP were identified. Four of them were detected in the majority of the HCCs, designated hARLP-1, hARLP-2, hARLP-3, and hARLP-4. An additional form, hARLP-5, which differs from hARLP-1 by a single amino acid substitution at position 313 was observed in only a few HCCs. A novel



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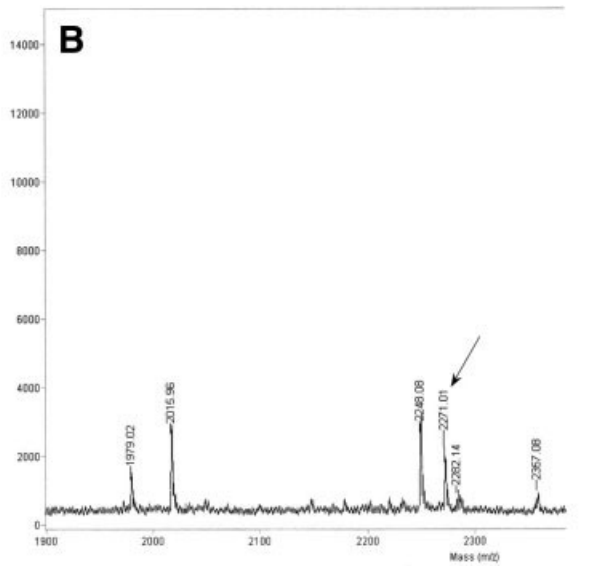
1979.04	1979.0425	0.0025	0		102-117	LSYLDVYLIHWPQGF K
2248.15	2248.1067	-0.0432	0	Cys_PAM: 187	178-195	YKPVTNQVECHPYLT QEK
2266.11	2266.0947	-0.0152	0		136-155	ATFLDAWEAMEELVD EGLVK
2272.03	2271.9863	-0.0436	0	Cys_PAM: 299	298-316	ACNVLQSSHLEDYPF DAEY
2282.17	2282.0896	-0.0803	0	MSO: 145	136-155	ATFLDAWEAMEELVD EGLVK



user mass	matching mass	Δmass (Dalton)	#MC	modification	position	peptide
1979.02	1979.0425	0.0225	0		102-117	LSYLDVYLIHWPQGF K
2248.08	2248.1067	0.0267	0	Cys_PAM: 187	178-195	YKPVTNQVECHPYLT QEK
2271.01	2271.0023	-0.0076	0	Cys_PAM: 299	298-316	ACNVLQSSHLEDYPF NAEY

Fig. 3. Detail of MALDI-mass spectra obtained from trypsin in gel digestion of hARLP-1 and hARLP-5. (A) hARLP-1 was different in 1 mass peak (2,272.03) from (B) hARLP-5 (2,271.01) (**arrows**). This difference showed a D at amino position 313 for hARLP-1 and an N for hARLP-5. cDNA sequencing[24,37] confirmed this difference.

antibody detected these different hARLP forms in immunoblots and in immunohistochemical sections of HCC.

A subfamily of the AKR superfamily comprises the aldose reductases (AKR1B). They play an important role in catalyzing the conversion of glucose to the hyperosmotic sugar sorbitol in the sorbitol pathway. Sorbitol has been implicated in the development of diabetic complications.[36] AR shares a 70% to 90% sequence identity with ARLPs, which form a distinct subgroup in the AKR1B group. ARLPs may be separated into different groups of specifically acting proteins depending on their response to exogenous factors.[5] In previous experiments, 4 different rat ARLPs (rARLPs) were found in nitroso-compound-induced rat hepatomas.[5] The amino acid sequence of rARLP (acc AJ277957)[5] is 82% identical with human ARL-1 (aldose reductase-like-1; acc O60218).[24] Congruent location in 2-DE gels and high sequence identity led us to assume that human hARLPs represent protein analogues to rat rARLPs.

Based on our MS results, hARLP-1 was characterized by aspartic acid (D) at position 313, while asparagine (N) was found at position 313 in hARLP-5. Database comparison revealed that hARLP-1 was identical to human ARL-1 (acc O60218),[24] and hARLP-5 was identical to human small intestine protein (HSI; acc O75890).[37,38] These data were confirmed by complementary DNA sequencing. For ARL-1, a G was sequenced at triplet position 313-1 (acc U37100),[24] leading to the triplet GAT coding for D; for HSI at triplet position 313-1, an A was identified,[37] leading to the triplet AAT coding for N. This data indicates 2 different allelic forms of the hARLP gene. The different allelic forms were found in 12 HCC samples for hARLP-1 and in 2 for hARLP-5.

It is of interest to compare results from proteome studies with mRNA expression. Due to posttranslational modifications, a single mRNA transcript may give rise to more than one protein.[39] This was also shown for hARLPs. Cao et al.[24] found 1 band for ARL-1 mRNA in Northern blots from human HCC. We used some of these tumor samples for proteome analysis. Comparisons of our 2-DE gels with Northern blots of Cao et al.[24] revealed comparable results. However, we found 4 different forms of hARLPs (hARLP-1 to hARLP-4), suggesting that these protein variants are the result of posttranslational modifications. Interestingly, hARLP-5 and AR appeared as only 1 spot each.

HCC of Caucasian patients was characterized by hARLP-1 to hARLP-4 in 80%, and by hARLP-5 in 20%. AR as a tumor-associated variant[40] was detected in 60% of HCC. This is comparable with the results of Cao et al.[24];

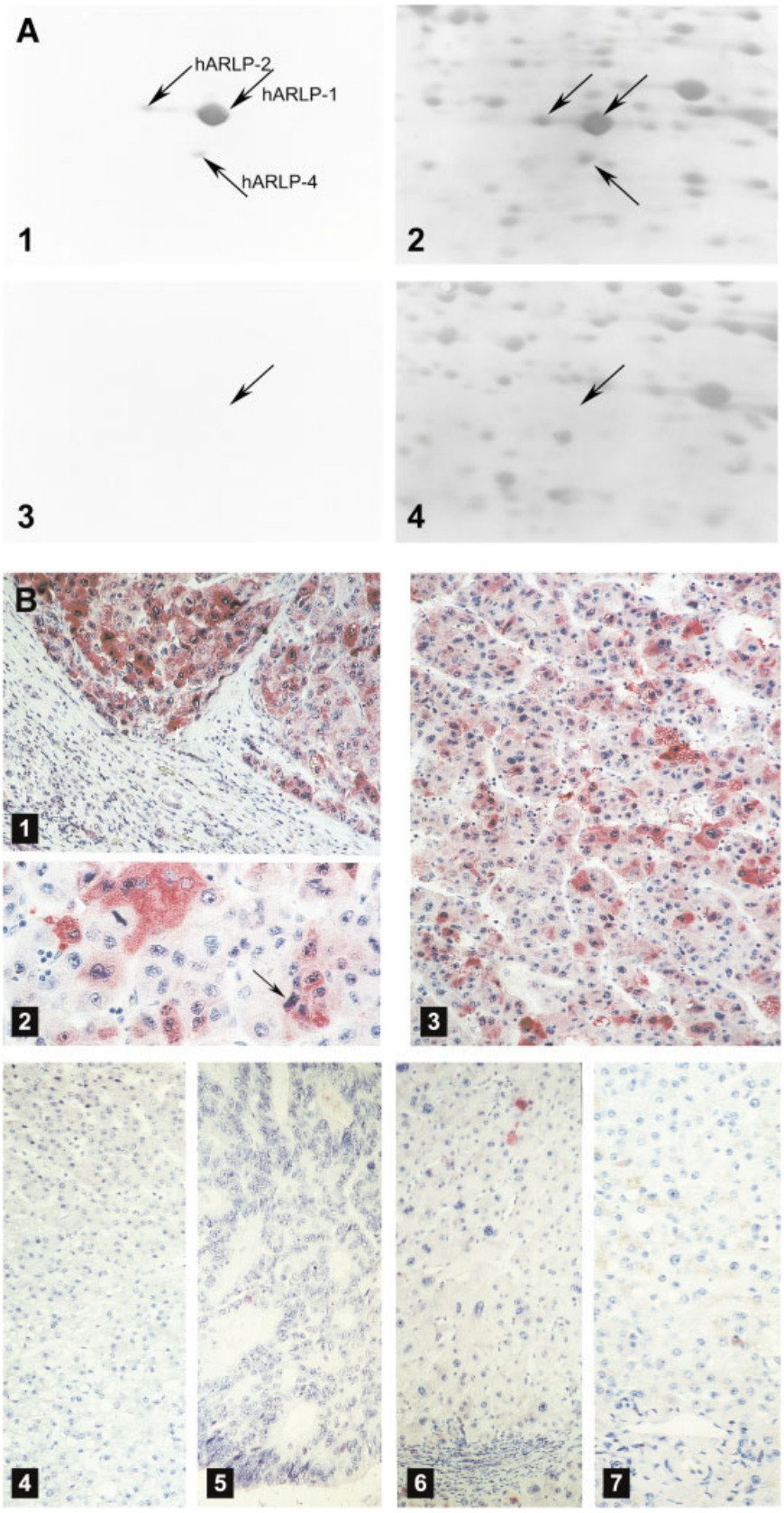


Fig. 4. Immunodetection of hARLPs with hARLP antibody in Western blots and in paraffin sections of human HCCs. (A) Human ARLPs were detectable by immunoblotting in HCC. After separation of 400 μg soluble protein from HCC samples T23 and 29HLN as a control by 2-DE, and after membrane transfer, membranes were incubated with hARLP antibody (1:1,000). (1) BCIP/NBT-stained spots (hARLP-3 not seen) were counterstained with CBB (2). (3) BCIP/NBT-stained membrane of normal liver tissue were counterstained with CBB (4).[35] (B) Immunohistochemistry (APAAP) of paraffin sections of human HCCs was performed on formalin-fixed sections with hARLP antibody (1:80). (1) Positive HCC with adjacent cirrhotic septa; (2) in HCC, hARLP also positively stained apoptotic cells (**arrow**, magnification $\times$ 400); (3) HCC with varying staining intensity; (4) negative hepatocellular adenoma; (5) negative metastasis of a colonic adenocarcinoma in the liver; (6) large liver cell dysplasia in cirrhotic liver with single faintly positive cell; and (7) noncirrhotic reference liver (in (1), (3)–(7) magnification $\times$100).

they found that in the Asian population, 54% of investigated HCC expressed ARL-1 mRNA and 29% expressed AR mRNA. The expression of a hepatoma-specific aldose reductase-related protein (HARP) mRNA was registered in 5 HCC samples.[25] The partial amino acid sequence deduced from HARP mRNA (positions 232-316)[25] was identical with ARL-1.[24]

We detected hARLP-1 and hARLP–4 in fetal liver. This differs from the results of Cao et al.[24] They did not find ARL-1 mRNA expression in fetal liver tissue. This difference might be due to the expression of hARLP at specific stages of development. However, hARLP obviously represents an additional embryonic liver enzyme, which, similar to the rat analogue rARLP,[5] the GST-P (glutathione-S-transferase-P),[41] or the α-fetoprotein,[3] is reactivated in human HCC.

Human ARLP could be used as a diagnostic marker in immunohistochemistry. The antibody directed against hARLP detected HCC in all sections from paraffin-embedded human material but adjacent cirrhotic or noncirrhotic reference liver was negative. Some hARLP-positive cells revealed condensed nuclei, typical of apoptosis, indicating that hARLP might be involved in apoptotic processes, a possible new aspect of hARLP function. AR, with 71% sequence identity to hARLP, was described as playing a key role in apoptosis.[42,43]

Early changes in energy metabolism, including enzymatic alterations associated with excessive storage of glycogen in altered hepatocytes, are typical for experimental hepatocarcinogenesis induced in rats by various chemicals.[44] The excessive glycogen storage reflects changes in various enzymes of carbohydrate metabolism.[44] The sorbitol pathway leading from glucose to sorbitol, catalyzed by AR, is not or only slightly active in normal adult liver cells.[45]With altered conditions, the sorbitol pathway might be upregulated by an increased AR amount and/or by the constitutive reactivation of embryonic hARLPs. The increased amount of AR and/or hARLPs, as seen in 2-DE of human HCC samples, may lead to an excess of sorbitol production in HCC cells, and, in consequence, to an imbalance of organic intracellular osmolytes. This may lead to deleterious effects on hepatocytes, including enlargement, due to increased intracellular osmotic pressure and hydration.[46]

As a member of the AKR family, AR is involved in the reduction of a variety of xenobiotic and endogenous aldehydes and ketones.[22,23] The AR-related ARLPs also appear involved in detoxification processes. Induction of the different hARLPs and enhancement of AR in human HCC might reflect different mechanisms of tumor induction. In most of the HCC samples investigated, hARLPs were induced independent of the HBV/HCV status. AR was described as elevated in livers with alcohol-associated injury and disease.[47] This agrees with our findings, that 2 HCCs originating from alcoholics show not only a remarkable increase of AR but also high hARLP amounts. In these cases, toxic acetaldehyde, a degradation metabolite of alcohol, might have played a role in the induction of these human HCCs. The tumor cells themselves, however, appear resistant to the toxic action of acetaldehyde because of an enhanced AR and/or hARLP amount. In addition, the induction of hARLPs in humans could reflect the detoxification of other factors (*e.g.*, ingested nitroso compounds), as was already proven in the rat model.[5] Proteome analysis of cell cultures of human HCC might help to clarify the role of differently acting drugs or xenobiotics in specifying a carcinogen-dependent protein pattern in HCC.

The increased amount of AR and/or the induction of hARLPs in all the HCC investigated might reflect an acquired resistance of the initiated liver cells to the toxic action of carcinogenic factors. While the overexpression of AR leaves cells more resistant to drugs,[48] the inhibition of AR enhances sensitivity to chemotherapeutic agents.[49]

Remarkably, in all investigated HCC, at least one of the various types of the described tumor-associated proteins of the AKR superfamily was induced or clearly enhanced. For these 2-DE investigations we used 10 HCC samples from Caucasian patients and 5 samples from Asian patients. Six additional HCC samples were used for immunohistochemistry. Of these human HCC samples, 95% (20 of 21) were positive for hARLPs as proven by 2-DE analysis or by the use of the antibody directed against hARLP, while the normal control liver was negative. We conclude that hARLP might play a role as an immunohistochemical diagnostic marker of human HCC.

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References

1. Henry SH, Bosch FX, Troxell TC, Bolger PM. Policy forum: public health. Reducing liver cancer—global control of aflatoxin. Science 1999; 286:2453–2454.
2. Seow TK, Liang RC, Leow CK, Chung MC. Hepatocellular carcinoma: from bedside to proteomics. Proteomics 2001;10:1249–1263.
3. Marwoto W, Miskad UA, Siregar NC, Gani RA, Boedihusodo U, Nurdjanah S, Suwarso, et al. Immunohistochemical study of p53, PCNA and AFP in hepatocellular carcinoma, a comparison between Indonesian and Japanese cases. Kobe J Med Sci 2000;46:217–229.
4. Qin LX, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. World J Gastroenterol 2002;8:385–392.
5. Zeindl-Eberhart E, Klugbauer S, Dimitrijevic N, Jungblut PR, Lamer S, Rabes HM. Proteome analysis of rat hepatomas: carcinogen-dependent tumor-associated protein variants. Electrophoresis 2001;22:3009–3018.

6. Zeindl-Eberhart E, Jungblut PR, Otto A, Rabes HM. Identification of tumor-associated protein variants during rat hepatocarcinogenesis. Aldose reductase. J Biol Chem 1994;269:14589–14594.
7. Zeindl-Eberhart E, Jungblut PR, Rabes HM. Expression of tumor-associated protein variants in chemically induced rat hepatomas and transformed rat liver cell lines determined by two-dimensional gel electrophoresis. Electrophoresis 1994;15:372–381.
8. Zeindl-Eberhart E, Jungblut PR, Otto A, Kerler R, Rabes HM. Further characterization of a rat hepatoma-derived aldose-reductase-like protein. Eur J Biochem 1997;247:792–800.
9. Wirth PJ. Two-dimensional polyacrylamide gel electrophoresis in experimental hepatocarcinogenesis studies. Electrophoresis 1994;15:358–371.
10. Newsholme SJ, Maleeff BF, Steiner S, Anderson NL, Schwartz LW. Two-dimensional electrophoresis of liver proteins: characterization of a drug-induced hepatomegaly in rats. Electrophoresis 2000;21:2122–2128.
11. Wirth PJ, Hoang TN, Benjamin T. Micropreparative immobilized pH gradient two-dimensional electrophoresis in combination with protein microsequencing for the analysis of human liver proteins. Electrophoresis 1995;16:1946–1960.
12. Yu LR, Zeng R, Shao XX, Wang N, Xu YH, Xia QC. Identification of differentially expressed proteins between human hepatoma and normal liver cell lines by two-dimensional electrophoresis and liquid chromatography-ion trap mass spectrometry. Electrophoresis 2000;21:3058–3068.
13. Seow TK, Ong S-E, Liang RCMY, Ren E-C, Chan L, Ou K, Chung MCM. Two-dimensional electrophoresis map of the human hepatocellular carcinoma cell line, HCC-M, and identification of the separated proteins by mass spectrometry. Electrophoresis 2000;21:1787–1813.
14. Liang RC, Neo JC, Lo SL, Tan GS, Seow TK, Chung MC. Proteome database of hepatocellular carcinoma. J Chromatogr B Analyt Technol Biomed Life Sci 2002;771:303–328.
15. Yoon GS, Lee H, Jung Y, Yu E, Moon HB, Song K, Lee I. Nuclear matrix of calreticulin in hepatocellular carcinoma. Cancer Res 2000;60:1117–1120.
16. Park KS, Kim H, Kim NG, Cho SY, Choi KH, Seong JK, Paik YK. Proteomic analysis and molecular characterization of tissue ferritin light chain in hepatocellular carcinoma. HEPATOLOGY 2002;35:1459–1466.
17. Park KS, Cho SY, Kim H, Paik YK. Proteomic alterations of the variants of human aldehyde dehydrogenase isozymes correlate with hepatocellular carcinoma. Int J Cancer 2002;97:261–265.
18. Lim SO, Park SJ, Kim W, Park SG, Kim HJ, Kim YI, Sohn TS, et al. Proteome analysis of hepatocellular carcinoma. Biochem Biophys Res Commun 2002;291:1031–1037.
19. Le Naour F, Brichory F, Misek DE, Brechot C, Hanash SM, Beretta L. A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis. Mol Cell Proteomics 2002;1:197–203.
20. Cho SY, Park KS, Shim JE, Kwon MS, Joo KH, Lee WS, Chang J, et al. An integrated proteome database for two-dimensional electrophoresis data analysis and laboratory information management system. Proteomics 2002;2:1104–1113.
21. Hanash S. Disease proteomics. Nature 2003;422:226–232.
22. Jez JM, Flynn TG, Penning TM. A new nomenclature for the aldo-keto reductase superfamily. Biochem Pharmacol 1997;54:639–647.
23. Maser E. Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. Biochem Pharmacol 1995;49:421–440.
24. Cao D, Tat Fans S, Chung SSM. Identification and characterization of a novel human aldose reductase-like gene. J Biol Chem 1998;273:11429–11435.
25. Scuric Z, Stain SC, Anderson WF, Hwang J-J. New member of aldose reductase family proteins overexpressed in human hepatocellular carcinoma. HEPATOLOGY 1998;27:943–950.
26. Ellis EM, Judah DJ, Neal GE, Hayes JD. An ethoxyquin-inducible aldehyde reductase from rat liver that metabolizes aflatoxin B1 defines a subfamily of aldo-keto reductases. Proc Natl Acad Sci USA 1993;90:10350–10354.
27. Praml C, Savelyeva L, Perri P, Schwab M. Cloning of the human aflatoxin B1-aldehyde reductase gene at 1p35-1p36.1 in a region frequently altered in human tumor cells. Cancer Res 1998;58:5014–5018.
28. Klose J. Fractionated extraction of total tissue proteins from mouse and human for 2-D electrophoresis. Methods Mol Biol 1999;112:67–85.
29. Klose J, Kobalz U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis 1995;16:1034–1059.
30. Anderson NL, Anderson NG. Analytical techniques for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: multiple gradient-slab gel electrophoresis. Anal Biochem 1978;85:341–354.
31. Anderson NG, Anderson NL. Analytical techniques for cell fractions. XXI. Two-dimensional analysis of serum and tissue proteins: multiple isoelectric focusing. Anal Biochem 1978;85:331–340.
32. Zeindl-Eberhart E, Rabes HM. Variant protein patterns in hepatomas and transformed liver cell lines as determined by high resolution two-dimensional gel electrophoresis (2DE). Carcinogenesis 1992;13:1177–1183.
33. Jungblut PR, Seifert R. Analysis by high-resolution two-dimensional electrophoresis of differentiation-dependent alterations in cytosolic protein pattern of HL-60 leukemic cells. J Biochem Biophys Methods 1990;21:47–58.
34. Lamer S, Jungblut PR. Matrix-assisted laser desorption-ionization mass spectrometry peptide mass fingerprinting for proteome analysis: identification efficiency after on-blot or in-gel digestion with and without desalting procedures. J Chromatogr B Biomed Sci Appl 2001;752:311–322.
35. Zeindl-Eberhart E, Jungblut PR, Rabes HM. A new method to assign immunodetected spots in the complex two-dimensional electrophoresis pattern. Electrophoresis 1997;18:799–801.
36. Carper DA, Wistow G, Nishimura C, Graham C, Watanabe K, Fujii Y, Hayashi O, et al. A superfamily of NADPH-dependent reductases in eukaryotes and prokaryotes. Exp Eye Res 1989;49:377–388.
37. Hyndman DJ, Flynn TG. Sequence and expression levels in human tissues of a new member of the aldo-keto reductase family. Biochim Biophys Acta 1998;1399:198–202.
38. Hyndman D, Flynn TG. The aldo-keto reductases and their role in cancer. Adv Exp Med Biol 1999;463:427–434.
39. Alaiya AA, Franzen B, Auer G, Linder S. Cancer proteomics: from identification of novel markers to creation of artificial learning models for tumor classification. Electrophoresis 2000;21:1210–1217.
40. Takahashi M, Fujii J, Miyoshi E, Hoshi A, Taniguchi N. Elevation of aldose reductase gene expression in rat primary hepatoma and hepatoma cell lines: implication in detoxification of cytotoxic aldehydes. Int J Cancer 1995;62:749–754.
41. Tsuchida S, Sato K. Glutathione transferases and cancer. Crit Rev Biochem Molec Biol 1992;27:337–384.
42. Murata M, Ohta N, Sakurai S, Alam S, Tsai J, Kador PF, Sato S. The role of aldose reductase in sugar cataract formation: aldose reductase plays a key role in lens epithelial cell death (apoptosis). Chem Biol Interact 2001;130–132:617–625.
43. Svanberg B, Ling C, Svensson PA, Johnson M, Carlsson B, Billig H. Isolation of differentially expressed aldose reductase in ovaries after estrogen withdrawal from hypophysectomized diethylstilbestrol treated rats: increased expression during apoptosis. Mol Cell Endocrinol 2000;164:183–190.
44. Bannasch P. Pathogenesis of hepatocellular carcinoma: sequential cellular, molecular, and metabolic changes. Prog Liver Dis 1996;14:161–197.
45. Jeffery J, Jörnvall H. Enzyme relationships in a sorbitol pathway that bypasses glycolysis and pentose phosphates in glucose metabolism. Proc Natl Acad Sci U S A 1983;80:901–905.
46. Stevens MJ, Henry DN, Thomas TP, Killen PD, Greene DA. Aldose reductase gene expression and osmotic dysregulation in cultured human retinal pigment epithelial cells. Am J Physiol 1993;265:E428–438.
47. O'connor T, Ireland LS, Harrison DJ, Hayes JD. Major differences exist in the function and tissue-specific expression of human aflatoxin B1 aldehyde reductase and the principal human aldo-keto reductase AKR1 family members. Biochem J 1999;343:487–504.
48. Lee KW, Ko BC, Jiang Z, Cao D, Chung SS. Overexpression of aldose reductase in liver cancers may contribute to drug resistance. Anticancer Drugs 2001;12:129–132.
49. Lee EK, Regenold WT, Shapiro P. Inhibition of aldose reductase enhances HeLa cell sensitivity to chemotherapeutic drugs and involves activation of extracellular signal-regulated kinases. Anticancer Drugs 2002;13:859–868.