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CHARACTERIZATION OF HUH6, HEP3B, HEPG2 AND HLE LIVER CANCER CELL LINES BY WNT/□- CATENIN PATHWAY, MICRORNA EXPRESSION AND PROTEIN EXPRESSION PROFILE

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Abstract – Somatic mutations in the genes members of WNT/β-catenin pathway, especially in *CTNNB1* codifying for β-catenin, have been found to play an important role in hepatocarcinogenesis. The purpose of this work is to characterize alterations of the WNT/β-catenin signalling pathway, and to study the expression pattern of a panel of microRNAs and proteins potentially involved in the pathogenesis of liver cancer. In this respect, the molecular characterization of the most used liver cancer cell lines HuH6, Hep3B, HepG2, and HLE, could represent a useful tool to identify novel molecular markers for hepatic tumour. A significant modulation of *FZD7*, *NLK*, *RHOU*, *SOX17*, *TCF7L2*, *TLE1*, *SLC9A3R1* and *WNT10A* transcripts was observed in all the four liver cancer cell lines. The analysis of selected microRNAs showed that miR-122a, miR-125a and miR-150 could be suitable candidates to discriminate tumoural versus normal human primary hepatocytes. Finally, Grb-2 protein expression resulted to be increased more than two-fold in liver cancer cell lines in comparison to normal human primary hepatocytes. These advances in the knowledge of molecular mechanisms involved in the pathogenesis of liver cancer may provide new potential biomarkers and molecular targets for the diagnosis and therapy.

Key words: Hepatoblastoma; Hepatocellular carcinoma; Molecular markers; WNT pathway; *CTNNB1*.

INTRODUCTION

Hepatoblastoma (HB) and hepatocellular carcinoma (HCC) are liver malignancies accounting for up to 96% of total liver tumours in humans, representing one of the major cause of death by cancer worldwide (*64,73*). HB is a very rare primary liver malignancy of infants and young children, likely originating from liver stem cells (*71*). Its annual incidence is estimated at 0.5–1.5 diagnoses per 1 million children below the age of 15 years in Western countries (*73,79*). HCC is one of the most common cancer ranking at the eighth level for incidence among human cancers in Asia, Africa and South Europe, causing about 1 million deaths annually (*53*). The HCC etiological factors are mostly related to infections by hepatitis B and C viruses (HBV and HCV, respectively), environmental exposure to natural carcinogens as aflatoxin B1 (AFB1), and life-style factors including cigarette smoking and alcohol intake (*77*).

The WNT/β-catenin signalling pathway, also named WNT/Frizzled (FZD) signalling cascade, is important for the determination of the cell fate during the embryonic development, as

Abbreviations: AFB1: aflatoxin B1; **AML:** acute myeloid leukaemia; **APC:** adenomatous polypolis coli; **AXIN:** axis inhibitor; **CDK:** cyclin-dependent kinase; **CK1:** casein kinase 1; **Cy3:** indocarbocyanine; **Cy5:** indodicarbocyanine; **DKK:** Dickkopf; **DVL:** Dishevelled; **EGF:** Epidermal Growth Factor; **FZD:** Frizzled; **GSK3β:** glycogen synthase kinase 3β; **HB:** hepatoblastoma; **HBV:** hepatitis B virus ; **HCC:** hepatocellular carcinoma; **HCV:** hepatitis C virus; **HGF:** Hepatocyte Growth Factor; **HGM:** high-mobility-group; **IGF:** Insulin-like Growth Factor; **ITF-2:** immunoglobulin transcription factor-2; **LPR:** lipoprotein receptor-related proteins; **miR:** microRNA; **MMP-7:** matrix metalloproteinase-7; **NFAT:** nuclear factor of activated T cells; **NLK:** Nemo-like kinase; **qPCR:** quantitative real-time RT-PCR; **TCF/LEF:** T-cell factor/lymphoid enhancer factor; **TGFα:** Transforming Growth Factor α; **TGFβ:** Transforming Growth Factor β; **uPAR:** urokinase-type plasminogen activator receptor.

well as in maintaining tissue homeostasis in the adult (*36*,*38*,*49*). WNT signals are transduced to the nucleus through two possible pathways (Fig. 1). The canonical WNT/□ β-catenin signalling pathway is initiated by the binding of WNT ligands to the transmembrane receptors. The resulting signals prevent β-catenin phosphorylation by a multiprotein complex composed by adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase 1 (CK1), and axis inhibitors (AXINs), and its subsequent proteosomal degradation (*3*, *14*, *63*). Nuclear β-catenin is then complexed with the T-cell factor/lymphoid enhancer factor (TCF/LEF) to activate the transcription of target genes (*10*,*27*,*37*,*44*,*70*,*76*,*88*). As observed in several types of tumours, the aberrant activation of the canonical WNT/β-catenin signalling pathway is an important contributor to tumourigenesis (*69*,*84*). On the contrary, the non canonical WNT signals are transduced through FZD family receptors and co-receptors (*55*,*56*,*67*) to either the Dishevelled (DVL)-dependent (*i.e.*, RHOA, RHOU, RAC, CDC42, and JNK (*4*,*87*)), or to the Nemo-like kinase (NLK) and nuclear factor of activated T cells (NFAT) (*21*,*31*) signalling cascades (Fig. 1). NLK is a serine/threonine kinase that suppresses the transcription activity of the β-catenin/TCF complex through the phosphorylation of TCF/LEF family transcription factors, thus inhibiting the non canonical WNT signalling pathway (*31*).

Approximately 80% of HBs and 20% of HCCs are characterized by somatic mutations in *CTNNB1*, which codifies for β-catenin (*41*,*65*,*94*,*95*). Recently, mutations in *AXIN1* and *AXIN2* have been demonstrated to be important in an additional 10% of HBs and HCCs, respectively (*86*). Note that an altered expression of specific WNT/β-catenin target genes, playing key roles in proliferation and survival of cancer cells, has been reported (*82*,*88*). There is increasing evidence that regulatory mechanisms different from mutations either in *CTNNB1* or in proteins involved in the maintenance of β-catenin stability may play a major role in hepatocarcinogenesis. Despite the identification of several target genes of the β-catenin/TCF transcription complex (*e.g.*, *c-MYC* (*27*,*49*,*82*), *cyclin D1* (*27*,*82*,*88*), *AXIN2* (*33*,*57*), *matrix metalloproteinase-7* (*MMP-7*; (*15*)), *FRA-1*, *c-JUN*, *urokinase-type plasminogen activator receptor* (*uPAR*; (*59*)), and *immunoglobulin transcription factor-2* (*ITF-2*; (*43*))), their role in liver cancer development is still not clear. Moreover, there is a robust evidence that an important role in hepatocarcinogenesis is also played by a dysregulated signalling induced by pro-tumorigenic growth factors, such as insulin-like growth factor (IGF), hepatocyte growth factor (HGF), transforming growth factor α (TGFα)/epidermal growth factor (EGF) and transforming growth factor β (TGFβ) signalling (*6*).

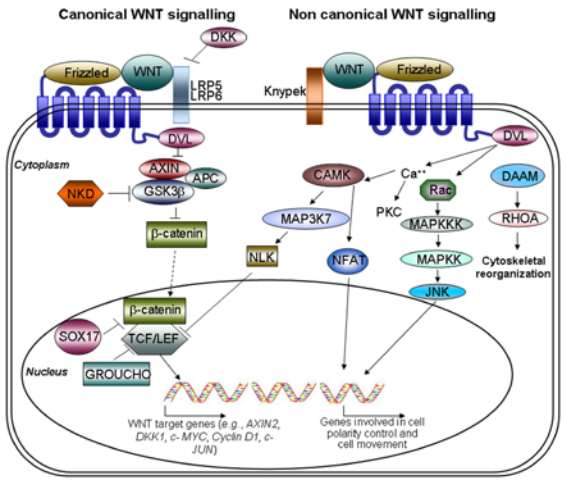

Figure 1. WNT signalling cascades. WNT signals are transduced to the canonical Wnt pathway for cell fate determination, and to the non canonical WNT pathway for control of cell movement and tissue polarity. Canonical WNT signals are transduced through FZD family receptors and LRP5/LRP6 co-receptor to the β-catenin signalling cascade. Non canonical WNT signals are transduced through FZD family receptors and the Knypek co-receptor to the DVL-dependent or the Ca^{2+}-dependent (NLK and NFAT) signalling cascades. Both pathways promote the transcription of a specific set of genes involved in cell proliferation, cell polarity control and cell motility. For details, see text.

MicroRNAs (miRs), an abundant class of non-coding small RNAs composed of approximately 21-26 nucleotides, have been shown to play significant roles in several cellular and pathogenic processes, including cellular development, immunological response, and carcinogenesis (*1*,*13*,*16*,*48*). MicroRNAs can modulate gene expression at a post-transcriptional level, by binding sequences located at the 3' UTR of specific mRNAs (*16*,*47*,*50*). MicroRNAs can target oncogenes or tumour suppressor genes, contributing to the initiation and progression of many human cancers (*24*,*54*). For instance, miR-15a and miR-16 expression have been associated to chronic lymphocytic leukaemia (*8*), miR-145 to colon, lung, breast and prostate cancer (*30*,*96*), and

miR-21 to glioblastoma (*11*). Moreover, it has been demonstrated that some microRNAs (*i.e.*, miR-21, miR-122a, miR-125a, miR-145, miR-150, miR-199a and miR-214) are down-regulated in HCC, whilst other (*i.e.*, miR-21 and miR-148a) are up-regulated (*46*,*89*). Interestingly, miR-122 expression is limited to the human liver, where it constitutes about 70% of the total microRNA content. In the absence of miR-122, liver functions and cholesterol levels are consistently compromised, probably because miR-122 regulates the expression of genes involved in cholesterol biosynthesis (*23*,*45*). In HCV infection miR-122 is required for efficient viral RNA expression: indeed, the virus can replicate in miR-122 expressing cells, such as HuH7 liver carcinoma cells, but not in HepG2, which does not express miR-122 (*35*). We recently demonstrated that different microRNAs are able to discriminate HB from HCC, as well as tumour versus non tumour tissues (*58*).

Even if alterations of the WNT/β-catenin pathway are recurrent in HB and HCC, molecular markers for early diagnosis and prognosis are still inadequate. So far, many studies on misregulation of the WNT/β-catenin target genes and mRNAs expression have been conducted on colorectal tumours and ovarian endometrioid adenocarcinomas (*42*).

Here, we present data on a pilot comparative molecular characterization of commercially available HB-like and HCC-like cancer cell lines at gene, microRNA and protein expression levels. A comparative molecular characterization of the most used liver cancer cell lines may represent a useful basis for both those researchers who deal with such biological material, and to gain new insights in the pathogenesis of HB and HCC, providing novel biomarkers for these types of tumours.

MATERIALS AND METHODS

Cell lines

HepG2 and Hep3B cell lines (ATCC, Middlesex, UK) were cultured in Eagle medium (PBI International, Italy), containing 15% foetal bovine serum (Lonza, Milan, Italy), 100 µg/ml penicillin and 100 µg/ml streptomycin (Biological Industries, Israel). HLE and HuH6 cell lines (Riken Bioresource Centre Cell Bank, Ibaraki, Japan) were grown in Dulbecco Modified Eagle's medium (PBI International) supplemented with 15% foetal bovine serum, 2mM L-glutamine (Biological Industries), 100 µg/ml penicillin and 100 µg/ml streptomycin.

Human primary hepatocytes (Lonza) were rapidly thawed and resuspended at a cell density of 1×10^6 in HCMT BulletKit® medium (Lonza), composed of HBMT Basal Medium and HCMT SingleQuots® (Lonza).

Oligo GEArray® Expression Analysis

Total RNA was isolated from the cultured cell lines using the ArrayGrade Total RNA isolation kit (SuperArray Bioscence, Frederick, MD). RNA quality and quantity were assessed both by agarose gel electrophoresis and by UV spectrophotometer. Using a TrueLabeling-AMP Linear RNA amplification kit (SuperArray Bioscience), 3 µg of total RNA were reverse-transcribed to obtain cDNA, and then converted by *in vitro* transcription into biotin-labeled cRNA using biotin-16-UTP (Enzo Life Sciences, Inc., Farmingdale, NY). Prior to hybridization, the cRNA probes were purified with the ArrayGrade cRNA clean-up kit (SuperArray Bioscience). Four µg of the purified cRNA probes were then hybridized to pre-treated Oligo GEArray® Human WNT Signalling Pathway Microarrays (OSH-043) (SuperArray Bioscience), which profiles the expression of 113 genes related to the WNT/β-catenin-mediated signal transduction. Following several washing steps, cRNA-bound array spots were detected using alkaline phosphatase-conjugated streptavidin and CDP-Star as a chemiluminescent substrate (SuperArray Bioscience). Chemiluminescence was detected by exposing the array membranes to X-ray film. The image data were transformed into numerical data using GEArray Expression Analysis Suite software (SuperArray Bioscience). Data evaluation, including background correction (subtraction of minimum value) and median normalization, were performed for each array. Moreover, gene expression ratio between each normalized tumour cell line profile and normalized human primary hepatocytes profile was calculated. The data-filtering criteria were: *i*, at least one of the spot intensities had to be more than twice compared to the background intensity, and *ii*, the spot intensity ratios between each cell line analysed and normal human primary hepatocytes had to be $\geq +2.0$ (for over -expression) or ≤ -2.0 (for under-expression).

Validation of microarray by quantitative real-time RT-PCR (qPCR)

PCR primers were designed using the prime software (http://www.autoprime.de/AutoPrimeWeb) taking into account the special design criteria for qPCR primers. Forward and reverse primer sequences of the genes analysed are listed in Table 1.

RNA was reverse-transcribed using MMLV reverse transcriptase (New England BioLabs, Inc., Ipswich, MA). An oligo-dT primer (Sigma-Aldrich, Saint Louis, USA) was used to prime the reverse transcription. qPCR was performed using a Rotor-Gene 6000 thermal cycling system (Corbett Research, Sidney, Australia), and the Quantimix Easy Syg kit (Biotools, Madrid, Spain) as the detection system. The thermal cycling conditions for all the transcript analysed were: 95°C for 4 min and 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec. *β-actin* was chosen as the reference gene for normalization of the results. As quality control, a melting curve was run immediately after the above cycling program, thus generating a first derivative dissociation curve for each transcript analysed. In all the melting curves obtained, no more than one peak appeared at temperature higher than 80°C. The fold change of transcripts expression in cancer cell lines relative to human primary hepatocytes was calculated using the $2^{-\Delta\Delta CT}$ method.

MicroRNAs expression analysis

MicroRNAs were extracted from the cell lines using the Ambion mirVana™ miRNA isolation kit (Ambion, Austin, TX USA) according to the manufacturer's

instructions. The TaqMan®MicroRNA Assays (Applied Biosystems, Foster City, CA) were used to detect and accurately quantify the following mature microRNAs: miR-21, miR-122a, miR-125a, miR-145, miR-148a, miR-150, miR-199a and miR-214. The Taqman® MicroRNA Assays for U6 RNA (Applied Biosystems) was used to normalize the relative abundance of microRNAs. In a first step, cDNA was reverse transcribed from total RNA samples using specific microRNA primers and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). In a second step, PCR products were amplified from cDNA samples using the TaqMan® MicroRNA Assay together with the TaqMan® Universal PCR Master Mix (Applied Biosystems). The fold change of transcripts expression in tumour, relative to normal human primary hepatocytes, was calculated using the $2^{-\Delta\Delta CT}$ method. For details, refer to the ABI user's bulletin "Relative Quantitation of Gene Expression: ABI PRISM 7700 Sequence Detection System: User Bulletin 2: Rev B".

Protein array

Protein arrays were performed using the Panorama™ Cell Signalling Antibody Microarray (Sigma-Aldrich). Each slide contained 224 antibodies spotted in 16 sub-arrays each containing duplicated spots of each antibody, as well as a single positive control spot for indocarbocyanine (Cy3) and indodicarbocyanine (Cy5), and a single negative control. Protein extracts were obtained by cell lysis in Buffer A (Sigma-Aldrich), supplemented with 0.6 units/ml of benzonase (Merck KGaA, Darmstadt, Germany), and with the supplied protease inhibitor cocktails (Sigma-Aldrich), according to the manufacturer's instructions. After protein quantification by Bradford protein assay (Bio-Rad, Munich, Germany), samples were diluted to 1 mg/ml of protein in Buffer A, and labelled for 30 min at room temperature with either Cy3 (emission peak 570 nm) or Cy5 (emission peak 670 nm) monofunctional reactive dyes (GE Healthcare, Buckinghamshire, UK). In each microarray, proteins extracted from human primary hepatocytes were labelled with Cy3 dye, whereas proteins extracted from one of the cell lines under investigation were labelled with Cy5 dye. Excess Cy3/Cy5 dye was eliminated from the labelled samples by SigmaSpin columns (Sigma-Aldrich), following the manufacturer's instructions. After assessing that the dye (D) to protein (P) molar ratio (D/P ratio) was higher than 2.3, 5 µg of each of the Cy3 and Cy5 labelled samples were added to the Array Incubation Buffer (Sigma-Aldrich), and the mixture was incubated for 30 min at room temperature with the microarray. Scanning was performed using the Scan Array™ Lite-Microarray Scanner (Perkin Elmer, Massachusetts, USA). Signals obtained were quantified using the ScanArray Express GenePix Pro 5.0 (Molecular Devices, Sunnyvale, CA, USA). The Cy5/Cy3 ratios for all spots on the microarray were normalized by dividing them by the median values. The data-filtering criteria were: *i*, at least one of the spot intensities had to be more than twice compared to the background intensity, and *ii*, only spot intensity ratios $\geq +1.5$ (for over-expression) or ≤ -1.5 (for under-expression) were considered.

Western blot

Twenty-five µg of whole protein extracts were loaded onto a 7-15% SDS-PAGE, and then transferred to a PVDF membrane (Immobilon Millipore, MA, USA). The blots were probed with either anti-TLE1 (2 µg/ml; Santa Cruz Biotecnology, Inc., California, USA), or anti-NLK (2 µg/ml; Santa Cruz), or anti-Grb-2 (2 µg/ml; Sigma-Aldrich), or anti-Cyclin D3 (1 µg/ml; Abcam), or anti-Cdc25C (1 µg/ml; Santa Cruz) antibodies. To verify that equal amounts of protein samples were loaded, filters were blotted with either β-actin (1 µg/ml; Sigma-Aldrich) or β-tubulin (1 µg/ml; Santa Cruz) antibodies. ECL detection solutions (Amersham Pharmacia) were used to visualise antibody reaction.

Statistical analysis

Statistical analysis was performed by means of Student's *t*-test, using the GraphPad InStat program.

RESULTS

Profiling of the expression of 113 genes related to the WNT/ β-catenin pathway

In order to confirm their genotype, we checked the presence or absence of the well known mutations characterizing the HuH6, Hep3B, HepG2 and HLE liver cancer cell lines used in the present study (data not shown) (*20*, *29*,*41*,*98*).

The expression of 113 genes was investigated in normal human primary hepatocytes and in HuH6, Hep3B, HepG2 and HLE liver cancer cell lines, using an Oligo GEArray® Human WNT Signalling Pathway Microarray (Fig. 2). No signals were visible in the blank spots and negative control spots, indicating that the array hybridization was highly specific. The intensity of housekeeping genes was comparable in all the experiments performed. The *β-actin* gene was used to normalize the intensities. The ratio values between genes expressed in HuH6, Hep3B, HepG2, and HLE cell lines and normal human primary hepatocytes have been summarized in Table 2. In particular, only the values of gene expression ratio $\geq +2.0$ and ≤ -2.0 were considered significant. In comparison to human primary hepatocytes, the expression of eight genes significantly changed in the liver cancer cells: four genes resulted up-regulated (*i.e.*, *FZD7*, *NLK*, *RHOU*, and *SOX17*) and four down-regulated (*i.e.*, *TCF7L2*, *TLE1*, *SLC9A3R1* and *WNT10A*).

Validation of the Oligo GEArray® Human WNT Signalling Pathway Microarray data

To confirm that the gene expression changes observed by microarray analysis were associated with the hepatic cell transformation phenotype, we examined the expression of four out of eight deregulated genes (two for each class, arbitrary chosen) by qPCR (*i.e.*, *NLK*, *RHOU*, *TCF7L2*, and *WNT10A*). qPCR confirmed an increased

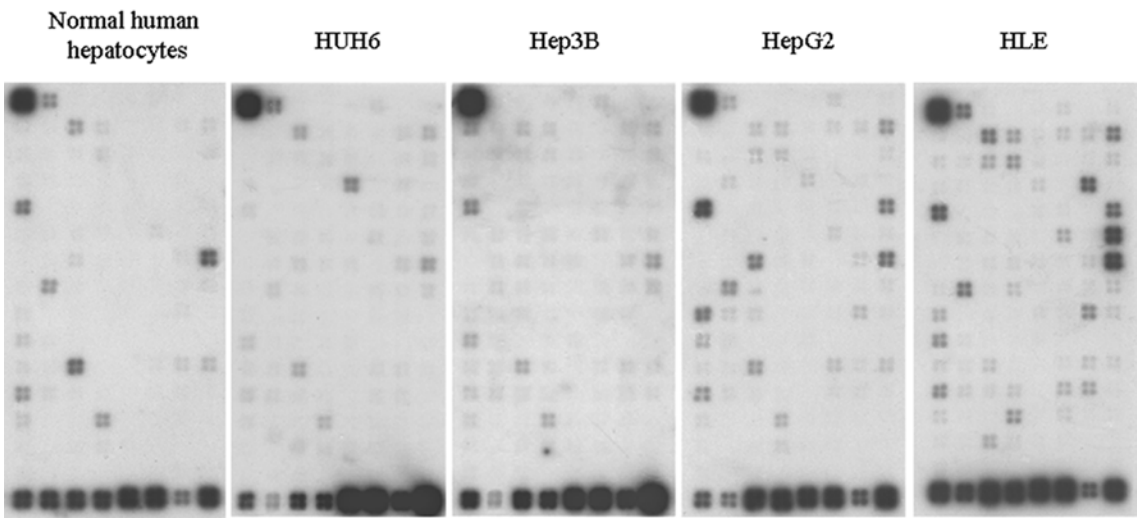

Figure 2. Parallel analysis of the gene expression profile in normal human primary hepatocytes, HuH6, Hep3B, HepG2, and HLE liver cancer cell lines. The human Oligo GEArray® WNT/β-catenin Signalling pathway microarray was hybridized with biotin-labeled cRNA obtained by *in vitro* transcription of each sample. For details, see text.

Table 1. Primer sequences used in qPCR experiments.

Primer	5'→3' sequence
RHOU	Forward: AGGCCTCTCTGCTACACCAA Reverse: TCAGGCACTGGCTTTTCTTT
NLK	Forward: GAAGTTTGAAGAGCTCCAGTG Reverse: GTCAAAGTCATACAGGGTGAAG
WNT10A	Forward: GGTTGCTCCACACCCTAAAA Reverse: ATGATGAAGGGAATGGTGGA
TCF7L2	Forward: TTAGTACCACAGCAAGGTCAAC Reverse: TACGACCTTTGCTCTCATTTC
β-actin	Forward: ATCTGGCACCACACCTTCTACAATGAGCTGC Reverse: CGTCATACTCCTGCTTCGTGATCCACATCTGC

Table 2. Gene expression profile by Oligo GEArray® Human WNT Signalling Pathway Microarray in the four cell lines analysed. In bold the genes commonly dysregulated in all the liver cancer cell lines analysed.

Gene	Protein encoded	Fold change
HUH6		
DKK1	Dickkopf homolog 1 (Xenopus laevis)	11,54
T	T, brachyury homolog (mouse)	8,83
RHOU	**Ras homolog gene family, member U**	**5,78**
NKD1	Naked cuticle homolog 1 (Drosophila)	5,78
NLK	**Nemo-like kinase**	**4,35**
SOX17	**SRY (sex determining region Y)-box 17**	**4,18**
NKD2	Naked cuticle homolog 2 (Drosophila)	3,23
PYGO1	Pygopus homolog 1 (Drosophila)	2,82
FZD7	**Frizzled homolog 7 (Drosophila)**	**2,74**
FZD6	Frizzled homolog 6 (Drosophila)	2,46
WNT5B	Wingless-type MMTV integration site family, member 5B	2,36
WIF1	WNT inhibitory factor 1	2,17
DAAM2	Dishevelled associated activator of morphogenesis 2	2,08
WNT8A	Wingless-type MMTV integration site family, member 8A	2,07
LRP5	Low density lipoprotein receptor-related protein 5	2,06
FZD8	Frizzled homolog 8 (Drosophila)	2,05
WNT10B	Wingless-type MMTV integration site family, member 10B	2,00
SFRP4	Secreted frizzled-related protein 4	-2,08
TCF7L1	Transcription factor 7-like 1 (T-cell specific, HMG-box)	-2,22
WNT10A	**Wingless-type MMTV integration site family, member 10A**	**-2,27**
SFRP1	Secreted frizzled-related protein 1	-2,33
GSK3A	Glycogen synthase kinase 3 alpha	-2,38
SLC9A3R1	**Solute carrier family 9 (sodium/hydrogen exchanger), member 3 1 regulator**	**-2,70**
PPP2CA	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	-2,78
WNT1	Wingless-type MMTV integration site family, member 1	-3,57
TLE1	**Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)**	**-3,57**
JUN	Jun oncogene	-3,57
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	-6,67
TCF7L2	**Transcription factor 7-like 2 (T-cell specific, HMG-box)**	**-9,09**
Hep3B		
NKD1	Naked cuticle homolog 1 (Drosophila)	9,89
DVL3	Dishevelled, dsh homolog 3 (Drosophila)	4,63
NKD2	Naked cuticle homolog 2 (Drosophila)	4,07
DAAM2	Dishevelled associated activator of morphogenesis 2	4,00
CSNK2A1	Casein kinase 2, alpha 1 polypeptide	3,84
RHOU	**Ras homolog gene family, member U**	**2,92**
CSNK2A2	Casein kinase 2, alpha prime polipeptide	2,70
CCND1	Cyclin D1	2,63
CCND2	Cyclin D2	2,58
DKK3	Dickkopf homolog 3 (Xenopus laevis)	2,32
BTRC	Beta-transducin repeat containing	2,32
SOX17	**SRY (sex determining region Y)-box 17**	**2,29**
NLK	**Nemo-like kinase**	**2,21**
FZD7	**Frizzled homolog 7 (Drosophila)**	**2,19**
CSNK1G1	Casein kinase 1, gamma 1	2,01

WNT16	Wingless-type MMTV integration site family, member 16	-2,00
WNT5B	Wingless-type MMTV integration site family, member 5B	-2,00
CCND3	Cyclin D3	-2,17
WNT10A	**Wingless-type MMTV integration site family, member 10A**	**-2,20**
TCF7	Transcription factor 7 (T-cell specific, HMG-box)	-2,32
WNT7B	Wingless-type MMTV integration site family, member 7B	-2,50
AES	Amino-terminal enhancer of split	-2,63
GSK3A	Glycogen synthase kinase 3 alpha	-2,70
WNT9B	Wingless-type MMTV integration site family, member 9B	-2,86
PYGO1	Pygopus homolog 1 (Drosophila)	-3,13
WNT2	Wingless-type MMTV integration site family member 2	-3,23
TCF7L2	**Transcription factor 7-like 2 (T-cell specific, HMG-box)**	**-3,33**
WNT3	Wingless-type MMTV integration site family, member 3	-3,33
WNT3A	Wingless-type MMTV integration site family, member 3A	-3,45
SLC9A3R1	**Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1**	**-3,45**
WNT10B	Wingless-type MMTV integration site family, member 10B	-3,45
SFRP1	Secreted frizzled-related protein 1	-3,57
WNT11	Wingless-type MMTV integration site family, member 11	-4,00
TLE1	**Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)**	**-4,17**
WISP1	WNT1 inducible signaling pathway protein 1	-4,17
WNT5A	Wingless-type MMTV integration site family, member 5A	-4,76
WNT2B	Wingless-type MMTV integration site family, member 2B	-5,26
JUN	Jun oncogene	-5,88
FZD3	Frizzled homolog 3 (Drosophila)	-5,88
LEF1	Lymphoid enhancer-binding factor 1	-6,25
T	T, brachyury homolog (mouse)	-7,14
PITX2	Paired-like homeodomain transcription factor 2	-100,00

HepG2

FOSL1	FOS-like antigen 1	6,80
RHOU	**Ras homolog gene family, member U**	**6,61**
DKK3	Dickkopf homolog 3 (Xenopus laevis)	6,58
WISP1	WNT1 inducible signaling pathway protein 1	4,50
T	T, brachyury homolog (mouse)	4,17
FZD2	Frizzled homolog 2 (Drosophila)	3,77
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	3,56
SOX17	**SRY (sex determining region Y)-box 17**	**3,03**
PORCN	Porcupine homolog (Drosophila)	2,97
NLK	**Nemo-like kinase**	**2,81**
FZD7	**Frizzled homolog 7 (Drosophila)**	**2,05**
BCL9	B-cell CLL/lymphoma 9	-2,00
CSNK1G3	Casein kinase 1, gamma 3	-2,13
AES	Amino-terminal enhancer of split	-2,17
WNT4	Wingless-type MMTV integration site family, member 4	-2,22
DAAM1	Dishevelled associated activator of morphogenesis 1	-2,27
WNT16	Wingless-type MMTV integration site family, member 16	-2,27
CCND2	Cyclin D2	-2,38
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	-2,50
FZD5	Frizzled homolog 5 (Drosophila)	-2,70
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	-2,70
TCF7L2	**Transcription factor 7-like 2 (T-cell specific, HMG-box)**	**-2,94**
WNT10A	**Wingless-type MMTV integration site family, member 10A**	**-3,13**
DVL2	Dishevelled, dsh homolog 2 (Drosophila)	-5,56

FOXN1	Forkhead box N1	-5,88
EP300	E1A binding protein p300	-6,25
TLE1	**Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)**	**-7,14**
SLC9A3R1	**Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1**	**-7,14**

HLE

NKD1	Naked cuticle homolog 1 (Drosophila)	16,84
RHOU	**Ras homolog gene family, member U**	**11,48**
NLK	**Nemo-like kinase**	**8,00**
T	T, brachyury homolog (mouse)	7,86
NKD2	Naked cuticle homolog 2 (Drosophila)	5,77
SOX17	**SRY (sex determining region Y)-box 17**	**5,34**
KREMEN2	Kringle containing transmembrane protein 2	5,25
SFRP4	Secreted frizzled-related protein 4	4,66
PYGO1	Pygopus homolog 1 (Drosophila)	4,29
DAAM2	Dishevelled associated activator of morphogenesis 2	3,78
FZD8	Frizzled homolog 8 (Drosophila)	3,75
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	3,49
SFRP1	Secreted frizzled-related protein 1	3,35
FZD7	**Frizzled homolog 7 (Drosophila)**	**3,08**
PPP2R1B	Protein phosphatase 2 (formerly 2A), regulatory subunit A beta isoform	2,78
PITX2	Paired-like homeodomain transcription factor 2	2,65
DVL3	Dishevelled, dsh homolog 3 (Drosophila)	2,64
KREMEN1	Kringle containing transmembrane protein 1	2,41
FZD6	Frizzled homolog 6 (Drosophila)	2,33
DKK3	Dickkopf homolog 3 (Xenopus laevis)	2,27
LEF1	Lymphoid enhancer-binding factor 1	2,21
FBXW4	F-box and WD repeat domain containing 4	2,14
DVL2	Dishevelled, dsh homolog 2 (Drosophila)	2,07
WNT16	Wingless-type MMTV integration site family, member 16	-2,00
SLC9A3R1	**Solute carrier family 9 (sodium/hydrogen exchanger) member 3 regulator1**	**-2,00**
PPP2CA	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	-2,04
CSNK1G1	Casein kinase 1, gamma 1	-2,04
CSNK2B	Casein kinase 2, beta polypeptide	-2,08
WNT3A	Wingless-type MMTV integration site family, member 3A	-2,27
CSNK1A1	Casein kinase 1, alpha 1	-2,44
APC	Adenomatosis polyposis coli	-2,44
CSNK1G2	Casein kinase 1, gamma 2	-2,63
WNT10A	**Wingless-type MMTV integration site family, member 10A**	**-2,78**
AXIN1	Axin 1	-2,78
CSNK1G3	Casein kinase 1, gamma 3	-2,94
CSNK1D	Casein kinase 1, delta	-3,23
AXIN2	Axin 2 (conductin, axil)	-3,23
GSK3A	Glycogen synthase kinase 3 alpha	-3,45
APC2	Adenomatosis polyposis coli 2	-3,57
BTRC	Beta-transducin repeat containing	-3,70
AES	Amino-terminal enhancer of split	-3,70
WNT9B	Wingless-type MMTV integration site family, member 9B	-4,00
WNT4	Wingless-type MMTV integration site family, member 4	-4,55
TCF7L2	**Transcription factor 7-like 2 (T-cell specific, HMG-box)**	**-4,55**
JUN	Jun oncogene	-4,55
CCND3	Cyclin D3	-4,55

TLE1	**Transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)**	**-5,26**
WNT9A	Wingless-type MMTV integration site family, member 9A	-5,56
DVL1	Dishevelled, dsh homolog 1 (Drosophila)	-7,14
BCL9	B-cell CLL/lymphoma 9	-8,33
WNT5B	Wingless-type MMTV integration site family, member 5B	-33,33

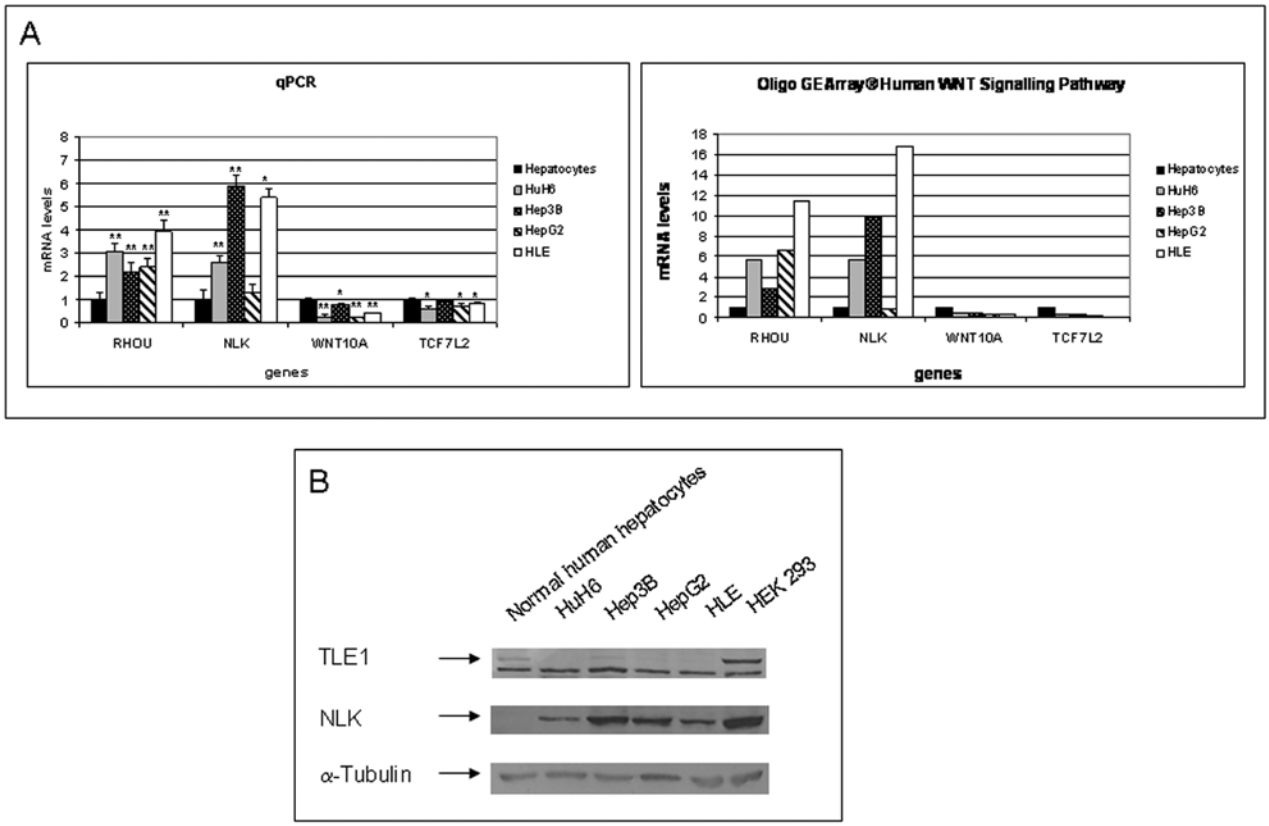

Figure 3. (A) Comparative analysis and validation of the Oligo GEArray® Human WNT Signalling Pathway Microarray data on selected genes (*i.e.*, *RHOU*, *NLK*, *CSNK2A2*, *WNT10A*, and *TCF7L2*). The qPCR graphs represents the mean of mRNA expression values in three repeated experiments with their respective standard deviations. Student's *t* -test: *p<0.05; **p<0.01. (B) Western Blot analysis to confirm TLE1 and NLK proteins expression. The protein lysate derived from HEK 293 cell line was used as positive control. β-tubulin was used to ascertain equal loading of proteins.

expression of *NLK* and *RHOU*, and a decreased expression of *TCF7L2* and *WNT10A* in HuH6, Hep3B, HepG2, and HLE cell lines. Data obtained were normalized with the expression levels of *β-actin* housekeeping gene in each cell line (Fig. 3A). These results clearly indicated a competency of the microarray analysis for the detection of changes in gene expression in the hepatic tumour cell lines investigated compared to normal human primary hepatocytes.

To further confirm gene expression changes observed, the expression levels of one down-regulated transcript (*i.e.*, TLE1) and one up-regulated transcript (*i.e.*, NLK) were further analysed by immunoblotting in normal human primary hepatocytes and in the liver cancer cell lines (Fig. 3B). The protein lysate derived from HEK 293 cell line was used as positive control. Data obtained indicated a reliability of the microarray results.

MicroRNAs expression analysis

Selected microRNAs were chosen according to previously published data (*89*). Among the 8 microRNAs analysed, miR-122a, miR-125a and miR-150 resulted to be dysregulated. In detail, miR-122a expression was down-regulated, with values ranging from 45- (in HuH6) to 13.000- (in HLE) fold compared to normal human primary hepatocytes. On the contrary, miR-125a

expression was up-regulated in liver cancer cell lines compared to normal hepatocytes (22-fold in HuH6; 18-fold in Hep3B; 6-fold in HepG2; and 94-fold in HLE). The miR-150 expression levels were reduced in the tumour cell lines, with values ranging from 9.8- (in Hep3B) to 159- (in HepG2) fold (Fig. 4). The expression of miR-21, miR-145, miR-148a, miR-199a, and miR-214 did not result significantly dysregulated (data not shown).

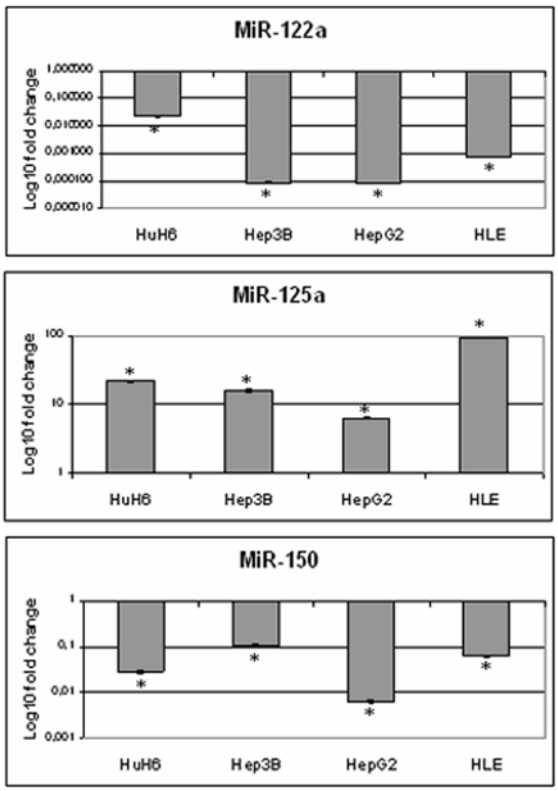

Figure 4. MicroRNAs showing an altered pattern in liver cancer cell lines compared to normal human primary hepatocytes. Each histogram plots the U6-normalized $\log_{10}$ fold change (ratio of tumours *versus* normal human primary hepatocytes). Data error bars represent standard deviation of three replicates. Student's *t* -test: *p<0.05.

Profiling of the expression of 224 proteins involved in the cell signalling pathway

In order to further characterize the four liver cancer cell lines, the expression of 224 proteins involved in biological pathways such as apoptosis, cell cycle, and signal transduction was analyzed using a protein array system (Fig. 5). In Table 3 the results obtained in HuH6, Hep3B, HepG2 and HLE cell lines have been summarized. Even if each liver cancer cell line displayed a significantly different protein expression profile, four proteins resulted commonly modulated: three of them (*i.e.*, c-Abl, Cdc25C and cyclin D3) are involved in the cell cycle regulation, whereas the fourth (*i.e.*, Grb-2) is involved in the signal transduction pathway.

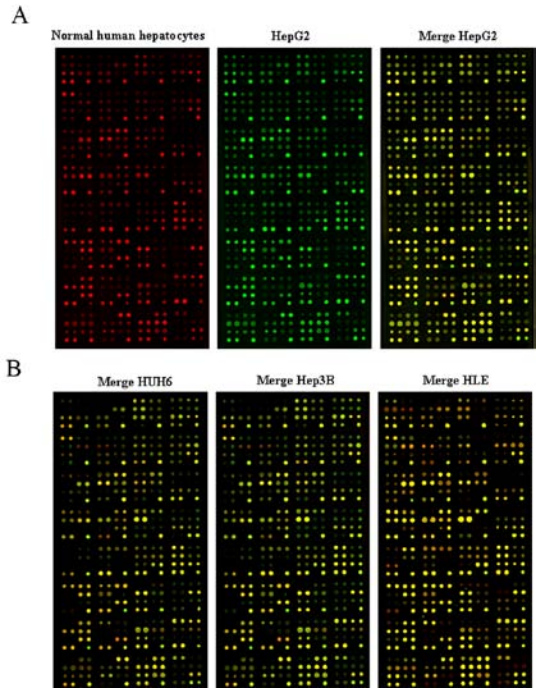

Figure 5. Protein array analysis using the Panorama® Cell Signalling Antibody Microarray containing 224 antibodies. Protein extracted from normal human primary hepatocytes were labeled with Cy5, whereas proteins obtained from each liver cancer cell line were labeled with Cy3. (A) As an example, here we show the protein array obtained from normal human primary hepatocytes and HepG2 cell line. In order to determine protein expression modulation, the two images were merged and further analyzed. (B) Protein array observed in HuH6, Hep3B and HLE cell lines, after merging with the normal human primary hepatocytes profile. For details, see text.

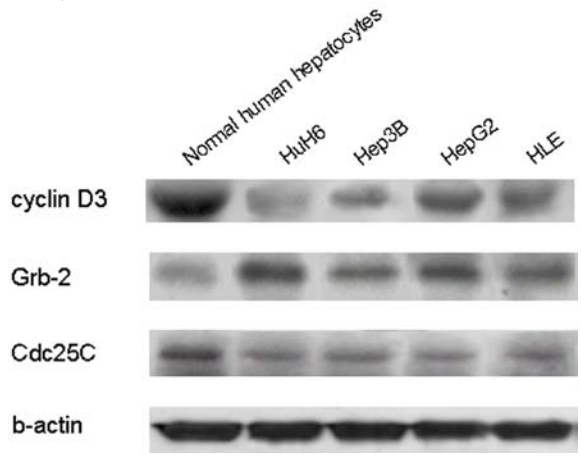

Figure 6. CyclinD3, Grb-2, and Cdc25C protein expression levels. In order to confirm the results obtained by protein array analysis, cyclin D1, Grb-2 and Cdc25C were analyzed by Western blot in normal human primary hepatocytes and in liver cancer cell lines. β-actin was used to ascertain equal loading of proteins.

Table 3. Protein expression profile. In bold the proteins commonly dysregulated in all the liver cancer cell lines analysed.

Protein	Functional group	Fold change
HUH6		
Cdc27	Cell Cycle	1,86
Bcl-XL	Apoptosis	1,57
Chk1	Cell Cycle	1,52
GRB-2	**Signal Transduction**	**1,50**
Phosphotyrosine	Signal Transduction	-1,35
Apoptosis Inducing Factor (AIF)	Apoptosis	-1,37
p53	Cell Cycle	-1,37
Connexin 32	Cytoskeleton	-1,37
ARNO (Cytohesin 2)	Signal Transduction	-1,39
b-NOS	Neurobiology	-1,43
PKB phosphoserine 473 (pS473)	Signal Transduction	-1,43
Cdk4	Cell Cycle	-1,45
Cdc25	**Cell Cycle**	**-1,47**
Cdk-7/cak	Cell Cycle	-1,47
i-NOS	Neurobiology	-1,47
Caspase 5	Apoptosis	-1,49
Dystrophin	Cytoskeleton	-1,49
Pan Cadherin	Cytoskeleton	-1,49
Cytokeratin pep 18	Cytoskeleton	-1,52
EGF receptor	Signal Transduction	-1,52
c-Abl	**Cell Cycle**	**-1,54**
GAP1	Signal Transduction	-1,56
Cyclin A	Cell Cycle	-1,59
Neurofilament 200	Neurobiology	-1,59
p38 MAPK activated diphosphorylated p38)	Signal Transduction	-1,59
MAP2 (2a+2b)	Cytoskeleton	-1,61
MAP Kinase activated protein kinase-2	Signal Transduction	-1,64
Topoisomerase-1	Nuclear	-1,72
PAK phospho (pS212)	Signal Transduction	-1,75
Cytokeratin pep 4	Cytoskeleton	-1,82
DAPK phospho (pS308)	Apoptosis	-1,89
Tyrosin hydroxylase	Neurobiology	-1,89
Tropomyosin	Cytoskeleton	-1,92
Cytokeratin pep 13	Cytoskeleton	-2,08
Cyclin D3	**Cell Cycle**	**-2,13**
Hep3B		
Chk1	Cell Cycle	1,87
GRB-2	**Signal Transduction**	**1,77**
Bcl-XL	Apoptosis	1,71
p19INK4d	Cell Cycle	1,70
Cdc27	Cell Cycle	1,67
Clathrin Light Chain	Cytoskeleton	1,57
Chondroitin sul fate	Cytoskeleton	-1,37
α-Catenin	Cytoskeleton	-1,37
Apoptosis Inducing Factor (AIF)	Apoptosis	-1,37
Calcineurin	Calcium	-1,39

MAP Kinase (ERK-l)	Signal Transduction	-1,39
DOPA Decarboxylase	Neurobiology	-1,41
Synaptotagmin	Neurobiology	-1,41
Pyk2 Phospho (pY881)	Signal Transduction	-1,41
Cdk-7/cak	Cell Cycle	-1,41
PKB phosphoserine 473 (pS473)	Signal Transduction	-1,43
Pan Cadherin	Cytoskeleton	-1,45
p53	Cell Cycle	-1,45
Caspase 5	Apoptosis	-1,45
i-NOS	Neurobiology	-1,47
CNPase	Neurobiology	-1,49
Cdh1	Cell Cycle	-1,49
Cdc25	**Cell Cycle**	**-1,49**
Ezrin	Cytoskeleton	-1,52
SMAD4	Cell Cycle	-1,52
Fibronectin	Cytoskeleton	-1,54
p38 MAPK activated (diphosphorylated p38)	Signal Transduction	-1,59
Pan Cytokeratin	Cytoskeleton	-1,59
MAP Kinase activated protein kinase-2	Signal Transduction	-1,61
ARNO (Cytohesin 2)	Signal Transduction	-1,64
Nerve Growth Factor Receptor	Neurobiology	-1,64
Dystrophin	Neurobiology	-1,64
c-Abl	**Cell Cycle**	**-1,64**
PAK phospho (pS212)	Signal Transduction	-1,67
Ap-1/cjun	Nuclear	-1,69
Plakoglobin	Cytoskeleton	-1,75
PKB/AKT	Signal Transduction	-1,75
Cdk4	Cell Cycle	-1,79
b-NOS	Neurobiology	-1,79
EGF receptor	Signal Transduction	-1,79
NF-κB	Signal Transduction	-1,79
Cathepsin D	Apoptosis	-1,85
MAP2 (2a+2b)	Cytoskeleton	-1,85
Caspase 4	Apoptosis	-1,89
Tropomyosin	Cytoskeleton	-1,92
c-myc	Cell Cycle	-2,00
Caspase 8	Apoptosis	-2,04
β Tubulin polyglutamylated	Cytoskeleton	-2,04
Cystatin A	Apoptosis	-2,08
Cyclin A	Cell Cycle	-2,08
GAP1	Signal Transduction	-2,08
Cyclin D2	Cell Cycle	-2,17
Neurofilament 200	Neurobiology	-2,17
DAPK	Apoptosis	-2,22
Caspase 4	Apoptosis	-2,27
Caspase 10	Apoptosis	-2,33
Cytokeratin pep 18	Cytoskeleton	-2,33
Internexin α	Cytoskeleton	-2,33
Cdc7 Kinase	Signal Transduction	-2,38
DAPK phospho (pS308)	Apoptosis	-2,50
Cytokeratin 8.12	Cytoskeleton	-2,56
Topoisomerase-1	Nuclear	-2,63
Cyclin D3	**Cell Cycle**	**-2,70**
NAK	Signal Transduction	-2,70
Cytokeratin pep 4	Cytoskeleton	-2,78

Tyrosin hydroxylase	Neurobiology	-2,86
Dystrophin	Cytoskeleton	-2,94
MAP1	Cytoskeleton	-3,03
Cytokeratin pep 19	Cytoskeleton	-3,33
Adaptin β1+β2	Cytoskeleton	-3,85
Spectrin (alfa+beta)	Cytoskeleton	-3,85
Cdk6	Cell Cycle	-4,76
Trf-1	Nuclear	-5,88
Cytokeratin pep 8.60	Cytoskeleton	-5,88

HepG2

GRB-2	**Signal Transduction**	**1,55**
Chondroitin sul fate	Cytoskeleton	-1,33
MAP Kinase activated protein kinase-2	Signal Transduction	-1,33
Cdk-7/cak	Cell Cycle	-1,35
Connexin 32	Cytoskeleton	-1,35
PAK phospho (pS212)	Signal Transduction	-1,35
Cyclin D3	**Cell Cycle**	**-1,39**
Cdk4	Cell Cycle	-1,41
SUV39H1 Histone Methyl Transferase	Nuclear	-1,41
Synaptotagmin	Neurobiology	-1,41
Caspase 8	Apoptosis	-1,43
Cystatin A	Apoptosis	-1,43
DAPK phospho (pS308)	Apoptosis	-1,43
SMAD4	Cell Cycle	-1,47
Cyclin A	Cell Cycle	-1,49
Neurofilament 200	Neurobiology	-1,49
Cdh1	Cell Cycle	-1,52
Tropomyosin	Cytoskeleton	-1,54
Cdc25	**Cell Cycle**	**-1,56**
Ap-1/cjun	Nuclear	-1,59
ARNO (Cytohesin 2)	Signal Transduction	-1,61
GAP1	Signal Transduction	-1,61
Cytokeratin pep 4	Cytoskeleton	-1,67
Cytokeratin pep 13	Cytoskeleton	-1,67
DAPK	Apoptosis	-1,69
Cytokeratin pep 18	Cytoskeleton	-1,69
c-Abl	**Cell Cycle**	**-1,75**
Cdk6	Cell Cycle	-2,00

HLE

i-NOS	Neurobiology	2,01
GRB-2	**Signal Transduction**	**1,93**
Chk1	Cell Cycle	1,77
Clathrin Light Chain	Cytoskeleton	1,61
Connexin 32	Cytoskeleton	-1,35
NTF2	Nuclear	-1,37
Topoisomerase-1	Nuclear	-1,37
DAPK phospho (pS308)	Apoptosis	-1,41
Cyclin D3	Neurobiology	-1,49
DAPK	Apoptosis	-1,52
Cdk4	Cell Cycle	-1,52
Cytokeratin pep 4	Cytoskeleton	-1,52

Cdc25	**Cell Cycle**	**-1,54**
Cytokeratin pep 13	Cytoskeleton	-1,56
c-Abl	**Cell Cycle**	**-1,67**

Validation of the Panorama™ Cell Signalling Antibody Microarray data by Western blot

To confirm the protein expression changes observed by the antibody microarray analysis, the expression of three out of four proteins commonly modulated (*i.e.*, Cdc25C, cyclin D3 and Grb-2) was further analysed by immunoblotting in normal human primary hepatocytes and in the liver cancer cell lines (Fig. 6). Data obtained indicated a reliability of the protein microarray analysis for the detection of changes in protein expression.

DISCUSSION

HB and HCC represent one of the major cause of death by cancer worldwide (*64,73*). Similarly to other type of tumours, mechanistic studies are currently performed using cell lines established from biopsies, assuming that growing them in laboratory for unlimited time does not alter their biological features. A comparative molecular characterization of the most used, among commercially available, liver cancer cell lines may represent a useful basis for those researchers who deal with such biological material. Furthermore, the molecular parameters chosen for these characterizations may be useful to gain new insights into pathogenesis of HB and HCC, providing novel biomarkers for these type of tumours.

Scientific knowledge about the WNT/β-catenin signalling target genes and their roles in hepatocarcinogenesis is limited, despite the discovery of several candidate genes. To determine whether the expression of genes involved in the WNT/β-catenin signalling pathway may be altered in HuH6, Hep3B, HepG2 and HLE liver cancer cell lines, a low-density WNT/β-catenin signalling-focused microarray was performed. Interestingly, the expression of *FZD7*, *NLK*, *RHOU*, *SOX17*, *TCF7L2*, *TLE1*, *SLC9A3R1* and *WNT10A* transcripts were significantly altered in all the four liver cancer cell lines analysed when compared to normal human primary hepatocytes

The canonical WNT/β-catenin signalling pathway is initiated by WNT ligands binding to the transmembrane receptors, FZDs and low-density lipoprotein receptor-related proteins (LPRs). Frizzled-7 (FZD7), a transmembrane WNT receptor, is one of the ten members of the frizzled gene family. While FZD7 expression is limited in normal tissues, its mRNA has been found at high level of expression in a wide variety of cancer cells, including melanoma, lung (*75*), esophageal (*85*), gastric (*40*) and colon cancer (*75,90,91*), as well as in HCC and lymphoblastic leukaemia (*39,60*). According to our results, Merle and collaborators (*60*) detected an over-expression of FZD7 in 90% of HCCs analysed, characterized by both a wild-type *CTNNB1* and a normal β-catenin accumulation.

An other important member of the WNT/β-catenin pathway is NLK, a serine/threonine kinase that suppresses the transcription activity of the β-catenin/TCF complex through the phosphorylation of the TCF/LEF family transcription factors, thus inhibiting the canonical WNT/β-catenin signalling pathway. According to our results, the NLK transcript up-regulation has been also observed in human ovarian cancer (*51*). Overall, the contemporary induction of *FZD7*, *RHOU* and *NLK* transcripts, as well as the up-regulation of genes codifying for proteins that inhibit the canonical WNT/β-catenin pathway, such as members of the Dickkopf (DKK) family (*i.e.*, *DKK1* in HuH6, and *DKK3* in Hep3B, HepG2 and HLE cell lines) and *NKD1* (up-regulated in HuH6, Hep3B and HLE cell lines), seem to indicate the activation of the non canonical WNT pathway in the four liver cancer cell lines investigated. In many instances, the canonical WNT/β-catenin signalling pathway is kept under control by a negative-feedback loop in which β-catenin/TCF activity induces the transcription of its own negative regulators, like AXIN and DKK1 (*9,52,74*). Even if we do not observe *NKD1* up-regulation in the HepG2 cell line by the gene array analysis, this gene results to be over-expressed by qPCR when compared to normal human primary hepatocytes (data not shown). Furthermore, the significant down-regulation of the *TCF7L2/TCF4* transcript supports the hypothesis that the non canonical WNT/β-catenin signalling pathway is activated. Although high expression levels of *TCF7L2/TCF4* have been reported in HCC (*34*), very recently it has been demonstrated that the increased expression of the tumour suppressor

gene *HINT1* in Hep3B and HepG2 hepatoma cells inhibits the transcriptional activities of β-catenin/TCF4 (*93*).

The Groucho/TLE family members binds to all known TCF/LEF family members acting as inhibitor of the WNT/β-catenin signalling pathway in the absence of nuclear β-catenin (*5,28,61,62*). The molecular basis of the switch from transcriptional repression to activation during the WNT/β-catenin signalling is not clear, and it is not known whether factors other than β-catenin are required to disrupt the interaction between Groucho/TLE and TCF/LEF (*18*). The ability of Groucho/TLE family members to interact with many different transcription factors suggests that its function might be probably dependent on the cell type and on the timing of expression during development. A possible role of the TLE proteins in tumour suppression has been suggested, since low levels of expression of both TLE1 and TLE4 has been described in myeloid cell lines and in a subset of acute myeloid leukaemia (AML) (*19*). Indeed, our data show a clear down-regulation of TLE1 in all the liver cancer cell lines investigated, supporting the speculation that TLE1 might behave as a tumour suppressor gene.

Structurally related to TCF/LEFs, several members of the Sox family of high-mobility-group (HMG) box transcription factors have been found expressed in different cell types (*78*). While several Sox factors (*i.e.*, Sox2, Sox9 and Sox17) are antagonists of the canonical WNT/β-catenin signalling pathway, others (*i.e.*, Sox4 and Sox5) are able to promote the WNT/β-catenin signalling activity (*83*). Note that the expression of several Sox proteins has been tightly correlated with cancer progression (*22,68,72*). In particular, Sox17, which is able to directly interact with β-catenin and TCF/LEF proteins, can either utilize β-catenin as a transcriptional co-factor or promote the degradation of β-catenin and TCFs (*83,100*). This finding is relevant because Sox17 may antagonize the WNT/β-catenin activity by competing with TCFs for β-catenin binding (*83,100*). Our data show that *Sox17* transcript levels are higher in liver cancer cells compared to normal human primary hepatocytes, thus supporting its role in antagonizing the canonical WNT/β-catenin pathway by promoting the degradation of TCF/LEF and β-catenin proteins, *via* a GSK3β-independent mechanism (*83*).

A further result that supports the hypothesis that the WNT/β-catenin canonical pathway may be repressed is the down-regulation of the *SLC9A3R1* and *WNT10A* transcripts in all the cell lines analysed. WNT10A has been recently recognized as a main activator of the WNT/β-catenin canonical signalling pathway since its up-regulation correlates to the re-activaction of massive proliferation in liver oval cells (*32*). The SLC9A3R1 protein associates with β-catenin, acting as a positive regulator of the canonical WNT/β-catenin signalling pathway (*81*). Moreover, it has been demonstrated that it participates in the β-catenin-mediated transactivation, recruiting other transcriptional activators to the β-catenin/TCF complex in the nucleus (*81*).

In the last years, microRNAs have assumed a central role in cancer research since they result dysregulated in several human malignancies (*26,66,92*). Identification of cancer-specific microRNAs and their targets is critical to understand their role in tumorigenesis, and may be important for defining novel therapeutic targets (*7,92*). Analysis of microRNAs expression revealed that miR-122a, miR-125a and miR-150 are useful in discriminating tumoural versus normal human hepatocytes. According to published data (*25*), MiR-122a, which plays a crucial role in liver disease (*89*), results down-regulated in all the tumour cell lines investigated. It has been also demonstrated that miR-122a is involved in the modulation of cyclin G1 expression in HCC-derived cell lines, as indicated by the inverse correlation between miR-122a levels and cyclin G1 expression (*25*). MiR-125a has been found to be up-regulated from 6- to 94-fold in tumour cell lines compared to normal human primary hepatocytes. Scott and colleagues (*80*) showed that ErbB2 protein, a component of the *ERBB* oncogene family, is affected by the overexpression of miR-125a, suggesting a role of this miR in tumour suppression. Remarkably, a reduced expression of miR-150 has been observed in all the liver cancer cell lines investigated. Chang and co-workers (*12*) have recently demonstrated that *myc* levels are affected by miR-150, supporting a possible role of this miR as a tumour suppressor. Conversely, over-expression of miR-150 has been observed in hematopoietic progenitor/stem cells (*99*). A computer-based prediction algorithm reveals that the Grb-2 protein could be a putative target of miR-150, while the Cdc25C protein could be a target of miR-125a. Comparing the results obtained by microRNA analysis and protein array strategy, we

hypothesize that miR-122a, miR-125a and miR-150 could represent suitable candidates in distinguishing tumoural from normal hepatocytes.

As previously mentioned, the protein array strategy revealed that Cdc25 phosphatases is down-regulated and Grb-2 is over-expressed in the cell lines investigated, compared to normal human primary hepatocytes. Cdc25C is responsible for the dephosphorylation events that activate the cyclin-dependent kinases (CDK) at specific stages of the cell cycle (*2*). The expression and activity of Cdc25 is finely regulated by multiple mechanisms including post-translational modifications. Moreover, alterations in phosphatases expression may lead to checkpoint dysfunction and genetic instability, as described during tumourigenesis (*2*). Grb-2 is an ubiquitously expressed adapter, which recruits a variety of signalling molecules and plays important roles in cell proliferation and cancer. Increased Grb-2 expression has been correlated with increased oncogenic signalling in breast tumours (*17*) and HCC (*97*). Interestingly, immunoblot analysis of proteins extracted from cryopreserved samples of three HB patients indicated a far higher level of Grb-2 in tumour with respect to the normal healthy counterpart (data not shown), suggesting that such marker is not restricted to liver cancer cells. In addition, the gene expression analysis by means of qPCR indicated that Grb-2 accumulation in hepatic tumour cell lines does not occur at the transcription level, but rather at the level of the mRNA stability or eventually post-translationally (data not shown). In this respect, the observation that Cdc25 and Grb-2 contain putative sequences for miR-125a and miR-150, respectively, let to hypothize that the down-regulation of miR-150 could be associated with the increase of Grb-2 protein levels, whereas the high levels of miR-125a could account for the reduced Cdc25C protein expression levels. Further investigations need to be performed to ascertain the fine tuning of these proteins through the interaction with the specific microRNAs.

In conclusion, we performed a pilot comparative molecular characterization of commercially available HB-like and HCC-like cancer cell lines at gene, microRNA and protein expression levels. In particular our data suggest a specific role of the WNT non canonical pathways in the establishment and/or maintenance of the hepatocarcinogenic phenotype, thus highlighting that the molecular parameters chosen might be also useful to gain new insights into the pathogenesis of HB and HCC, providing potential novel biomarkers involved in the pathogenesis of liver cancer.

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