Differential proteomic analysis of hepatocellular carcinoma

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Abstract. The principal aim of the present study consisted in the identification of the disregulated proteins associated with the development of hepatocellular carcinoma (HCC). The differences in protein expression between hepatocellular carcinoma (HCC) and the corresponding non-HCC liver tissues were investigated in a cohort of 20 patients using two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) coupled with mass spectrometry (MS). The up- and down-regulated protein spots that exhibited 1.5-fold difference signal intensity with statistical significance ($p<0.05$, t-test, confidence intervals 95%) were excised from the gel and identified by peptide mass fingerprinting using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Thirty-six protein spots corresponding to 29 different disregulated proteins, belonging to heterogeneous metabolic pathways, have been identified. Down-regulated proteins (n=23) were found superior in number than the up-regulated proteins (n=6). Detoxification, carbohydrate metabolism and amino acid biotrasformation represented the main disregulated pathways in HCC. Up-regulation of aldo-keto reductase 1C2, thioredoxin and transketolase, involved in metabolic and regulatory cellular processes including proliferation, differentiation and carcinogenesis were remarkable. These proteins could represent useful biomarkers to provide new insights into global pathophysiologic changes of HCC and for the development of new pharmacological approaches to HCC therapy.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third deadliest neoplastic disease with nearly one million deaths per year (1). The incidence of HCC has a wide geographic variability with peaks in Asia and sub-Saharan Africa but over the last two decades a substantial increase was reported in Europe and North America (2). Liver cirrhosis degeneration, chronic and persistent infection of hepatitis B (HBV) and hepatitis C virus (HCV), aflatoxin B1 exposure, alcohol drinking or genetic defects like hemochromatosis are the most important etiological factors (3). Although there are many options to treat HCC, the patients outcome is poor. Surgical resection and liver transplantation, if applicable, are the standard treatment and the only options that offer cure or long-term survival (4,5). However, most patients at the time of diagnosis present an advanced stage of the (disease): this precludes local ablative or surgical interventions. For patients with non-resectable HCC, pharmacological treatment protocols can be used but the impact on overall survival, even with the new targeted therapy, is very low (4). Further advances in our understanding of the molecular basis of HCC development are needed to identify both biomarkers for early diagnosis and novel potential pharmacological targets. Recently, different systematic approaches have been reported to study HCC at genetic, transcriptional and translational levels (6-8). The proteomic-profiling investigation offers a unique advantage allowing the delineation of global changes in expression patterns resulting from transcriptional and post-transcriptional control and post-translational modifications. The transformation of a healthy cell into a neoplastic cell can be defined as an altered flow of the functional information carried by the proteome. Thus, the identification of changes in protein expression may correlate with the appearance of the tumor and may reveal important molecular information on HCC pathogenetic mechanisms.

In order to increase our knowledge on the protein signatures that characterize HCC disease, in this study we have investigated the proteome expression changes between tumor (HCC) and distal normal liver tissues (non-HCC) in 20 Caucasian HCC patients who underwent resection and/or orthotopic liver transplant by using two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) technique combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). This study confirmed most of the previously found protein HCC biomarkers. In addition, we identified novel differentially expressed proteins that may prove useful for diagnosis and prognosis of HCC as well as for pharmacological applications.

Patients and methods

Patients and tissue collection. Tissue samples were obtained from 20 patients who underwent surgical liver resection or orthotopic liver transplant for HCC at the Department of Surgery and Transplantation Unit of the University of Udine, Italy and of the University of Torino, Italy. Matched sets of neoplastic HCC and of normal liver non-HCC tissue, distal from tumor site, were obtained during surgery under supervision of a qualified pathologist and immediately frozen at -80˚C until processing. All patients were thoroughly informed about the study and gave written consent for the investigation in accordance with the ethical guidelines of the Local Ethics Committee.

Sample preparation. A tissues sample of 100 mg was suspended and homogenated in 5 volumes of ice-cold lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) containing 4% (w/v) PMSF and protease inhibitor cocktail (GE Healthcare Bio-Sciences, Uppsala, Sweden). The homogenate was centrifuged twice for 30 min at 2000 g at 4˚C to remove the nuclei fraction and the supernatant proteins were precipitated with cold acetone overnight at -20˚C. The protein pellet was vacuum-dried and solubilized in 50 μl of denaturating buffer [7.0 M urea, 2.0 M thiourea, 4% (w/v) CHAPS, 10 mM DTT, 30 mM Tris-HCl and protease inhibitor cocktail] and immediately stored in aliquots at -80˚C before use to perform 2-dimensional polyacrylamide gel electrophoresis (2-DE). Protein concentration of samples was measured using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA).

DIGE analysis and study design. One of the key points of proteomic investigations is represented by the quantitative protein expression analysis. This requires specific technology able to efficiently provide accurate and reproducible separation and quantification of the proteins present in biological samples. A modification of conventional 2-DE is represented by the 2D-DIGE developed by Unlu *et al* (9) in which multiple protein samples are pre-labeled with different fluorescence dyes (Cy2, Cy3 and Cy5), mixed together and run on a 2D gel. The co-separation of different samples on the same gel has the evident advantage to reduce the number of gels and to eliminate the problems associated with the inter-gel variation. Finally, the use of an internal standard run on the background of all gels also facilitates gel-to-gel spot matching and allows powerful statistically reliable comparisons of protein abundance among tumor and control samples. For the design of the present study we used as internal standard a protein sample obtained by the mix of equal amounts of all protein extracts derived from 20 patients (n=40 total samples tissue) labeled with Cy2 dye. The HCC and non-HCC samples were labeled with Cy3 and Cy5 dyes respectively. In order to take into account the different cyanine reactivity all samples were also labeled using the dye swap approach (Cy3 and Cy5 switched).

Protein labeling. The extracted protein fractions from HCC and non-HCC tissues (50 μg) were pH adjusted to pH 8.5 by 50 mM NaOH. Then proteins were labeled with the three different N-hydroxysuccinimide cyanine dyes reconstituted in anhydrous dimethylformamide at a ratio of 100 pmol CyDye per 25 μg of protein (30 min on ice). The reaction was stopped by adding 1 μl of 10 mM lysine for 10 min on ice.

DIGE analysis. Twenty-five micrograms of Cy3- and Cy5-labeled samples from each patient were combined before mixing with 25 μg Cy2-labeled internal standard and the total volume of the sample was made up to 200 μl with rehydration buffer (7.0 M urea, 2.0 M thiourea, 4% (w/v) CHAPS, 2% (w/v) Bio-lyte, 40 mM DTT). Samples were actively rehydrated into 11 cm 3-10 immobilized non-linear pH gradient (IPG) strips (Bio-Rad) at 20˚C for 12 h using a Protean IEF Cell (Bio-Rad). Isoelectric focusing was performed for a total of 46 kVh (ramp to 100 V in 2 h, ramp to 500 V in 1 h, hold at 1000 V for 2 h, ramp to 8000 V in 5 h, hold at 8000 V for 3 h). The IPG strips were conditioned in equilibration buffer (6.0 M urea, 2% (w/v) SDS, 50 mM Tris-Cl, pH 8.8, 30% (v/v) glycerol) supplemented with 1% (w/v) DTT for 15 min at room temperature, followed by 2.5% (w/v) iodoacetamide in equilibration buffer for another 15 min incubation at room temperature. IPG strips were placed on the top of 8-16% gradient polyacrylamide pre-cast Criterion gels (Bio-Rad).

DIGE quantification analysis. After 2-DE, gels were scanned on the Typhoon Trio scanner (GE Healthcare Bio-Sciences) using excitation/emission wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm) and Cy5 (633/670 nm). The PMT voltage intensity was adjusted to ensure that pixel intensity of each image was within 60000-90000. Quantitative differential expression analysis was performed by DeCyder™ 6.5 software (GE Healthcare Bio-Sciences). Scanned images of fluorescently labeled proteins were sequentially analyzed by differential in-gel analysis (DIA-module) during which the Cy5:Cy2 and Cy3:Cy2 normalization of protein spot was performed. The Log abundance ratios of each protein spot were then compared between HCC and non-HCC from all gels by Biological Variation Analysis (BVA-module). Due to an intrinsic variability associated to the patient peculiarities and the tissue heterogeneity, we chose a stringent criterion: thus we evaluated only those protein spots present in the 80% of the paired samples investigated with intensity differences between HCC and non-HCC greater than 1.5-fold and

Table I. Patient characteristics.

Characteristic	n (%)
No. of patients	20
Gender	
Male	19 (95)
Female	1 (5)
Race	
Caucasian	20 (100)
Age, years (median, range)	63.5 (53-82)
Serology	
Negative	5 (25)
HCV+	8 (40)
HBV+	7 (35)
Cirrhosis	10 (50)
Clinical intervention	
Surgical excision	6 (30)
Transplant	8 (40)
OLT[a]	6 (30)

[a]OLT, orthotopic liver transplantation.

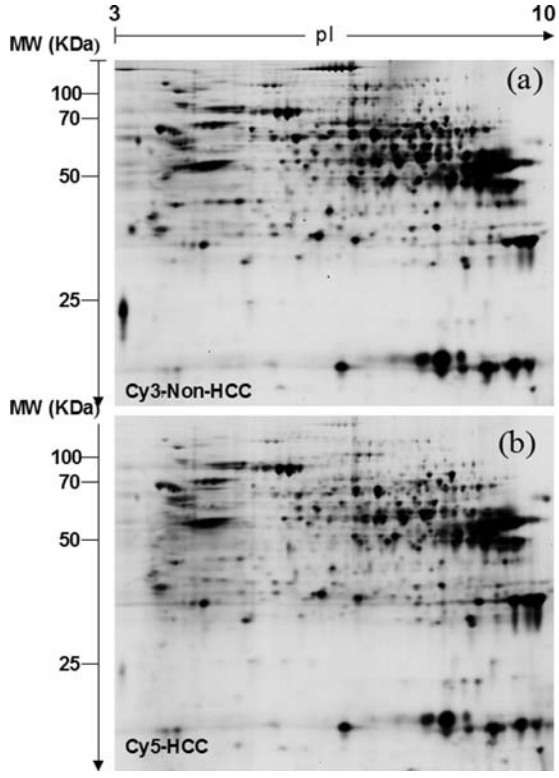

Figure 1. Representative results of analytical 2D-DIGE maps for non-HCC tissue labelled with Cy3 (a) and the corresponding HCC tissue labelled with Cy5 (b). The intensity of Cy3 -non-HCC and Cy5-HCC spots in the 2D map was referred to the intensity of the corresponding spots in the mixed-sample internal standard labelled with Cy2 dye running in the same experiment.

confidence intervals of at least 95% with statistical significance ($p<0.05$), by paired Student's t-test. The statistical significance of the difference in protein expression among more than two groups was assessed by analysis of variance by ANOVA test.

Mass spectrometry protein identification. The proteins that based on the previous quantitative expression analysis resulted differentially expressed in HCC as compared to non-HCC tissues (Student's t-test $p<0.05$) were identified by MALDI-TOF MS analysis. A preparative gel containing 200 μg of pooled protein extracts was prepared and stained with colloidal coomassie G250 (Bio-Rad). It was scanned and protein spots of interest were precisely positioned by software assisted matching between preparative and analytical gel spot pattern. These protein spots were than excised from the gel and destained with 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile following overnight in-gel trypsin digestion. The tryptic peptides were extracted with 1% (v/v) TFA, purified with ZipTip Pipette Tips (Millipore, Billerica MA, USA) and analyzed by MALDI-TOF MS on a Voyager-De-Pro Biospectrometry Workstation (Applied Biosystems-MDS/Analytical Technologies, Foster City, CA, USA). Peptide mass spectra were processed using Data Explorer version 5.1 software (Applied Biosystems). The NCBInr and Swiss Prot were used as the protein sequence databases, to produce a standardized probabilistic measure of confidence, limiting the search to human proteins, allowing for one trypsin missed cleavage and with a 150 parts per million (ppm) mass tolerance error. The fixed modification selected was cysteine carbamidomethylation, while the variable modification selected was the methionine oxidation. We included only protein identifications with a Mowse (Molecular Weight Search) score >66 ($p<0.05$).

Western blot analysis. Cytoplasmatic samples (50 μg) from pooled normal (C2) and tumor tissues (NT) were separated by SDS-PAGE with 4-20% gradient polyacrylamide gel. The proteins were transferred to nitrocellulose membranes and blotted with antibodies against Glutathione S transferase A1 (GST-A1) (1:250 dilution, Abnova GmbH, Heidelberg Germany), Casein kinase 1 ε isoform (CK1ε) (Dil 1:250, Tebu-Bio SrL, Milan, Italy) and thioredoxin (TRX) (1:200 dilution, dilution Santa Cruz Biotechnology, Heidelberg, Germany). Vinculin, detected by monoclonal antibody at 1:500 dilution (Sigma-Aldrich, Milan, Italy), was used as a house-keeping protein. The signal on the blots was detected with an ECL (GE Healthcare Bio-Sciences) using a GS800 densitometer (Bio-Rad) image analyzer.

Results

During the period 2005-2007 twenty patients with histologic diagnosis of primary HCC who underwent surgery were enrolled for this proteomic study. None of the 20 patients had received any prior anticancer treatment before surgery. The demographic and clinical characteristics of the patients investigated are reported in Table I. Ten patients presented HCC on liver cirrhosis, 8 and 7 presented HCV and HBV serology, respectively; none of the patients was positive for both HCV and HBV. In Fig. 1 a typical HCC/non-HCC liver 2D-DIGE

Table II. Differentially expressed proteins between HCC and non-HCC tissues as identified by peptide mass fingerprinting.

Spot	Fold change	Protein description	Accession no.	MW	IP	Matching peptides	Coverage %
659	+2.32	Aldo-keto reductase family 1 member C2	P52895	36735	7.14	14	49
312	+1.80	Transketolase	P29401	67877	7.58	14	26
1318	+1.79	Thioredoxin	P10599	11606	4.81	5	43
1178, 1180	+1.56	Peptidyl-prolyl cis-trans isomerase A	P62937	17881	7.83	8, 8	47, 39
1309	+1.55	Fatty acid-binding protein liver	P07148	14208	6.60	11	65
389	+1.55	Protein disulfide-isomerase	P0723	55293	4.67	15	36
516	-2.36	Glutamate dehydrogenase 1	P00367	56008	6.71	23	37
635, 527, 515	-2.36	Betaine-homocysteine S-methyltransferase 1	Q93088	44998	6.59	21, 7, 15	49, 20, 39
520	-2.02	Argininosuccinate synthase	P00966	46530	8.08	13	26
612, 621, 617, 613	-1.96	Fructose-bisphosphate aldolase B	P05062	39341	8.07	13, 11, 8, 14	29, 25, 17, 30
595	-1.78	Selenium-binding protein 1	Q13228	52312	6.13	12	32
448	-1.70	Casein kinase I isoform epsilon	P49674	47314	9.68	5	19
629	-1.59	Fructose-1,6-bisphosphatase 1	P09467	36683	6.60	20	42
866	-1.57	Enoyl-CoA hydratase	P30084	28342	5.88	6	26
410, 417	-1.56	UTP-glucose-1-phosphate uridylyltransferase 2	Q16851	56808	8.14	22, 12	45, 28
279	-1.56	78 kDa glucose-regulated protein	P11021	70477	5.01	18	35
285	-1.56	Heat shock cognate 71 kDa protein	P11142	70897	5.38	20	33
918	-1.55	Glutathione S-transferase A2	P09210	25532	8.54	10	29
457	-1.54	Calreticulin	P27797	46466	4.29	8	24
352	-1.53	Dihydroxyacetone kinase	Q3LXA3	58976	7.13	13	28
573	-1.52	Aspartate aminotransferase, cytoplasmic	P17174	46116	6.57	16	42
915	-1.52	Glutathione S-transferase A1	P08263	25500	8.92	13	40
555	-1.51	Isocitrate dehydrogenase [NADP] cytoplasmic	O75874	46659	6.54	17	48
712	-1.51	Bile salt sulfotransferase	Q06520	33648	5.71	9	36
770	-1.51	Annexin A4	P09525	35751	5.85	20	47
424, 425	-1.51	Retinal dehydrogenase 1	P00352	54730	6.30	16, 16	35, 33
357	-1.50	Catalase	P04040	59624	???	13	30
451	-1.50	Aldehyde dehydrogenase	P05091	54444	5.69	15	33
551	-1.50	Glutamine synthetase	P15104	41933	6.41	12	23

experiment is reported where we highlighted over 1500 protein spots. Decyder software DIGE Image comparison tracked a total of 480 protein spots that matched the 80% of the DIGE gels from HCC and non-HCC samples. After statistical analysis of the normalized quantities of matched protein spots, the BVA software module detected 82 spots whose intensity changed significantly between HCC and non-HCC samples ($p<0.05$, confidence interval 95%). Sixty-two of these proteins showing more than 1.5 fold change were selected for the protein identification step by running preparative gels of pooled tissue samples. The matching between analytical and preparative gel resulted confident for 44 of the 62 selected protein spots (71%) (Fig. 2). The protein identification by MALDI-TOF MS fingerprint analysis was successful in 37 of the 44 excised proteins spots. A comprehensive list of identified proteins with the relative expression ratio between HCC and non-HCC is reported in Table II. Some

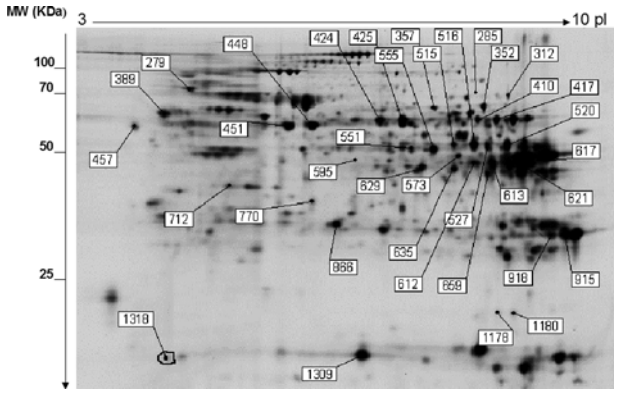

Figure 2. Preparative 2D-PAGE map. The spots evidenced by a number are those resulting from the matching between preparative and analytical gel protein maps. The protein spots of interest were excised from the gel, digested with trypsin and identified by MALDI-TOF MS peptide mass fingerprinting.

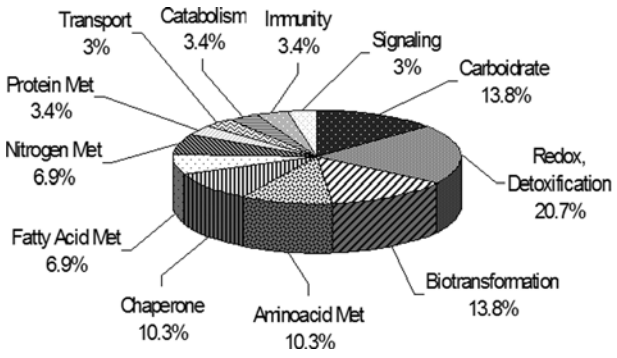

Figure 3. Functional classification of the 29 differential expressed proteins identified in HCC.

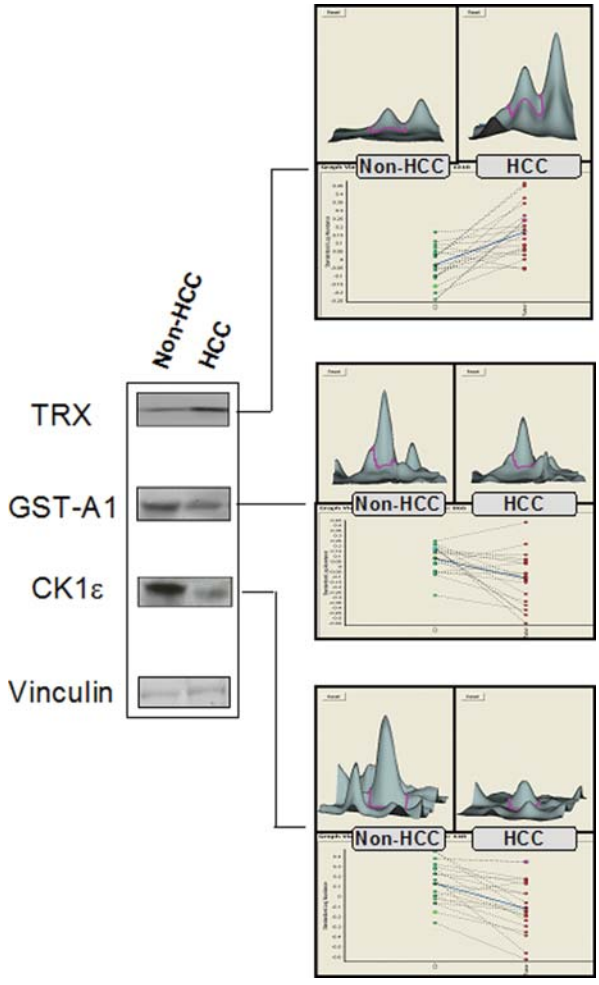

Figure 4. Western blotting and DIGE comparison for TXR,GSTA1 and CK1ε expression. On the left we reported the Western blot expression of proteins in HCC and non-HCC pooled extracts tissues together with the vinculine expression used as housekeeping protein. For each protein on the upper right we report the pair of pixel Cy2 normalized volume intensity of Cy3 and Cy5 fluorescence as a representative sample. While on the lower right the intensity distribution across all 20 patients investigated is reported.

proteins were found as multiple spots: peptidyl-prolyl cis-trans isomerase A (n=2), betaine-homocysteine S-methyl-transferase 1 (n=3), fructose-bisphosphate aldolase B (n=2) and UDP-glucose-1-phosphate uridylyltransferase 2 (n=2). Such redundancy is likely due to proteolysis and post-transational modifications as well as to the conformational equilibrium of proteins during electrophoresis (10). The HCC/non-HCC expression ratio of all multiple spots referred to the same protein showed concordance in the extent and direction of variation. The identified up- and down-regulated proteins in HCC belong to heterogeneous metabolic pathways according by their Gene Ontology Consortium classification (http://www.geneontology.org/index.shtml) (Fig. 3). Only 6 out of the 29 (21%) identified protein spots resulted over-expressed in HCC tissues while the remaining 23 (79%) were down-regulated. When ANOVA test was applied to HCV and HBV subgroup analysis the high inter-patients variability of the protein expression profile and the small number of patients probably prevented reaching statistical significance. The 2D-DIGE differential expression results were confirmed by Western blot analysis on HCC and non-HCC pooled extract tissues. In Fig. 4 a representative result for TRX, GSTA1 and CK1ε is reported. Overall the extent of different protein expression as investigated by immunoblotting confirmed the 2D-DIGE results.

Discussion

In this study we reported the most abundant proteins out of the whole metabolic alterations that characterize HCC. Network pathways are often difficult to establish because of the biochemical noise associated to tissues heterogeneity and inter-patient variation. This late feature also limited the possibility to statistically differentiate protein patterns associated with HBV- and/or HCV-positive HCC cases. Thus, the differentially expressed proteins identified in this study could be considered as common features of HCC tumor development and progression not specifically associated to HCV or HBV. In spite of these limitations we underlined that the major disregulated biochemical pathways in HCC involve carbohydrate metabolism and detoxification oxido-reduction processes in agreement with most of the previous proteomic and transcriptional investigations review by Su *et al* (11).

Among carbohydrate metabolism, the fructose-bis-phosphate aldolase B enzymes underwent the highest down-regulation in HCC followed by fructose-1,6-bisphosphatase (Table II). The down-regulation of glucose metabolism in HCC appears in contrast to what is generally observed in almost all cancers where an increment of anaerobic glycolysis activity is instead commonly reported as 'Warburg effect' (12). Hypothetically, a down-regulation of fructose-bisphosphate aldolase B, which is involved in the conversion of fructose-1,6-bisphospate to glyceraldehyde 3-phosphate and dihydroxy-acetone phosphate, would hamper glucose utilization and reduce tumor growth. This conclusion is apparently in contrast with the recent findings that associate the fructose-bisphosphate aldolase B down-regulation with a poor differentiated high grade and aggressive stages of HCC (13). This discrepancy suggests that HCC may use alternative metabolic pathways as source of energy for tumor growth. In the same context we reported a significant up-regulation of transketolase which is also involved in the control of glucose degradation through the non-oxidative pentose phosphate pathway. The high transketolase expression in HCC may imply a strong

activation of this alternate pathway to glycolysis in the utilization of cellular glucose. The *in vitro* suppression of the transketolase gene by using a silencing approach effectively arrested cellular growth in human hepatoma cells (HepG2), suggesting a possible role in HCC development (14). The metabolic control of the non-oxidative pentose phosphate pathway by inhibition of transketolase may constitute a promising novel pharmacological target to be explored.

A consistent group of proteins significantly down-regulated in HCC are directly or indirectly involved in the detoxification pathways of endogenous and exogenous reactive molecular species, including alcohol metabolites: aldehyde dehydrogenase II, selenium-binding protein 1, GSTA1, glutathione S-transferase A2 isoforms (GSTA2) and catalase (Table II). The decreased levels of these proteins may lead to a reduced detoxification capability, which is a probable consequence to transcriptional factors secondary to HCC development. The selenium-binding protein 1 which is indirectly associated with the detoxification pathways was found to be significantly down-regulated in HCC. The cellular importance of this protein seems associated to its capacity to store selenium which is a key element for the function of many detoxification enzymes. The reduced expression of selenium-binding protein 1 in HCC may result significant because it directly impacts on the ability of selenium to regulate cell function and growth of HCC as already been reported for ovarian, colon and lung carcinoma (15).

The high level of cellular oxidant and reactive oxygen species that can arise from the down-regulation of the detoxification enzymes observed in HCC may be associated with the high expression of AKR1C2 whose transcription is dependent on a 5'-antioxidant responsive element activated by NRF2 (16).

In addition to detoxification enzymes we also reported down-expression of CK1ε further validated by Western blot analysis (Fig. 4). This protein is known to phosphorylate protein kinase D2; active protein kinase D2 is trapped in the nucleus by CK1ε-induced phosphorylation leading to derepression of the apoptosis-inducing Nur77 (17). For the first time the present finding reports a CK1ε reduction in HCC suggesting a decreased pro-apoptotic activity of Nur77 that may in turn promote malignant hepatocyte proliferation.

Gene expression and cellular growth are regulated by the cellular redox status (18). The up-regulation of TRX as well as the down-regulation of GSTA1 emerging from DIGE experiment and validated by Western blot analysis can reflect a disregulation of the cellular redox status which is balanced by the endogeneous thiols buffer system mainly from glutathione and TRX sources (19). Remarkably, high expression of TRX as evaluated by immunohistochemistry in HCC biopsies was recently reported (20). Moreover, higher serum levels of TRX were found in patients with HCC as compared with patients with chronic hepatitis or liver cirrhosis (21). These findings support a possible key role for TRX in HCC development and progression where it may represent a potential biomarker. In addition to protection against oxidative stress, TRX has also been involved in anti-apoptotic functions and growth factor and cytokine effects (22). These latter features contribute to make TRX a potential pharmacological target for HCC. It could also be a therapeutical approach that may take advantage from the development of selective TRX inhibitors which are currently under phase I clinical investigation (23).

In conclusion, our results shed light on few specific HCC metabolic signatures involving the carbohydrate and detoxification metabolism. Specifically, transketolase and TRX over-expression could represent new potential biomarkers as well as novel pharmacological targets.

Acknowledgements

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