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ANALYSIS OF THE MOLECULAR AND CELLULAR PATHWAYS INVOLVED IN THE GENESIS OF RARE HEPATIC TUMOURS

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Abbreviations

AFP	α-fetoprotein
Akt	Protein kinase B
AML	Acute myeloid leukaemia
APC	Adenomatous polypolis coli
BF	Benzofuran
BWS	Beckwith-Wiedemann Syndrome
CDK	Cyclin-dependent kinase
CLL	Chronic lymphoid leukemia
DEHP	Di-(2-ethylhexyl) phthalate
DKK	Dickkopf
DVL	Dishevelled
EGF	Epidermal Growth Factor
ELBW	Extremely low birth weight
FAP	Familial adenomatous polyposis
FFPE	Formalin-fixed paraffin-embedded
FZD	Frizzled
GD	Gestational day
Grb-2	Growth factor receptor-bound protein 2
GSK-3β	Glycogen synthase kinase-3β
HB	Hepatoblastoma
HCC	Hepatocellular carcinoma
HGF	Hepatocyte Growth Factor
IGF	Insulin-like Growth Factor
ITF-2	Immunoglobulin transcription factor-2
LBW	Low birth weight
LOAEL	Low-observed-adverse-effect level
LPR	Lipoprotein receptor-related proteins
MAPK	Mitogen-activated protein kinase
miR	microRNA
MMP-7	Matrix metalloproteinase-7
NFAT	Nuclear factor of activated T cells
NLK	Nemo-like kinase
NRs	Nuclear receptor
PAS	Periodic Acid Schiff
PI3K	Phosphoinositide 3-kinase/
PM	Plasmamembrane
PND	Post-natal day

PPARs	Peroxisome proliferator activated receptors
PPRE	Peroxisome Proliferator Response Element
Ps	Phthalate(s)
PVC	Polyvinyl chloride
qRT-PCR	Quantitative real-time reverse transcription PCR.
RD	Rare disease
RR	Relative Risk
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGFα	Transforming Growth Factor α
TGFβ	Transforming Growth Factor β
uPAR	Urokinase-type plasminogen activator receptor
UTR	Untranslated region
WNT	Wingless-type

Summary

1 Italian

In accordo con il Parlamento Europeo e con il Consiglio dell'Unione Europea (Regolamento EC n.141/2000), le malattie rare (*rare diseases*, RD) sono definite esclusivamente sulla base della loro prevalenza che non deve essere superiore a 5 casi su un totale di 10.000 cittadini europei. Le malattie rare costituiscono un gruppo ampio ed eterogeneo che include più di 6.000 malattie e coinvolge tutti gli organi e tessuti, spesso mostrando all'interno della stessa patologia subtipi clinici differenti.
La maggior parte delle malattie rare può causare la morte e/o la disabilità del paziente. Una diagnosi accurata e precoce è spesso di fondamentale importanza ai fini della prevenzione e della cura; molto spesso, però, la diagnosi e/o la prognosi di queste malattie risultano incerte.
Nel presente studio è stato affrontantato un approccio integrato sull'epatoblastoma (HB), un raro e severo tumore maligno del fegato che insorge in età pediatrica. Sebbene l'epatoblastoma, nei paesi occidentali, abbia un' incidenza di soli 1.5 casi per milione di individui di età inferiore ai 15 anni, rappresenta il tumore maligno del fegato più frequente in età pediatrica (Reynolds et al., 2004; McLaughlin et al., 2006; Roebuck and Perilongo, 2006). La sua prognosi è basata su diversi fattori clinici e istologici. Bambini che mostrano una prognosi infausta, sono spesso caratterizzati da livelli anomali di α-fetoproteina (AFP) (<100 oppure >1,000,000 ng/ml) e dalla presenza di metastasi distanti (ad esempio al polmone e ai linfonodi); una progressiva diminuzione dei livelli di AFP nel corso della chemioterapia, è invece caratteristica dei pazienti con prognosi fausta (Roebuck and Perilongo, 2006; De Ioris et al., 2007). Evidenze scientifiche dimostrano la natura multifattoriale dell'epatoblastoma, che risulta associato sia a malattie genetiche (la sindrome di Beckwith-Wiedemann (BWS) e la Poliposi Adenomatosa Familiare (FAP)) (Roebuck and Perilongo, 2006), sia a fattori non genetici e ambientali che possono essere costituiti dallo *status* endocrino e metabolico della madre (giovane età, sovrappeso, uso di anticoncezionali, fumo) e preclampsia (McLaughlin et al., 2006). È stata inoltre suggerita un'associazione con l'esposizione occupazionale paterna a metalli, a prodotti del petrolio e a vernici/pigmenti, e il possibile coinvolgimento di contaminanti ambientali, quali gli ftalati (Buckley et al., 1989; Reynolds et al., 2004; McLaughlin et al., 2006).

Inoltre dati epidemiologici indicano il basso peso alla nascita (LBW) e l'aumento dei tassi di sopravvivenza di bambini LBW, come fattori associati ad un incremento di incidenza di epatoblastoma (Reynolds et al., 2004).
Nella prima parte del lavoro, studi *in vitro* su 4 linee cellulari umane di tumore del fegato e 9 biopsie ottenute da pazienti affetti da HB, hanno permesso di individuare nuovi marcatori dei tumori infantili del fegato. La nostra attenzione si è focalizzata sul pathway canonico e non canonico di WNT (*Wingless-type*), e sul pathway promosso da IGF-II (*Insulin-Like Growth Factor II*); questi risultano infatti deregolati in FAP e in BWS rispettivamente, le malattie genetiche che predispongono allo sviluppo di HB. È noto che i pazienti FAP sono portatori della mutazione germinale nel gene *APC* (*adenomatosis polyposis coli*), che codifica una proteina facente parte del complesso, costituito anche da Axina e GSK-3β (*Glycogen synthase kinase-3β*) capace di regolare i livelli citoplasmatici di β-catenina (Orford et al., 1997). Quest'ultima rappresenta l'effettore centrale del pathway canonico di WNT e la presenza di β-catenina nel citoplasma, determina l'induzione della trascrizione di geni coinvolti nella proliferazione cellulare (Rubinfeld et al., 1996). I pazienti affetti da BWS sono caratterizzati dalla perdita di imprinting (*loss of imprinting, LOI*) nella regione 11p15 dove è localizzato il gene *IGF-II.* La LOI causa l'espressione biallelica del gene con conseguente overespressione della proteina; è stato suggerito che tale fenomeno possa determinare un aumento nel tasso di proliferazione cellulare, mancato differenziamento e sviluppo di tumore (Veronese et al., 2010).
Saggi di array di mRNA e proteine, effettutati nelle linee cellulari di tumore del fegato, hanno rivelato diversi profili di espressione genica e proteica rispetto alla linea di controllo di epatociti normali. L'array di mRNA ha analizzato l'espressione di diversi geni (96) coinvolti nei pathway canonico e non canonico di WNT ed ha mostrato, nelle linee cellulari di tumore del fegato rispetto alla linea di controllo, la contemporanea upregolazione di geni antagonisti del pathway canonico di WNT, come *NLK* e *SOX17*, e la downregolazione dei suoi agonisti, come *TCF7L2, TLE1, SLC9A3R1* e *WNT10A.* Queste evidenze suggeriscono l'inattivazione del pathway canonico di WNT, mentre la overespressione di *RHOU* dimostra l'attivazione del pathway non canonico.
La tecnologia innovativa dell'array di proteine ci ha permesso di valutare i livelli di espressione di 224 proteine coinvolte in diversi pathway biologici, come l'apoptosi, il ciclo cellulare e la trasduzione del segnale, rivelando che la proteina Grb-2 (*growth factor receptor-bound protein 2*) è overespressa

nelle linee cellulari di tumore del fegato rispetto alla linea di controllo. Grb-2 è un adattatore cellulare ubiquitario attivato, in condizioni normali dai pathway delle IGF (I e II). Mediante l'interazione con il pathway Raf/MAPK (*mitogen-activated protein kinase*), Grb-2 è capace di promuovere la proliferazione cellulare (Foulstone et al., 2005). In un secondo tempo abbiamo approcciato lo studio di 9 biopsie, 3 delle quali pervenute in forma congelata e 6 in paraffina, provenienti da pazienti affetti da HB. In questi rari campioni abbiamo potuto saggiare, i marcatori precedentemente individuati e validati nelle linee cellulari (trascitti di NLK, RHOU e WNT10A; la proteina Grb-2). Lo studio ha mostrato risultati simili tra le 3 biopsie congelate di HB e le linee cellulari di tumore del fegato, suggerendo la validità di questi marcatori anche nei campioni tissutali.
I microRNA (miR) sono piccole molecole di RNA, a singolo filamento di 20-22 nucleotidi, capaci di legare per complementarità il 3' UTR (*untranslated region*) di specifici mRNA target, e di reprimerne la traduzione. Poiché i profili di espressione dei microRNA sono alterati in diversi tumori rispetto alle loro controparti normali, queste molecole sono ampiamente utilizzate anche nel campo della classificazione dei tumori (Calin and Croce, 2006). Un array eseguito nelle 3 biopsie di HB congelate, ha permesso di evidenziare 51 microRNA specificatamente deregolati nella parte tumorale rispetto a quella normale. Dal confronto dei nostri risultati con dati precedentemente pubblicati (Varnholt et al., 2008), sono stati selezionati 9 microRNA da analizzare nei nostri 9 campioni. Dal confronto dei livelli dei microRNA tra la parte tumorale e quella non-tumorale, sono stati evidenziati 4 microRNA upregolati (miR-125a, -150, -199a e -214) e uno downregolato (miR-148a). Dati in letteratura dimostrano che il miR-214 è capace di legare l'mRNA di PTEN e di downregolare il suo prodotto proteico che rappresenta un importante repressore del pathway mitogenico PI3K (Phosphoinositide 3-kinase)/Akt (PKB, protein kinase B), attivato in condizioni normali dai pathway di IGF (Foulstone et al., 2005; Yang et al., 2008). Anche nei nostri campioni, è stata evidenziata una correlazione tra alti livelli di miR-214 e bassi livelli proteici di PTEN nel tessuto tumorale rispetto a quello sano.
Lo studio di linee cellulari umane di tumore del fegato e di biopsie ottenute da pazienti affetti da HB mostrano l'inattivazione del pathway canonico di WNT e la contemporanea induzione di quello non canonico. Inoltre l'attivazione dei pathway di IGF è ipotizzata sulla base della deregolazione dei livelli proteici di Grb-2 e PTEN.
Nella seconda parte del progetto è stata studiata l'associazione tra l'insorgenza di HB e l'esposizione al di-etil-esil-ftalato (DEHP).

Quest'ultimo è lo ftalato più utilizzato, ed ha tra i suoi principali target il fegato. Studi effettuati sia nel topo che nell'uomo, dimostrano che il DEHP è capace di alterare del metabolismo del glucosio nel fegato (Latini et al., 2008). Il DEHP interagisce con la famiglia proteica dei PPARs (*Peroxisome proliferator activated receptors*) (Feige et al., 2007; Latini et al., 2008), che mediano i pathway di IGF-I e II responsabili della sintesi di glicogeno (Lopez et al., 1999). L'esposizione *in utero* di ftalati è significativamente associata alla prematurità (Latini et al., 2008), che a sua volta è stata fortemente associata all'HB (McLaughlin et al., 2006). L'epatoblastoma può insorgere sia in seguito a disturbi che possono interessare le fasi critiche dell'organogenesi, sia in seguito all'interazione, durante le vita prenatale o nelle prime fasi di quella postatale, con fattori di rischio, quali il DEHP (Salvatore et al., 2008).
Al fine di studiare gli effetti dell'esposizione pre-natale al DEHP sullo sviluppo del fegato, femmine di topi CD-1 sono state esposte al DEHP (25 e 100 mg/kg al giorno) durante il periodo critico dell'organogenesi ed istogenesi del fegato, compreso tra l'11esimo e il 19esimo giorno di gestazione (Duncan, 2003). Maschi e femmine della generazione F1, sono stati sacrificati nel giorno post natale (PND) 21 e 35, rappresentando rispettivamente lo svezzamento e l'inizio dell'età puberale. Studi istopatologici, istochimici e di espressione genica, eseguiti sui campioni di fegato di topi PND35, non hanno rilevato differenze significative tra i topi trattati e i loro rispettivi controlli. Per quanto riguarda i topi PND21 trattati rispetto ai controlli, è stata denotata la presenza di alterazioni nei pathway metabolici del glucosio e dei lipidi. L'inattivazione della GSK-3β è stata speculata sulla base dell'assenza di accumulo di glicogeno e della presenza di β-catenina nel citoplasma. L'accumulo di glicogeno in età fetale è promosso da IGF-II, ed è essenziale nell'assicurare livelli stabili di glicemia alla nascita, momento in cui il neonato si adatta alla vita extra-uterina. (Lopez et al., 1999; Hui et al., 2006). La presenza di β-catenina citoplasmatica indica l'attivazione del pathway canonico di WNT e l'induzione della trascrizione dei geni coinvolti nella proliferazione cellulare (Rubinfeld et al., 1996). Topi PND21 trattati mostrano inoltre una vacuolizzazione degli epatociti (epatosteatosi), fenomeno indotto da un eccesso di sintesi dei lipidi (Shimano et al., 2007), correlato all'inibizione della sintesi di glicogeno. I livelli di α-fetoproteina nel topo si abbassano progressivamente dopo la nascita, fino a spegnersi entro la terza settimana di vita, momento in cui il fegato è pronto ad accumulare energia sottoforma di glicogeno anziché AFP (Rusyn et al., 2006; Heudorf et al., 2007; Latini et al., 2009). Data l'associazione dell'espressione di AFP con lo stato

embrionale, tale gene viene anche utilizzato come marcatore della maturazione degli epatociti (Qin and Tang, 2004).
I nostri risultati hanno rivelato alti livelli di espressione genica di *AFP* nei topi PND21 trattati, e suggeriscono come il DEHP possa essere coinvolto sia nell'alterare lo switch da AFP a glicogeno, sia nel ritardare la maturazione degli epatociti.
In ultima analisi, è stato verificato il possibile coinvolgimento dei microRNA nelle alterazioni indotte da DEHP. *IGF-II*, essenziale nella vita fetale nel promuovere la sintesi del glicogeno, contiene all'interno del suo secondo introne, il miR-483 (Fu et al., 2005; www.ensembl.org). L'analisi dell'espressione di questo microRNA, ci ha permesso di stabile che anch'esso rappresenta un marcatore della maturazione degli epatociti, in quanto i suoi livelli si abbassano progressivamente dopo la nascita, fino a spegnersi. In topi PND21 trattati con la dose più alta di DEHP, è possibile osservare alti livelli di miR-483 che confermano un ruolo, per questa sostanza chimica, nel causare il ritardo della maturazione degli epatociti. Abbiamo inoltre dimostrato che questo microRNA è capace di downregolare i livelli proteici di β-catenina, costituendo quindi un limite allo stimolo proliferativo indotto dalla sua presenza nel citoplasma.
L'approccio multidisciplinare, costituito da modelli *in vitro* ed *in vivo*, ci ha permesso di individuare marcatori precoci e tardivi di epatoblastoma. I nostri studi hanno dimostrato che l'esposizione pre-natale a ftalati causa il ritardo della maturazione del fegato, alterando i pathway dei carboidrati e dei lipidi coinvolti nella nutrizione fetale. L'inibizione della sintesi di glicogeno, dovuta dall'inattivazione della di GSK-3β, determina l'induzione della sintesi dei lipidi, come dimostrato dall'epatosteatosi. L'aumento dei livelli d'espressione di *AFP*, in seguito all'esposizione pre-natale al DEHP, suggerisce sia un'alterazione nello switch da AFP a glicogeno, sia un ritardo nella maturazione degli epatociti, confermata anche dalla presenza di alti livelli di miR-483, un altro marcatore fetale. Inoltre è stato proposto un ruolo biologico per questo miR, che consiste nel limitare lo stimolo proliferativo indotto dalla presenza di β-catenina nel citoplasma.
La caratterizzazione dei modelli *in vitro* (linee cellulari umane di tumore del fegato e biopsie di pazienti HB) ha permesso di identificare dei trascritti (WNT10A, NLK e RHOU), delle proteine (Grb-2 e PTEN) e un microRNA (miR-214) capaci di rappresentare dei nuovi marcatori tardivi dell'epatoblastoma.
L'analisi dell'espressione genica ha mostrato l'inattivazione del pathway canonico di WNT e la contemporanea attivazione di quello non canonico. A livello proteico, l'upregolazione di Grb-2 nei campioni tumorali rispetto ai

normali, fa speculare l'attivazione del pathway mitogenico Raf/MAPK. L'upregolazione del miR-214 osservata nei campioni tumorali rispetto ai normali, correla con i livelli proteici di PTEN, che risultano più bassi nella controparte tumorale. Questa evidenza suggerisce che l'attivazione del pathway di PI3K/Akt possa determinare la deregolazione dei processi biologici da questo controllati, quali il metabolismo del glicogeno e la sopravvivenza cellulare (Foulstone et al., 2005).
Sebbene le alterazioni a carico dei pathway di WNT e delle IGF osservate negli studi *in vivo* che *in vitro,* interessino meccanismi differenti, l'importanza di questi e delle loro interazioni risulta di fondamentale importanza nell'insorgenza di epatolastoma e nella sua caratterizzazione.

2 English

According to the European Parliament and the Council of the European Union (Regulation EC n.141/2000), rare diseases are defined solely on the basis of low prevalence and affect not more than five individuals per 10.000 in the European population. RD are a large and diverse group of disorders; they include more than 6.000 conditions and involve all organs and tissues, often with several clinical subtypes within the same disease.
Almost all RD may cause early mortality and/or long-term disability and accurate and timely diagnosis is often of great importance for prevention and treatment. Very often information on many RD are insufficient, concerning either diagnosis and/or prognosis.
An integrated approach was set on a very rare and severe liver malignancy of childhood, hepatoblastoma. Although its annual incidence in western countries is 1.5 cases per million of individuals younger than 15 years, HB represents the most common liver cancer of childhood (Reynolds et al., 2004; McLaughlin et al., 2006; Roebuck and Perilongo, 2006). Its prognosis depends on numerous clinical and histological factors. Children with a poor prognosis are usually characterized by abnormal α-fetoprotein levels (<100 or >1,000,000 ng/ml) and distant metastases (i.e. lung and lymph nodes), whereas patients with good prognosis appear to have, among others, a decline in circulating AFP levels during chemotherapy (Roebuck and Perilongo, 2006; De Ioris et al., 2007). Scientific evidence points out HB as a multi-factorial condition associated with: genetic conditions (i.e. Beckwith-Wiedemann Syndrome and Familial Adenomatous Polyposis) (Roebuck and Perilongo, 2006), non genetic and environmental factors such as the endocrine-metabolic status of the mother (young age, higher body mass index, use of infertility treatments, smoking) and (pre)eclampsia (McLaughlin et al., 2006). An association with occupational paternal exposure to metals, petroleum products and paints/pigments as well the possible involvement of ubiquitous environmental contaminants, such as phthalates, have been suggested (Buckley et al., 1989; Reynolds et al., 2004; McLaughlin et al., 2006). Moreover, epidemiological data indicate low birth weight and increased survival of LBW newborns as consistently associated with increased risk of HB (Reynolds et al., 2004).
In the first part of the project, *in vitro* studies on 4 liver cancer cell lines and 9 matched biopsis collected from HB patients were performed in order to individuate new molecular markers of childhood liver cancers. Special

attention was dedicated to the canonical and non canonical WNT pathways and to the IGF-II signalling, since they are involved in the pathogenesis of FAP and BWS, the genetic disorders that predispose to HB onset. FAP patients carry a germline mutation in *APC* gene, which codifies for a protein that is part, together with Axin and GSK-3β, of the complex that controls the cytoplasmic levels of β-catenin (Orford et al., 1998), the central effector molecule of the canonical WNT pathway (Rubinfeld et al., 1996). BWS patients show a loss of imprinting LOI in the region 11p15, where *IGF-II* gene is localized. The LOI causes the biallelic expression and therefore, an increase of IGF-II levels, that could lead, as suggested by Veronese and coworkers, to enhanced cellular proliferation, differentiation failure and tumour development (Veronese et al., 2010).

An mRNA- and protein-array, performed on the liver cancer cell lines; showed different signatures in the expression levels of genes and proteins, compared to normal human hepatocytes. The mRNA array analyzed the expression of several genes (96) involved in canonical and non canonical WNT signallings and revealed, in liver cancer cell lines compared to normal human hepatocytes, the contemporary upregulation of antagonists genes of the canonical WNT pathway, such as *NLK* and *SOX17*, and the downregulation of its agonists genes, such as *TCF7L2, TLE1, SLC9A3R1* and *WNT10A.* These evidences suggest an inactivation of the canonical WNT signalling; at the same time the overexpression of RHOU transcript indicate the activation of the non canonical WNT signalling.

The very innovative protein array technology evaluated the epression levels of 224 proteins involved in biological pathways such as apoptosis, cell cycle, and signal transduction, and revealed that Grb-2 is over-expressed in the cell lines investigated, compared to normal human primary hepatocytes. Grb-2 is an ubiquitously expressed adapter induced by IGF (I and II) signallings, in normal conditions; through the interaction with Raf/MAPK, Grb-2 activation leads to the induction of cellular proliferation (Foulstone et al., 2005). In a second step, we approached the study of 3 snap-frozen and 6 paraffin-embedded matched tissues obtained from HB patients. In these rare samples we could evaluate the markers previously evidenced and validated in liver cancer cell lines (NLK, RHOU and WNT10A transcripts; Grb-2 protein). This study showed similar results in HB matched tissues when compared to liver cancer cell lines, suggesting that these markers are reliable also in HB tissue samples.

MicroRNAs are small RNA sequences, 20-22 nucleotides long, that can bind to the 3'UTR of specific mRNAs, and regulate their expression by leading to translational repression, mRNA cleavage, and mRNA decay.

Since microRNA expression profiles are altered in several tumours compared to their normal counterparts, these molecules are largely used also in the field of cancer classification (Calin and Croce, 2006). A microRNA-array, performed in the three snap-frozen HB tissues, revealed that 51 microRNAs could distinguish tumour from non-tumour samples. By comparing our results with previously published data (Varnholt et al., 2008), we selected 9 microRNAs to be analysed in our 9 matched samples. The comparison between these microRNAs expression in tumour and in non-tumour counterparts, highlighted four up-regulated microRNAs (miR-125a, -150, -199a and -214), and a down-regulated one (miR-148a). It has been demonstrated that miR-214 binds to the PTEN mRNA and downregulates its protein levels (Yang et al., 2008). PTEN is a negative regulator of the PI3K/Akt pathway which is activated by IGF signalling, in normal conditions (Foulstone et al., 2005). In our samples as well, the comparison of tumour and normal counterparts indicated a correlation between high miR-214 levels and low PTEN protein levels in the tumour samples.

The study of liver cancer cell lines and HB biopsis revealed the inactivation of the canonical WNT pathway and the contemporary induction of the non canonical one. Furthermore the activation of IGF-signalling is also speculated on the basis of the deregulation of Grb-2 and PTEN protein levels.

In the second part of the project the association between HB onset and the exposure to a particular phthalate, DEHP, was investigated. DEHP is the most abundant phthalate in the environment and liver represents one of its main targets. Studies performed on mice and humans demonstrated that DEHP alters the glucose metabolism (Latini et al., 2008). This process involve a family of proteins called PPARs that mediates the IGF-promoted signalling (Feige et al., 2007; Latini et al., 2008), responsible of glycogen storage (Lopez et al., 1999). *In utero* exposure to phthalates has been shown to be significantly associated with prematurity (Latini et al., 2008), that in turn has been strongly associated with HB (McLaughlin et al., 2006). HB can derive either from developmental disturbances during critical phases of organogenesis or from the interplay of risk factors (e.g. DEHP) during prenatal or early neonatal life (Salvatore et al., 2008).

In order to investigate the post-natal effects of DEHP prenatal exposure on liver development, pregnant CD-1 mice were exposed to DEHP (25 and 100 mg/kg BW pro die) during the critical period of liver organogenesis and histogenesis (starting from the 11th day of gestation and till the 19th) (Duncan, 2003). Male and female F1 mice were sacrificed at post natal day

21 and 35, respesenting weaning and puberty, respectively. Histopathological, histochemical and gene expression studies performed on livers of PND35 treated mice did not show significant differences compared to controls, while at PND21 treated mice showed alterations in glucose and lipid metabolism that were absent in controls. The inactivation of GSK-3β was speculated since these mice were characterized by lack of glycogen storage and presence of cytoplasmic β-catenin. Glycogen storage is triggered by IGF-II in fetal life and ensures stable levels of glycaemia at birth when the newborn makes the adjustment to extra-uterine life (Lopez et al., 1999; Hui et al., 2006). The presence of cytoplasmic β-catenin indicates that the canonical WNT pathway is active, and that the transcription of genes involved in cell proliferation is promoted (Rubinfeld et al., 1996). PND21 mice exposed to DEHP showed hepatocytes vacuolization (hepatosteatosis), a feature of an ecess of fatty acid synthesis, as a consequence of glycogen synthesis inhibition (Shimano et al., 2007). AFP levels in mice progressively decrease after birth and are almost completely switched off in the third week of life, when liver accumulates energy as glycogen instead of AFP (Rusyn et al., 2006; Heudorf et al., 2007; Latini et al., 2009) On the basis of the association between *AFP* expression and embryonal status, this gene is also used as a marker of hepatocytes maturation (Qin and Tang, 2004). Our results showed high levels of *AFP* gene expression in treated mice compared to controls, suggestable of a role for DEHP exposure in determining both the alteration of post-natal AFP to glycogen switch off, and a delay of hepatocytes maturation.

Furthermore the possible role of microRNAs in the DEHP-induced alterations was speculated. *IGF-II* gene, fundamental in the promotion of glycogen storage during fetal life (Lopez et al., 1999; Hui et al., 2006), harbours a microRNA, miR-483, within its second intron (Fu et al., 2005; www.ensembl.org). The analysis of miR-483 expression showed that this miR can be considered a fetal marker, since its levels progressively decrease after birth. High levels of miR-483 were detected in PND21 mice treated with DEHP 100 mg/kg bw pro die, in confirmation with a role of DEHP exposure in delaying the hepatocytes maturation. We also demonstrated that this miR is able to target and downregulate β-catenin, thus representing a limit in the proliferation stimulus induced by high levels of this protein within the cytoplasm (Rubinfeld et al., 1996).

The multidisciplinary approach, performed by using *in vivo* and *in vitro* studies, enabled us to identify early and tardive markers of hepatoblastoma. Our studies indicated that the prenatal exposure to DEHP delays liver maturation by affecting carbohydrate and lipid pathways involved in fetal

nutrition. The incapability to accumulate glycogen, due to the inactivation of GSK-3β function, is counterbalanced by an enhanced synthesis of lipid, as demonstrated by the presence of hepatocyte vacuolization. Furthermore the improper increased levels of *AFP* gene expression, suggested that DEHP alters the post-natal AFP-to-glycogen switch and may delay the post natal maturation of hepatocytes, as suggested also by high levels of miR-483, another fetal marker. A biological role for miR-483 in limiting the proliferation activation induced by β-catenin has been also proposed.

The characterization of *in vitro* models (liver cancer cell lines and HB matched tissue samples) has identified new molecular tardive markers of chilhood liver cancers, at mRNA- (WNT10A, NLK and RHOU trascripts), protein- (Grb-2 and PTEN), and at microRNA- (miR-214) level. Gene expression analysis showed an inactivation of the canonical WNT signalling; and the contemporary activation of the non canonical one. At protein level, the upregulation of Grb-2 in tumour compared to non tumour samples, lets speculate the activation of the Raf/MAPK signalling pathway. The upregulation of miR-214 in tumour compared to non tumour samples seemed to affect PTEN protein levels, which resulted to be downregulated in the tumour counterpart. This evidence suggests that the activation of PI3K/Akt pathway could lead to the misregulation of the cellular functions that it controls, such as glucose metabolism, cell proliferation and survival (Foulstone et al., 2005).

Even though in *in vivo* and *in vitro* models the patterns of deregulation of WNT and IGF-signalling pathways involve different mechanisms, the importance of these pathways and their crosstalks can be considered pivotal in the onset and in the characterization of hepatoblastoma.

Introduction

1 Childhood tumours

Malignant tumours are relatively uncommon in children, when one compares them to adult cancers. However, cancer is the main cause of death due to disease in adolescents and children aged over 1 year old, and its incidence is comparable to the cumulative incidence of relatively common conditions, such as cerebral palsy, diabetes and bacteria meningitis, that affect quality of life and survival of childhood population (Ferrari et al., 2007). The annual incidence of cancer is estimated to be around 1 in 7000 children younger than 15 years of age; more than 12,000 new cases of children and adolescents less than 20 years with malignancies are diagnosed every year in the United States of America (Gurney and Bondy, 2006) (and about 1800 new cases a year in Italy) (Ferrari et al., 2007).
Though cancer is the main cause of disease-related mortality in children, all paediatric tumours could be virtually defined as 'rare'. According to the European Parliament and the Council of the European Union (Regulation EC n.141/2000), rare diseases are defined solely on the basis of low prevalence and affect not more than five individuals per 10.000 in the European population. RD are a large and diverse group of disorders; they include more than 6.000 conditions and involve all organs and tissues, often with several clinical subtypes within the same disease. Almost all RD may cause early mortality and/or long-term disability and accurate and timely diagnosis is often of great importance for their prevention and treatment. Very often information on many RD are insufficient, concerning either diagnosis and/or prognosis. About 80% of RD have identified genetic origins; however in many cases the clinical phenotype and prognosis appear to be modulated by still unknown factors (e.g. different mutations on the same gene(s), epigenetic factors, etc.). Some important groups of RD, such as birth defects and childhood tumours, originate from gene-environment interactions still poorly clarified (www.eurordis.org).

1.1 Liver tumours in children

Malignacies of the liver account for slightly >1% of all pediatric

malignacies. Two cases of liver tumours out of three result malignants. While the predominant histology of liver tumours in adult are represented by hepatocellular carcinoma (HCC), in children hepatoblastoma (HB) accounts for the two thirds of liver tumours. Also sarcomas, germ cell tumours, rhabdoid tumours and the more familial hepatocellular carcinoma are liver pediatric tumours. The treatment and prognosis in children are guided by the histology and anatomy of the liver tumour (Litten and Tomlinson, 2008).

1.1.1 Hepatocellular carcinoma

Hepatocellular Carcinoma (HCC) represents the second most common malignant tumour in children. It is markedly distinct from HB and the age of diagnosis is typically observed after 10, representing the most common malignacy of liver in adolescents. HCC can be associated to known hepatic viral infections or cirrhosis and even though in some cases the tumour can take decades to develop, occasionally cases are seen in very young children (Litten and Tomlinson, 2008).
Previous reports from Southeast Asia cite an annual incidence of pediatric hepatic tumours that is roughly four times higher than western reports in children <15 years of age (Chen et al., 2005). This finding is largely based on the high hepatitis carrier rate, with a Taiwanese report stating that 80% of primary liver tumours in children were hepatocellular carcinoma.With the introduction of hepatitis B vaccine in Southeast Asia, however, there has been a marked reduction in the incidence of hepatocellular carcinoma, although the impact of the hepatitis B vaccine has mainly reduced the incidence of liver tumours in males (Chang et al., 1997). Occasionally, malignant tumours in children are seen with features of both hepatocellular carcinoma and hepatoblastoma. These tumours are more common in children with a diagnosis at later ages than that typical of hepatoblastoma (Litten and Tomlinson, 2008).

1.1.2 Hepatoblastoma: the most common pediatric liver tumour

HB is a very rare primary liver malignancy of infants and young children, with an annual incidence of 0.5–1.5 diagnoses per 1 million children below the age of 15 years in Western countries. Despite its low incidence,

hepatoblastoma represents the most common malignant tumour in children (Schnater et al., 2003; Roebuck and Perilongo, 2006). For poorly understood reasons, hepatoblastoma occurs in males significantly more frequently, compared to females (Weinberg and Finegold, 1983). HB is classified as an embryonal cancer, since these tumours are composed of cells resembling the developing fetal and embryonic liver. The cells comprising HB mark similarly to hepatic stem cells, defined as pluripotent hepatoblasts that can differentiate into hepatocytes or cholangiocytes (Ruck and Xiao, 1997; Ruck and Xiao, 2002). Hepatoblastoma tumour develops prenatally and is therefore diagnosed in very young children having an overall median age of 18 months; only 5% of new HB cases are diagnosed in children >4 years of age. Most commonly, these tumours are present in the right lobe of the liver (Exelby, 1975). These tumours can be histologically divided into epithelial or mixed epithelial/mesenchymal tissues. The majority of HB are epithelial and composed by embryonal and fetal cell types; HBs composed of small undifferentiated cells represent the 5% of HBs cases and are characterized by a worse prognosis (Haas et al., 2001). HB prognosis depends on numerous clinical and histological factors: children with a poor prognosis are usually characterized also by abnormal α-fetoprotein levels (<100 or >1,000,000 ng/ml) and distant metastases (e.g. lung and lymph nodes), whereas patients with good prognosis appear to have, among others, a decline in circulating AFP levels during chemotherapy (Roebuck and Perilongo, 2006; De Ioris et al., 2007).
Scientific evidences point out HB as a multifactorial condition associated with: genetic conditions (e.g. Beckwith-Wiedemann Syndrome and Familial Adenomatous Polyposis) (Roebuck and Perilongo, 2006), non genetic and environmental factors such as the endocrine-metabolic status of the mother (young age, higher body mass index, use of infertility treatments, smoking) and (pre)eclampsia (McLaughlin et al., 2006). An association with occupational paternal exposure to metals, petroleum products and paints/pigments as well the possible involvement of ubiquitous environmental contaminants such as phthalates have been suggested (Buckley et al., 1989; Reynolds et al., 2004; McLaughlin et al., 2006).

Conventional cytogenetic analyses of chromosomal aberrations in HB, performed using standard karyotyping,(Tonk et al., 1994; Sainati and Leszl, 1998; Nagata et al., 1999; Park et al., 1999; Yeh et al., 2000), fluorescence in situ hybridization (FISH),(Balogh et al., 1999; Parada et al., 2000; Sainati et al., 2002; Surace et al., 2002) and comparative genomic hybridization (CGH),(Hu et al., 2000; Weber et al., 2000), have been reported. Although

these analyses have identified several chromosomal aberrations in HB, predominantly the gains in chromosomes 1q, 2, 8q, 17q, and 20 and the loss in chromosome 4q, the tumour-associated genes of HB involved in these genomic copy number (CN) alterations are yet to be identified (Suzuky et al., 2008).

1.2 Dysregulation of growth factor signalling and microRNA expression in childhood hepatic tumours

HCC represents the most used model for the study of the pathogenesis of human childhood liver tumours, even though only limited knowledge has been gathered regarding genomic alterations occuring during its development and progression. There is vast evidence for the dysregulation in HCC of protumourigenic growth factor signalling affecting different signalling systems such as IGF-, WNT-, Hepatocyte Growth Factor (HGF)-, Transforming Growth Factor a (TGFα)/Epidermal Growth Factor (EGF)- and Transforming Growth Factor β (TGFβ) signalling. Dysregulation of these factors and their pathway components has been connected with essential tumour properties such as tumour cell proliferation, antiapoptosis neo-angiogenesis, invasive behavior and chemotherapy resistance (figure 1; Breuhahn et al., 2006).

Cancer is a complex genetic disease involving structural and expression abnormalities of both coding and non-coding genes. For almost three decades, the alteration of protein-coding oncogenes and/or tumour-suppressor genes (Bishop, 1991; Hunter, 1991; Weinberg, 1991) have been thought to be the causes of tumourigenesis.
With the discovery in the past few years of thousands of genes that produce non-coding RNA transcripts with no significant open reading frame, it has become evident that the genomic complexity of the cancer cell is far greater than expected.
These microRNAs are small non-coding RNAs of 20–22 nucleotides, typically excised from 60–110 nucleotide foldback RNA precursor structures (Ambros, 2004; Bartel, 2004; Pasquinelli et al., 2005). The biogenesis of miRNAs involves a complex protein system, including members of the Argonaute family, Pol II-dependent transcription and the RNase IIIs Drosha and Dicer (Kim and Nam, 2006). MiRNAs are involved in crucial biological processes, including development,

differentiation, apoptosis and proliferation (Bartel, 2004), through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression (figure 2) (Bartel, 2004; Lim et al., 2005; Rajewsky, 2006).

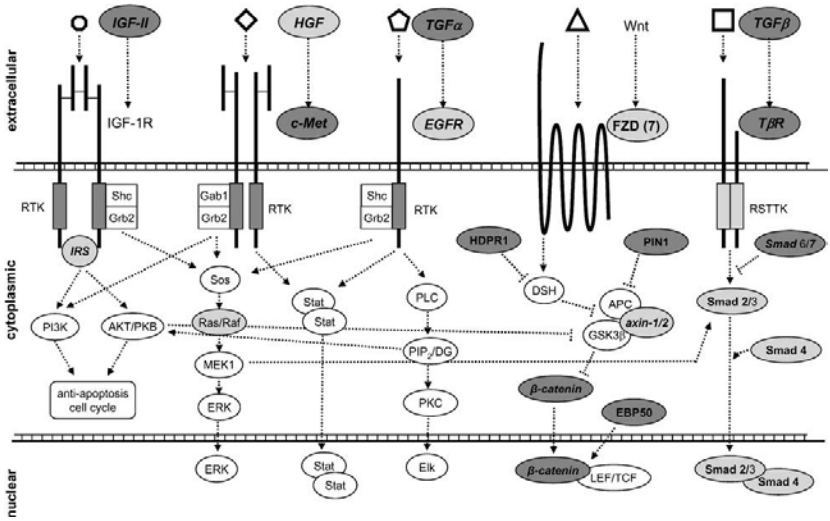


Figure 1. Schematic and simplified display of different growth factor signalling pathways frequently involved in the development and progression of human hepatocellular carcinogenesis (IGF/IGF-IR, HGF/MET, TGFα/EGFR, WNT/FZD, and TGFβ/TβR) and their potential cross-talks. Predominantly dysregulated signalling components are highlighted in dark gray. Seldom regulated components (e.g. Smad2), molecules not expressed by tumour cells (e.g. HGF) and distinct protein familiy members dysregulated in HCCs (e.g. FZD-7) are highlighted in light gray. (Breuhahn et al., 2006).

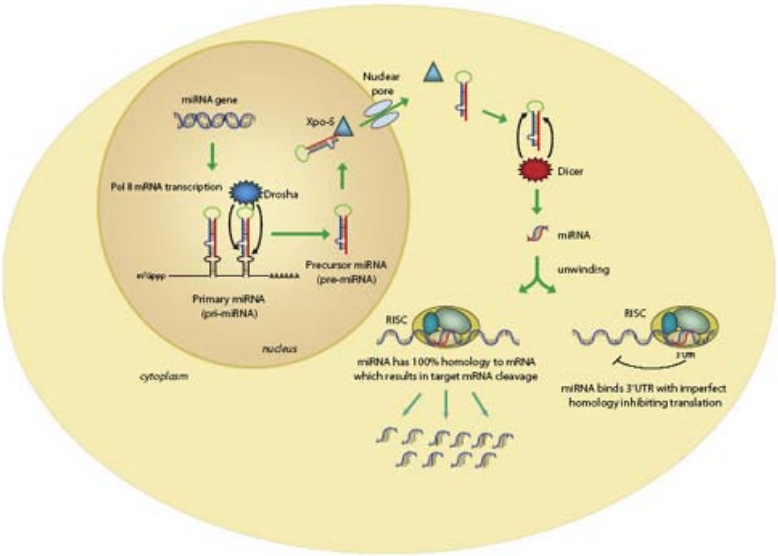


Figure 2. In the nucleus, the RNase III–type enzyme Drosha processes the long primary transcripts (pri-miRNA), yielding a hairpin precursors (pre-miRNA) consisting of approximately 70 nt. The pre-miRNA hairpins are exported to the cytoplasm where they are further processed into unstable, 19-25 nt miRNA duplex structures by the RNase III protein Dicer (Sontheimer 2005). The less stable of the two strands in the duplex is incorporated into a multiple-protein nuclease complex, the RNA-induced silencing complex (RISC), which regulates protein expression (www.invitrogen.com).

The number of human miRNAs reported is continuously updated by miRBase, the microRNA database which accounts so far over 940 human hairpin sequences (the April 2010 release of miRBase, at the Sanger Institute15, http://www.mirbase.org/).

Initially identified in B-cell chronic lymphocytic leukaemia (CLL) (Calin et al., 2002), changes in the expression level of miRNAs have subsequently been detected by different groups in many types of human tumours (several reviews give various details of the link between miRNAs and cancer, such as McManus, 2003; Berezikov et al., 2005; Caldas and Brenton, 2005; Calin et al., 2005; Chen, 2005; Croce and Calin, 2005; Gregory and Shiekhattar, 2005; Calin and Croce, 2006; Esquela-Kescher and Slack, 2006; Hammond, 2006; Hwang and Mendell, 2006). MiRNAs have been proposed to

contribute to oncogenesis because they can function either as tumour suppressors (as is the case for miR-15a and miR-16-1) or oncogenes (as is the case for miR-155 or members of the miR-17–92 cluster). The genomic abnormalities found to influence the activity of miRNAs are the same as those for protein-coding genes, such as chromosomal rearrangements, genomic amplifications or deletions and mutations. In a specific tumour, abnormalities both in protein-coding genes and miRNAs can be identified (Calin and Croce, 2006).

To date, every type of tumour analysed by miRNA profiling has shown significantly different miRNA profiles (for mature and/or precursor miRNAs) compared with normal cells from the same tissue (table 1, figure 3).

Table 1. Facts about microRNAs-expression profiling in human cancers (Calin and Croce, 2006)

Cancer type*	MiRNA profiling data	Significance
Chronic lymphocytic leukaemia	A unique signature of 13 genes associated with prognostic factors (ZAP70 and IgVH mutation status) and progression (time from diagnosis to therapy)	MiRNAs as diagnostic markers (the identification of two categories of patients)
Lung adenocarcinoma	Molecular signatures that differ with tumour histology; miRNA profiles correlated with survival (*miR-155* and *let-7*)	MiRNAs as prognostic and diagnostic markers
Breast carcinoma	MiRNA expression correlates with specific pathological features	MiRNAs as prognostic markers
Endocrine pancreatic tumours	A signature that distinguishes endocrine from acinar tumours; the overexpression of *miR-21* is strongly associated with both a high Ki67 proliferation index and the presence of liver metastases	MiRNAs as diagnostic and prognostic markers
Hepatocellular carcinoma	MiRNA expression correlated with differentiation	MiRNAs as prognostic markers
Papillary thyroid carcinoma	MiRNA upregulation (for example, *miR-221* and *miR-222*) in tumoral cells and normal cells adjacent to tumours, but not in normal thyroids without cancers	MiRNAs probably involved in cancer initiation
Glioblastoma	A specific signature compared with normal tissues	MiRNAs as diagnostic markers
Human cancers	MiRNA-expression profiles accurately classify cancers; an miRNA classifier classes poorly differentiated samples better than a messenger RNA classifier	MiRNAs as diagnostic markers
Human solid cancers	Common signature for distinct types of solid carcinomas	Specific miRNAs are involved in common molecular pathways

*Only data from microarray studies reporting results on human primary tumours were included in this table. IgV_H, immunoglobulin heavy-chain variable-reg MiRNA, microRNA. ZAP70, 70 kDa zeta-associated protein.

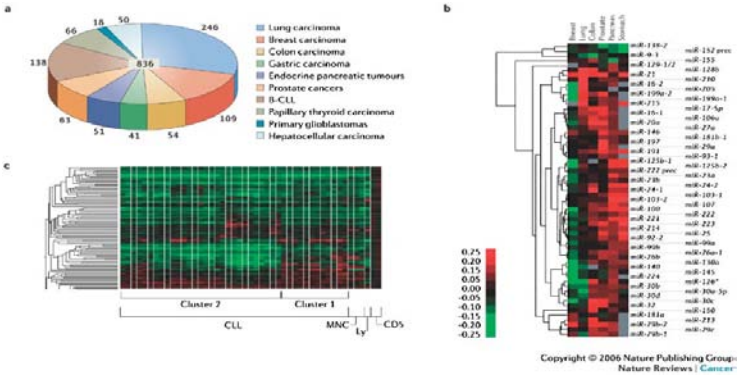


Figure 3. Examples of microRNA profiles in human solid and liquid cancers. The general consensus is that profiling enabled the signatures that are associated with diagnosis and prognois. (A) The profiling of 836 solid tissue and haematological primary cancers (and corresponding normal tissues samples) using the microarray technology (as described by Liu. et al., 2004) is shown. The exact number of analysed samples from each tumour type (corresponding normal controls included) is shown. (B) The common expression signature in six solid cancers indicates common altered regulatory pathways involving the same microRNA genes (named on the right side of the dendrogram) (Volinia et al., 2006). These miRNAs could be considered as 'cancer markers', because variations of their expression could identify the cancerous state. (C) The distinct clusters identified by miRNA profiles in chronic lymphocytic leukaemia (CLL) (Calin et al., 2004), were further confirmed to be associated with prognostic factors and disease progression (Calin et al., 2005). Note also that various normal haematopoietic samples cluster in a group that is distinct from all CLL samples. MNC, mononuclear cells; Ly, B lymphocytes; CD5, a subset of B lymphocytes largely accepted to represent the equivalent of malignant cells in CLL (Chiorazzi et al., 2005) (Calin and Croce, 2006).

In the case of HCC, the evaluation of the total content of microRNAs, showed that specific miRs are able to distinguish HCC from normal tissue with 97,8% accuracy. Also a correlation with differentiation of the tumours was found (Murakami et al., 2006).

Despite the abundance of studies focused on HCC alterations in pathways and microRNA expression, HB pathogenesis is poorly known. Several studies has focused their attention on the canonical WNT and IGF-II signallings, since their deregulation is involved in the pathogenesis of the genetic disorders, the Familial adenomatous polyposis and Beckwith-

Wiedermann Syndrome, that predispose to HB onset (Roebuck and Perilongo, 2006). FAP patients carry a germline mutation in *APC* gene, which codifies for a protein involved in the regulation of the cytoplasmic levels of β-catenin (Orford et al., 1997), the central effector molecule of the canonical WNT signalling pathway (Rubinfeld et al., 1996). BWS patients show a loss of imprinting in region 11p15 where *IGF-II* gene is localized. The LOI causes the biallelic expression and therefore, an increase of IGF-II levels, that could lead, as suggested by Veronese and coworkers, to enhanced cellular proliferation, differentiation failure and tumour development (Veronese et al., 2010).

1.2.1 WNT pathway and its role in cancer

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 well as in maintaining tissue homeostasis in the adult (Katoh, 2002; Lee et al., 2006; Khan et al., 2007). WNT signals are transduced to the nucleus through two possible pathways (figure 4). 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 APC, GSK-3β, casein kinase 1, and Axins, and its subsequent proteosomal degradation (Blaker et al., 1999; Moon et al., 2004; Clevers, 2006). Nuclear β-catenin is then complexed with the T-cell factor/lymphoid enhancer factor (TCF/LEF) to activate the transcription of target genes (He et al., 1998; Pennica et al., 1998; Tetsu and McCormick, 1999; Kramps et al., 2002; Katoh and Katoh, 2003; Chamorro et al., 2005; Sareddy et al., 2009;). As observed in several types of tumours, the aberrant activation of the canonical WNT/β-catenin signalling pathway is an important contributor to tumourigenesis (Peifer and Polakis, 2000; Takigawa and Brown, 2008). On the contrary, the non canonical WNT signals are transduced through FZD family receptors and co-receptors (Oishi et al., 2003; Lu W et al., 2004; Lu X et al., 2004) to the Dishevelled (DVL)-dependent (i.e., RHOA, RHOU, RAC, CDC42, and JNK; Boutros et al., 1998; Tao et al., 2001), or the Nemo-like kinase (NLK) and nuclear factor of activated T cells (NFAT; Ishitani et al., 2003; Dejmek et al., 2006) signalling cascades (figure 4). Particularly, NLK is a serine/threonine kinase that suppresses the transcription activity of the β-catenin/TCF complex through

phosphorylation of TCF/LEF family transcription factors to inhibit the non canonical WNT signalling pathway (Ishitani et al., 2003). Approximately 80% HBs and 20% HCCs are characterized by somatic mutations in *CTNNB1*, which codifies for β-catenin (Koch et al., 1999; Nhieu et al., 1999; Wei et al., 2000; Yamamoto et al., 2003). Mutations in *AXIN1* and *AXIN2* have been demonstrated to be important in an additional 10% of HBs and HCCs, respectively (Taniguchi et al., 2002). Note that an altered expression of specific WNT/β-catenin target genes, playing key roles in proliferation and survival of cancer cells, has been already reported (Shtutman et al., 1999; Tetsu and McCormick, 1999). 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; He et al., 1998; Shtutman et al., 1999; Lee et al., 2006), cyclin D1 (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999), AXIN2 (Jho et al., 2002; Lustig and Behrens, 2003), matrix metalloproteinase-7 (MMP-7; Crawford et al., 1999), FRA-1, c-JUN, urokinase-type plasminogen activator receptor (uPAR; Mann and Smart, 2002), and immunoglobulin transcription factor-2 (ITF-2; Kolligs et al., 2002); their role in liver cancer development is still not clear.

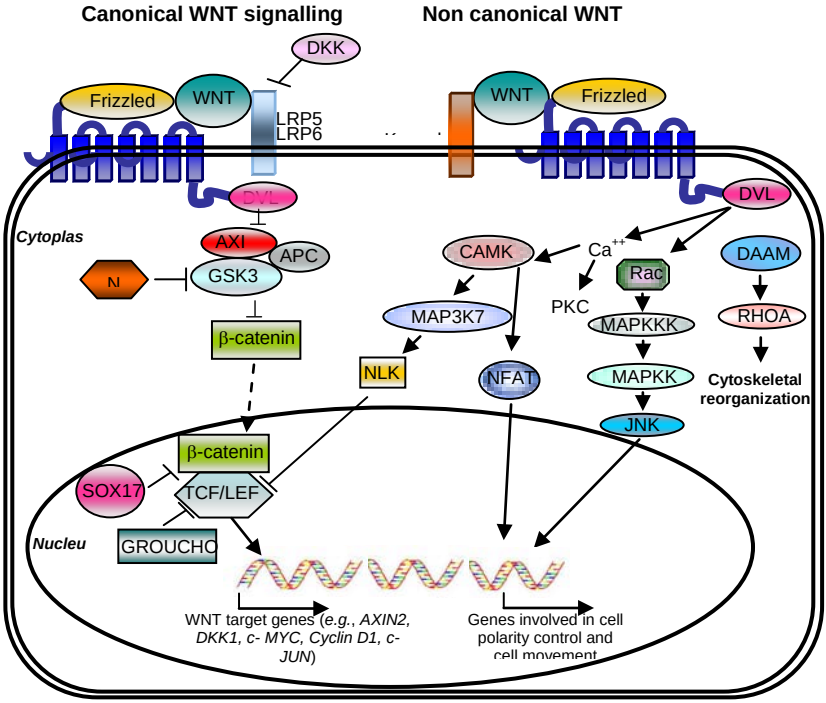


Figure 4. WNT signalling cascades. WNT signals are transduced to the canonical WNT pathway for cell fate determination, and to the non canonical WNT pathway for control of cell movement and tissue polarity. Canonical WNT signals are transduced through FZD family receptors and LRP5/LRP6 co-receptor to 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 Ca2+-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 (di Masi et al., 2010).

1.2.2 IGF system and its role in cancer

The IGF signalling pathway is of central relevance in embryogenesis as well as lifespan regulation, and exhibits potent proproliferative and antiapoptotic properties. Key regulatory molecules of this axis are small

ligands (IGF-I and IGF-II, 7–8 kDa), IGF binding proteins (IGFBP1–6) and membrane-bound receptors (IGF-I receptor (IGF-IR), as well as the mannose-6- phosphate receptor (IGF-II/M6PR, IGF-IIR). Bioavailability of both IGFs is influenced by the presence of secreted IGFBPs (Murphy, 1998) as well as IGF-IIR, which directs IGFs to lysosomal degradation (Braulke, 1999). However, the system is far more complex, as both ligands not only bind to their conventional receptors, IGF-IR (Esposito et al., 1997) and IGF-IIR, but also to the insulin receptor (isoform A, INSR; Frasca et al., 1999), and INSR/IGF-16F-IR hybrid molecules (Sakai and Clemmons, 2003). After ligand binding, rapid phosphorylation of distal intracellular targets commences specific cellular downstream effectors such as INSR-substrates IR5 (IRS1,2,4), leading to the activation of, for example PI3K/Akt pathway. The PI3K/Akt pathway is negatively regulated by the lipid phosphatase PTEN whose activity is often reduced in several cancers (Simpson and Parsons, 2001), HCC included, underlying the key role of this pathway in cancer biology (Meng et al., 2007). Moreover, binding of Grb-2 leads to the activation of the Raf/ MAPK signalling pathway. Together, this regulatory network results in increased proliferation, increased cell survival, increased cell mass, and metabolic effects (figure 5; Foulstone et al., 2005).

An increased level of *IGF-II* gene expression compared with normal livers has been reported in a high proportion of human HB. In the fetal liver, promoters P2, P3, and P4 are active and expressed monoallelically; P3 is the most active promoter and P1 is inactive. However, in the adult liver, P1 becomes dominant and is biallelically expressed, and P2, P3 and P4 activities are decreased or lost (Li et al., 1998). In addition, P4 hypomethylation has been shown in several human cancers, including HCC, suggesting that it may be an early end common event in hepatocarcinogenesis (Tang et al., 2006). Although the exact mechanism leading to these alterations is not entirely certain, it seems that LOI and/or promoter demethylation are at least partly involved (Gray et al., 2000). It has been suggested that increased IGF-II could lead to an enhaned cellular proliferation, differentiation failure, and tumour development (Veronese et al., 2010).

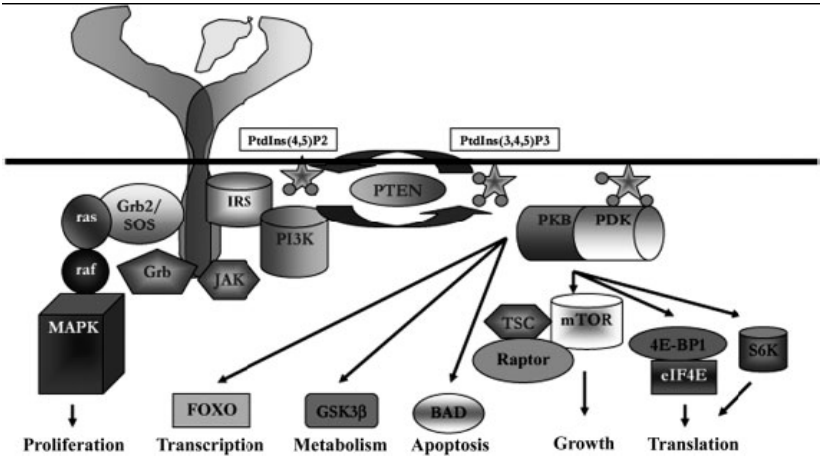


Figure 5. Intra-cellular components of the insulin-like growth factor system. A simplified figure of the intra-cellular pathway is presented. Phosphorylation of the activation loop of the cytoplasmic domain of the IR and IGF-IR results in enhanced catalytic activity of the tyrosine kinase domain. Protein–protein interactions with insulin receptor substrates (IRS) establish priming of the PI3K enzymatic conversion of membrane phospho-inositols, activation of phospho-inositide-dependent protein kinase (PDK), protein kinase B (Akt/PKB), and downstream substrates that control transcription (forkhead transcription factors, FOXO), metabolism (GSK-3β), apoptosis (bcl-associated death promoter, BAD), cell growth and translation (mammalian target of rapamycin, mTOR; tuberous sclerosis gene product, TSC; Raptor; eukaryotic initiation factor 4E, eIF4E and its binding protein, 4E-BP1; and ribososmal protein S6 kinase, p70S6K). Via similar protein–receptor interactions, activation of proliferation is mediated via the Ras GTPase-mediated pathway leading to cell proliferation (Raf, and mitogen activated protein kinase family, MAPK) (Foulstone et al., 2005).

1.2.3 Altered microRNAs in hepatocarcinogenesis

Several studies on microRNA expression profile in HCC samples have suggested that many of the miRNA changes, that occur during hepatocarcinogenesis, do so early, so that many changes that predispose to HCC have already taken place in liver cirrhosis and other premalignant lesions (Varnholt, 2008). Subsequent changes in the miRNA expression in the transition from cirrhosis to HCC seem to be much less marked (Jiang et

al., 2008). A progressive downregulation of miR-145 and miR-198 from cirrhotic tissue to dysplastic nodules and further to HCCs of increasing histological grades has been observed. The fact that abnormal miRNA expression patterns are already present in premalignant lesions has also been shown for other organ sites such as miR-143 and miR-145, which are downregulated in colonic adenomas as well as adenocarcinomas (Michael et al., 2003) and miR-221, which is upregulated in papillary thyroid carcinomas and also in peritumoural thyroid parenchyma (He et al., 2005). Changes of miRNA patterns have been demonstrated to occur before tumour formation in a HCC-model of rats exposed to tamoxifen (Pogribny et al., 2007). Therefore, it remains a tantalizing possibility that miRNAs could serve as early warning markers for cancer initiation or progression (Caldas and Brenton, 2005; Calin and Croce, 2006; Cummins and Velculescu, 2006).

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 (Hutvagner, 2006; Lee et al., 2008). 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 (Mott et al., 2007).

MiR-21 was found to be upregulated in seven different studies performed on HCCs. Meng and coworkers, have demonstrated that miR-21 targets the tumour suppressor gene PTEN, which is a key contributor to HCC pathogenesis and growth, whose protein product is frequently absent in HCCs (Meng et al., 2007).

While miR-122 is the most abundant miRNA in adult livers, other miRNAs such as miR-92a and miR-483 are highly expressed in fetal livers (Girard et al., 2008). MiR-483 is localized in the second intron of *IGF-II* gene, pointing out again the importance of 11p15 locus (Fu et al., 2005). Veronese and coworkers tested the potential oncogenic activity of this miR, proving evidence for a role as an antiapoptotic oncogene involved in human tumourigenesis. In fact they demonstrated that an anti-miRNA oligonucleotide against miR-483 could suppress the tumourigenicity of HepG2, a cell line that overexpresses both miR-483 and *IGF-II* gene. Conversely, no antitumour affect was elicited by inhibition of IGF-II (Veronese et al., 2010).

Despite the identification of several altered microRNAs in HCC, no data are available concerning HB.

1.3 Risk factors in HB: di(2-ethylhexyl)phthalate exposure and its biological effects

The exposure of the general population to phthalates, or phthalate esters, is a global problem due to their possible health impact (Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008). Phthalates are industrial chemicals commonly used to soften rigid polyvinyl chloride (PVC)-based plastics contained in food packaging, medical devices (e.g., intravenous bags and tubing), enteric coatings of some medications, consumer products (soaps, shampoos and other cosmetics), paints and pesticide formulations; as phthalates are not covalently bound to PVC, they may be released in the surrounding media, such as water, foods as well as blood (Reynolds et al., 2004).
Di(2-ethylhexyl)phthalate (DEHP) is the most abundant phthalate in the environment. In Europe, the exposure of general population to DEHP has been estimated at about 2 mg/day, based on ambient monitoring and scenario calculations (uptake of food, dust, water, etc.) (Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008). The age-related mean (and maximum) estimated exposure were as follows: 16.16 (135.28) μg/kg body weight (bw)/day in infants (< 1 yr; 5.5 kg bw), 6.31 (62.10) μg/kg bw/day in toddlers (1-3 yrs; 13 kg bw), 2.54 (14.71) μg/kg bw/day in female adults (>18 yrs; 60 kg bw) and 2.85 (16.32) μg/kg bw/day in male adults (> 18 yrs; 70 kg bw) (Heudorf et al., 2007). The European Risk Assessment Report for DEHP established a Tolerable Daily Intake (TDI) of 20 μg/kg bw/day for newborns (<3 mo) and women in childbearing age and of 48 μg/kg bw/day for general population (Heudorf et al., 2007). Therefore, a significant proportion of infants and toddlers may have DEHP intake higher than TDI. Moreover, medical devices using PVC-based materials can provide higher exposures than the environmental ones: in the case of blood transfusion, an exposure to 250-300 mg of DEHP may occur, equivalent to 4.2-5.0 mg/kg for an adult weighting 60 Kg. Neonates in intensive care units are the population group undergoing the highest exposure to DEHP relative to body weight; the daily dose may increase up to 20 folds the TDI. Small children have also a higher production of oxidized metabolites, which have to be

considered in the biomonitoring of DEHP (Heudorf et al., 2007; Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008).

1.3.1 Molecular mechanisms involved in DEHP-mediated toxicity in humans

In rodent toxicological studies, phthalates and their metabolites show several adverse effects in multiple organ systems, including the reproductive tracts, liver and thyroid; although some differences in potency and targets exist among the compounds, the developing organism appears consistently more vulnerable than the adult (Heudorf et al., 2007; Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008).
In humans, cumulating data point out the possible risk of exposure to DEHP and other phthalates. Most reported adverse effects concern reproductive health such as increased risk of poor sperm quality and endometriosis.
However, human studies start to pay attention also to consequences of *in utero* phthalate exposure: in particular, this can be associated with a shorter duration of pregnancy and with altered homeostasis of placental essential fatty acids, potentially leading to abnormal fetal development. (Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008).
DEHP can modulate, either directly or indirectly, different Nuclear Receptors (NRs), such as PPARs (Mulholland et al., 2005; Latini et al., 2008; Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008). In rodents, PPAR-α acts as a proliferative inducer and hepatocarcinogen, affecting multiple signalling pathways through the transcriptional activation of PPAR-regulated genes, and regulating other NRs such as the androgen receptor (Mulholland et al., 2005).
In humans, DEHP does not induce peroxisome proliferation suggesting that effectors other than PPAR-α might be involved in liver tumour induction or promotion (Latini et al., 2008; Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008). PPAR-γ is one major target of DEHP that mediates IGFs-promoted signalling (Feige et al., 2007; Latini et al., 2008). The molecule of PPAR-γ consists of an N-terminal domain (also called A/B domain), which is responsible for ligand-independent transcriptional regulation. The DNA-binding domain

(or C) contains two zinc-finger-like and an *α*-helical DNA binding motifs. Through the C domain, PPAR-*γ* recognizes PPRE (Peroxisome Proliferators Response Element) sequences in the regulated promoter regions. PPAR-γ interacts at different levels with IGF system and its downstream signalling pathways as MAPK, PI3K and mTOR. These crosstalks suggest that PPAR-γ acts as a an inhibitor of IGF-signalling(figure 6; Belfiore et al., 2009).

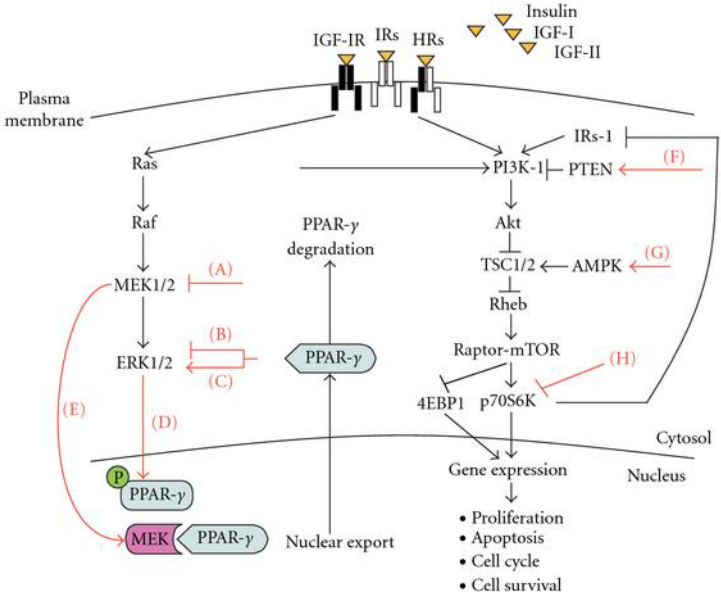


Figure 6. Crosstalk between PPAR-γ and IGF system downstream signalling pathways. The points of interaction between PPAR-γ and MAPK/PI3K pathways occur at different levels and are indicated as arrows (activation) or bars (inhibition). In some cell contexts PPAR-γ ligands reduce MEK1/2 protein expression (A) and inhibit ERK1/2 phosphorylation (B). However, in other cell systems, PPAR-γ agonists may activate ERK1/2 (C). Alternative mechanisms of interaction between PPAR-γ and MAPK/PI3K pathways include (D) ERKmediated PPAR-γ phosphorylation at Ser84/114; (E)MEK1/2-dependent PPAR-γ nuclear export followed by PPAR-γ degradation; (F) PTEN upregulation; (G) mTOR downregulation via activation of AMPK; (H) inhibition of p70S6K phosphorylation (Belfiore et al., 2009).

1.3.2 HB, altered fetal growth and exposure to phthalates

Significant exposure of human fetuses and newborns to DEHP has been demonstrated. Measurable concentrations of phthalates' metabolites have been found in the amniotic fluid, thus linking fetal to maternal exposure, direct neonatal exposure can also occur. In particular, newborns in neonatal intensive care units are a group at particularly increased risk of phthalate toxicity in this subpopulation. Exposure may reach 3 mg/kg bw/day for a 4-kg newborn due to DEHP leakage from multiple PVC-based devices for extra corporal membrane oxygenation and parenteral nutrition, and such exposures may occur for a period of weeks or even months (Scientific Commitee on Emerging and Newly-Identified Health Risks, 2008). The increased risk of HB in premature (<38 weeks) and/or LBW(<2,500 g) babies has been indicated by different authors since '90s (Reynolds et al., 2004; McLaughlin et al., 2006). Extremely low birth weight (ELBW, less than 1,000 g) was associated with a strongly increased risk of HB with an estimated 56.9 relative risk (RR) of HB in comparison to normal birth weight (> 2,500 g) (McLaughlin et al., 2006). ELBW infants diagnosed with HB had received relatively longer duration of intensive care treatments, whose diffusion has greatly improved the survival of premature infants in industrialized countries during the last decades (Bunin, 2004; Reynolds et al., 2004; McLaughlin et al., 2006). Although the link between long-term perinatal phthalates exposure and HB pathogenesis might be biologically plausible, no direct evidence has been so far reached. Epidemiological data collected by Spector and collaborators revealed that among paediatric cancers, only hepatoblastoma risk is strongly associated to children with LBW, and unlikely to be attributable to chance and bias, having been observed in the United Kingdom, in Japan and in USA (Spector et al., 2008).

As showed in 1.3.1 paragraph, PPAR-γ is one major target of DEHP and mediates IGFs-promoted signalling. The activation of PPAR-γ, as a consequence of DEHP exposure, leads to the inactivation of the IGF-signalling (Feige et al., 2007; Latini et al., 2008). In particular of IGF-II is a key regulator of fetal growth and plays important roles in placental nutrient transfer; it is abundantly expressed in fetal liver, its concentration declining after birth (Rodriguez et al., 2007). Two transitional key events are known to occur post-natally in mammalian liver and both of them involve IGF-II: the fetal-to-adult switch of the hepatic glycogen storage and the fetal-to-

adult switch of IGF-II developmental-regulated transcripts. Hepatic glycogen storage is IGF-II-dependent during fetal life whereas post-natally is carried over by insulin; at the end of gestation, glycogen storage is triggered via IGF-II to ensure stable levels of glycaemia at birth when the newborn makes the adjustment to extra-uterine life (Lopez et al., 1999; Hui Tang et al., 2006).
In case of premature babies, both the hepatic transitional events may be affected: i) the IGF-II-dependent hepatic glycogen storage is shortened by the reduced gestation, and in turn ii) the switch of IGF-II developmental-regulated transcripts takes place earlier when the major fetal IGF-II transcript is not prone yet to silencing and, concurrently, when the hepatic mitotic signalling is further triggered to replenish the insufficient hepatic glycogen storage.
Moreover, in premature babies these two crucial events take place in presence of, and may be perturbed by, phthalate exposure that can induce effects in a more susceptible tissue similarly to *in utero* exposure (Salvatore et al., 2008).

1.4 HB evidences in mouse

HB is mostly studied in mice where, although rare, it may occur spontaneously. Mouse HB derives from pluripotent endodermal stem-like cells; thus the pathogenesis is similar, but not identical, to the human cancer (Ruck and Xiao, 2002; Turusov et al., 2005; Roebuck and Perilongo, 2006). Unlike in humans, mouse HB generally occurs in aged rather than juvenile animals: this can be due to the standard testing schedule for carcinogenicity in rodents, where dosing starts in juveniles and lasts through to two years of age, i.e., the major part of the rodent lifespan. A few chemicals can increase HB incidence; likewise human HB, chemically-induced murine HB shows a higher incidence in males. In particular, the lifetime exposure to benzofuran (BF), a recognised hepatocarcinogen in adult male mice, causes HB with higher specificity in comparison to other liver tumours, although no information are available on the specific mechanism of action. (Turusov et al., 2005). In mice, the critical window for liver development and differentiation starts at gestational day (GD) 12 and is completed at birth (GD21) (Duncan, 2003). However, at present, no data do exist on the induction of HB-like alterations in rodents at early life stages and/or upon intrauterine exposure.
As in humans, the WNT/β-catenin signalling pathway plays a major role

in mouse HB: i) immunohistochemical studies have shown a loss of β-catenin membrane staining along with increased cytoplasmic and nuclear staining; ii) a high prevalence of deletion mutations affecting the GSK-3β-binding region of the β-catenin gene have been identified.
In mice, disruption of the IGF-I, IGF-II or IGF-IR genes retards fetal growth, whereas disruption of IGF-IIR or over-expression of IGF-II enhances fetal growth. On the other hand, high levels of IGF-II are often associated with perinatal lethality, whereas in humans the up-regulation of IGF-II has been described in a high percentage of HB (Gray et al., 2000; Kim et al.,.2005; Rodriguez et al., 2007). Finally. Moreover, in developing rodents, phthalates have been also shown to interfere with the IGF signalling (Bowman et al., 2005). So far, there are no data on DEHP-induced HB in mice; indeed, the range of chemicals inducing HB in mice has not been thoroughly investigated. Most important, no evidences do exist on whether prenatal chemical exposures might induce HB-related alterations in mice, especially in relation to intrauterine growth retardation and/or altered WNT/β-catenin signalling pathway.

Rationale and aim of the project

Hepatoblastoma is a rare hepatic malignant tumour of childhood and represents the most frequent primary liver cancer in children aged 6 months-3 years, accounting for up to 1% of all paediatric malignancies (Reynolds et al., 2004; McLaughlin et al., 2006; Roebuck and Perilongo, 2006). A valuable serum tumour marker, AFP, is used in the diagnosis and monitoring of hepatic tumours, since its levels are elevated in almost all hepatoblastomas (Roebuck and Perilonfo, 2006; De Ioris et al., 2007). HB shows an apparent association with familial inherited disorders such as Beckwith–Wiedemann Syndrome and Familial Adenomatous Polyposis (Roebuck and Perilongo, 2006). However, most cases are sporadic and the aetiology is likely multifactorial, e.g., a genetic predisposition interacting with environmental factors (Buckley et al., 1989; Reynolds et al., 2004; McLaughlin et al., 2006).

In order to gain new insights into the molecular and cellular mechanisms involved in the establishment and maintenance of HB, an integrated study, composed by *in vitro* and *in vivo* models, was performed.

The *in vitro* experiments were performed on 4 human liver cancer cell lines (HuH6, HepG2, Hep3B and HLE) and 9 matched liver tissue samples obtained from HB patients (aged 8-39 mo) recruited at Bambino Gesù Hospital. The aim of these studies was the identification of specific molecular markers with diagnostic and prognostic worthiness, in sporadic cases of HB. There is vast evidence for the dysregulation of protumourigenic growth factor signalling in childhood liver tumours affecting different signalling systems such as IGF-, WNT-, HGF-, TGFα/EGF- and TGFβ signalling (Breuhahn et al., 2006). We focused our attention on the canonical and non canonical WNT pathways and to the IGF-II signalling, since they are involved in the pathogenesis of FAP and BWS, respectively, the genetic disorders that predispose to HB onset (Roebuck and Perilongo, 2006). FAP patients carry a germline mutation in *APC* gene, which codifies for a protein that is part, together with Axin and GSK-3β, of the complex that controls the cytoplasmic levels of β-catenin (Orford et al., 1997), the central effector molecule of the canonical WNT pathway (Rubinfeld et al., 1996). BWS patients show a loss of imprinting in the region 11p15, where *IGF-II* gene is localized. The LOI causes the biallelic expression and therefore, an increase of IGF-II levels, that could lead, as suggested by Veronese and coworkers, to enhanced cellular proliferation, differentiation failure and tumour development (Veronese et

al., 2010).
Since sporadic cases of HB and childhood liver cancers in general, result to be very heterogeneous by the pathogenic point of view, the use of microarray technology was needed in order to perform a wide screening of gene expression, microRNA content, and protein levels. All the changes between normal and tumour samples observed in these parameters, could represent fundamental steps of oncogenesis and need to be understood. Array technology offers a global view and gives unprecedented opportunities to obtain molecular signatures of the state of activity of diseased cells and patient samples. Microarray analysis may provide invaluable information on disease pathology, progression, resistance to treatment, and response to cellular microenvironments and ultimately may lead to improved early diagnosis and innovative therapeutic approaches for cancer (Macgregor and Squire, 2002).
In a first step mRNA and protein arrays were performed on the liver cancer cell lines and using normal primary hepatocytes as a control. The mRNA array technology analyzed the expression of several genes (96) involved in WNT pathway. The very innovative protein array technology evaluated the expression levels of 224 proteins involved in biological pathways such as apoptosis, cell cycle and signal transduction.
The use of liver cancer cell lines was needed in order to select markers (transcripts and/or proteins) of childhood liver cancers, and to set the experimental parameters for their analysis; in a second step, the expression of these markers was also tested in HB matched tissues. This strategy was used in order to minimize the employment of biological materials extracted from hepatoblastoma matched tissues that are considered really precious, because of the rarity of the tumour. Paraffin-embedded samples show poor quality of biological materials because of the damages caused by this process of inclusion; therefore several analysis, such as PCR on DNA sequences longer than 200-300 bp and qRT-PCR on mRNAs, cannot be performed. Conversely, the evaluation of short non-codifing RNA sequences, the microRNAs, can be performed also in paraffin-embedded samples. MicroRNAs are small RNA sequences, 20-22 nucleotides long, that can bind to the 3'UTR of specific mRNAs, and regulate their expression by leading to translational repression, mRNA cleavage, and mRNA decay; microRNAs that are downregulated in cancer and target oncogenes act as tumour suppressors, while miRNAs that are upregulated in cancer and target tumour suppressor genes act as oncogenes (Varnholt, 2008). Furthermore microRNAs are of pivotal importance in cancer classification, since their expression profiles can distinguish tumours from

normal counterparts in several cancers (Calin and Croce, 2006).
The aim of the *in vivo* experiments was the elucidation of the association between risk factors and HB onset. Associations with factors related to the endocrine-metabolic status of the mother (young age, higher body mass index, use of infertility treatments, smoking) and to the paternal occupational exposure to metals, petroleum products and paints/pigments, have been suggested (McLaughlin et al., 2006). In particular HB appears to be linked to preterm children, characterized by low birthweight, that have to undergo intensive medical cures (e.g. treatments with oxygen and furosemide) (Spector et al., 2008). These children are exposed to phthalates, substances widely used to soften PVC, the plastic extensively employed in medical devises; DEHP is the most abundant phthalate and is considered a pollutant, since it is released from PVC, becoming an environmental contaminant (Heudorf et al., 2007; Latini et al., 2009). Peak of DEHP-exposure occurs in vulnerable subgroups, e.g., LBW newborns; epidemiological data collected by Spector and collaborators revealed that among paediatric cancers, only hepatoblastoma risk is strongly associated to children with low birthweight, and unlikely to be attributable to chance and bias, having been observed in the United Kingdom, in Japan and in USA (Spector et al., 2008). Liver is a main DEHP target, where it is responsible for the alteration of glucose and lipid metabolism. This latter mechanism is mediated by a particular family of nuclear receptors, the PPARs (Rusyn et al., 2006). In mice, DEHP exposure causes the proliferation of peroxisomes, that result in the elevation of fatty acid metabolism. This mechanism is mediated by PPAR-α, which acts also as a proliferative inducer and hepatocarcinogen. In humans, DEHP does not induce the proliferation of peroxisomes PPAR-α-mediated, but can interact with PPAR-γ, inactivating the IGFs-promoted signalling (Feige et al., 2007; Latini et al., 2008) and its downstream protein, GSK-3β (Foulstone t al., 2005).
In utero exposure to phthalates has been shown to be significantly associated with prematurity (Latini et al., 2008) that in turn has been strongly associated with HB (McLaughlin et al., 2006). The hypothesis that HB can derive either from developmental disturbances during critical phases of organogenesis or from the interplay of risk factors (i.e. DEHP) during prenatal or early neonatal life, has been already formulated (Salvatore t al., 2008). In order to investigate the post-natal effects of DEHP prenatal exposure on liver development, pregnant CD-1 mice were exposed to DEHP (25 and 100 mg/kg BW pro die) and BF (120 mg/kg BW pro die) during the critical period of liver organogenesis and histogenesis (starting from the 11th day of gestation and till the 19th) (Duncan, 2003). Male and

female F1 mice were sacrificed at weaning and puberty. Histopathological, histochemical and gene expression studies on livers were performed in order to detect alterations in glucose and lipid metabolism. As markers of glucose metabolism, the accumulation of glycogen and AFP expression levels were evaluated; in fact in mice, AFP gene expression is usually switched off during the third week of life, when liver accumulates energy as glycogen instead of AFP (Lopez et al., 1999; Mizejewski, 2001; Tan et al., 2005).

Glycogen storage is dependent on GSK-3β activation (Lopez et al., 1999), the protein responsible also for the control of β-catenin levels within the cytoplasm (Kim et al., 2005). β-catenin is the transcription factor of the canonical WNT, and induces the transcription of genes involved in cell proliferation (Rubinfeld et al., 1996). As a marker of alteration in lipid metabolism, the presence of hepatocyte vacuolization (hepatosteatosis) as a result of an enhanced fat synthesis (Shimano et al., 2007) was assessed. The analysis of the expression of fetal markers (AFP and miR-483) was performed in order to test if intrauterine exposure to DEHP may delay the post natal maturation of hepatocytes.

Results

1 Characterization of HuH6, Hep3B, HepG2 and HLE liver cancer cell lines by WNT/β-catenin pathway and protein expression profile

In this section, I present the data on a pilot comparative molecular characterization of commercially available HB-like and HCC-like cancer cell lines at gene and protein expression levels. A comparative molecular characterization of the most used liver cancer cell lines may represent a useful basis to gain new insights in the pathogenesis of HB and HCC, providing new biomarkers for childhood liver tumours.

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

In order to confirm their genotype, we checked the presence or absence of the well known mutations characterizing the HB (HuH6) and HCC (Hep3B, HepG2 and HLE) cell lines used in this study (de La Coste e t al., 1998; Koch et al., 1999; Ikenoue e t al., 2002; Zeng et al., 2007).
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 (figure 7). 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).

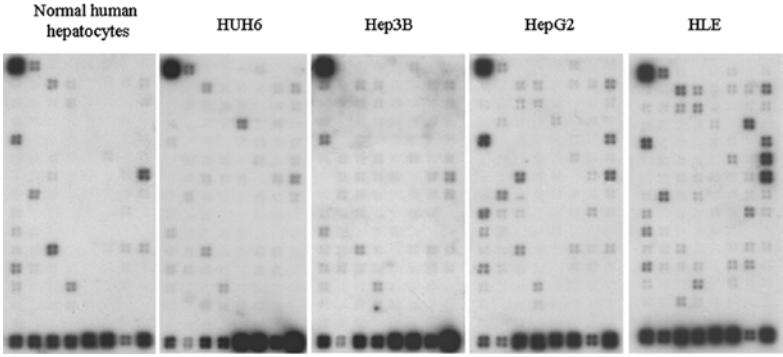


Figure 7. Parallel analysis of the gene expression profile in normal human primary hepatocytes, HuH6, Hep3B, HepG2, and HLE liver cancer cell lines. The human Oligo GEArray® WNT/β-catenin Signalling pathway microarray was hybridized with biotin-labeled cRNA obtained by in vitro transcription of each sample. For details, see text.

Table 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

1.2 Validation of the oligo GEAarray® 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 qRT-PCR (i.e., *NLK*, *RHOU*, *TCF7L2*, and *WNT10A*). qRT-PCR confirmed an increased 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 (figure. 8A). 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 (figure 8B). The protein lysate derived from Human Embrional Kidney (HEK) 293 cell line was used as positive control. Data obtained indicated a reliability of the microarray results.

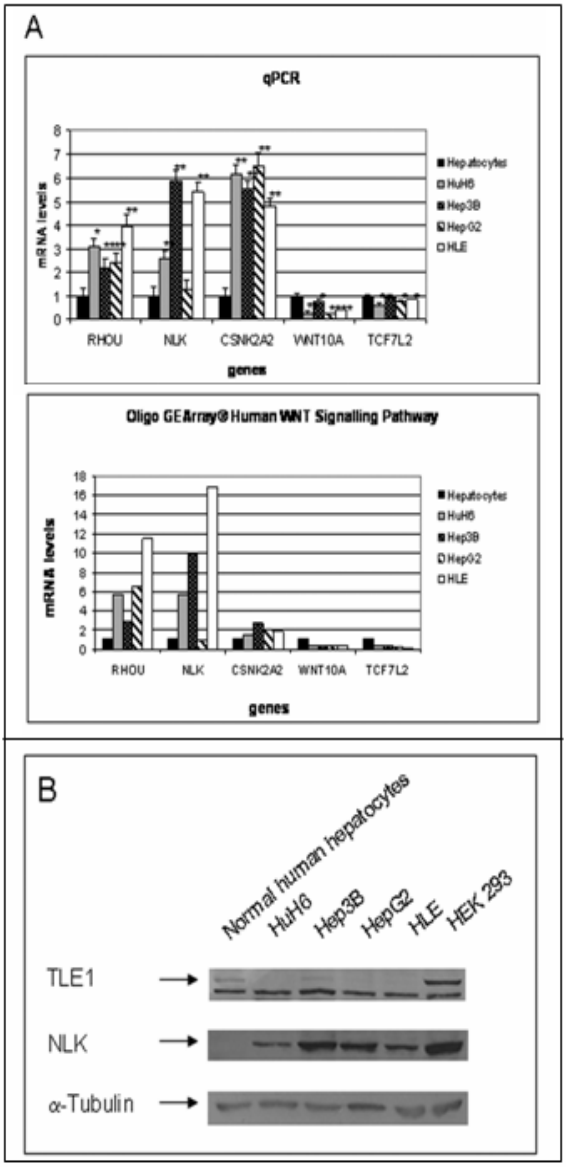


Figure 8. (A) Comparative analysis and validation of the Oligo GEArray® Human WNT Signalling Pathway Microarray data on selected genes (i.e., *RHOU*, *NKD1*,

CSNK2A2, *WNT10A*, and *TCF7L2*). The qRT-PCR 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 protein 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.

1.3 Profiling of the expression of 224 proteins involved in the cell signalling pathways

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 (figure 9). 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.

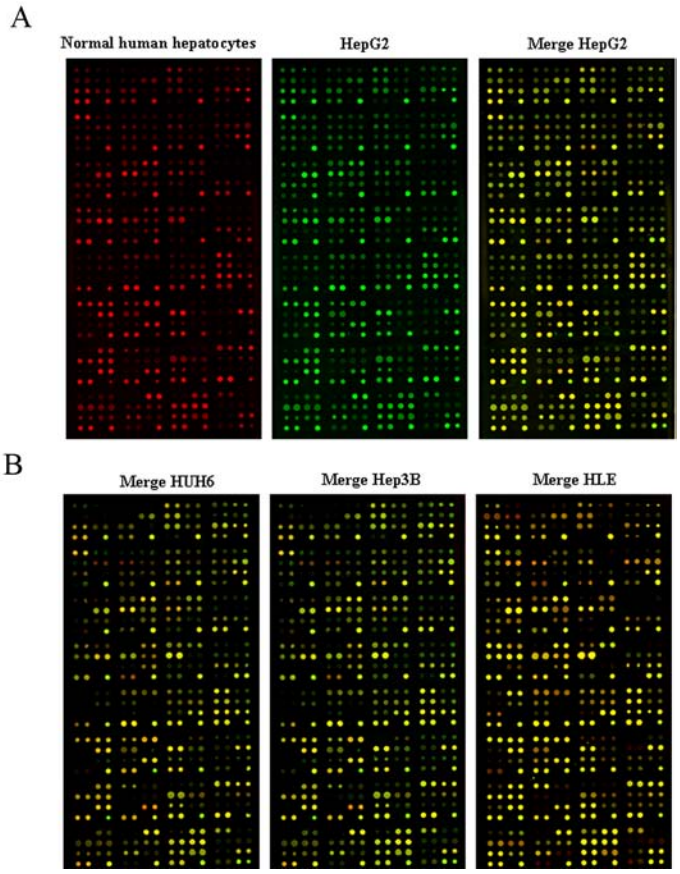


Figure 9. Protein array analysis using the Panorama^{TM} Cell Signalling Antibody Microarray containing 224 antibodies. Protein extracted from normal human primary hepatocytes were labeled with Cy5, whereas proteins obtained from each liver cancer cell line were labeled with Cy3. (A) Protein array obtained 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.

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

Protein change	Functional group	Fold
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**

1.4 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 (figure. 10). Data obtained indicated a reliability of the protein microarray analysis for the detection of changes in protein expression.

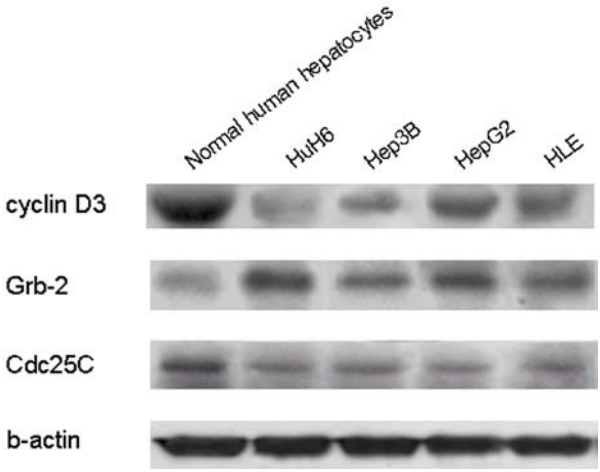


Figure 10. CyclinD3, Grb-2, and Cdc25C protein expression levels. In order to confirm the results obtained by protein array analysis, cyclin D1, Grb-2 and Cdc25C were analyzed by Western blot in normal human primary hepatocytes and in liver cancer cell lines. β-actin was used to ascertain equal loading of proteins.

2 Characterization of HB patients by the study of selected genes, mRNAs, proteins, and analysis of the total content of microRNAs

In this section, we analyzed samples from nine HB patients: three snap-frozen and six paraffin-embedded (FFPE) tissues. The dealing with rare diseases implicates a difficulty to collect a high number of cases. These samples are also usually archived for long times and then, as a result, have poor quality of extracted DNA, RNA and proteins. In this frame the use biological materials extracted from immortalized cell lines, allowed us to set the experimental conditions and to select few candidates (mRNAs and proteins) to be further investigated in tissue samples.
Because of the scarsity of quantity and/or quality of biological materials, FFPE tissues were not invastigated for: methylation specific PCR of *IGF-II* promoters, expression of mRNAs and proteins resulted to be deregulated in liver cancer cell lines (see paragraph 1 of the Results). In the three snap-frozen samples an array platform was carried out to evaluate the global content of microRNAs. Thank to the short sequence of microRNAs (about 20-22 nucleotides), they are not subjected to the strong degradation that occurs in mRNAs, which are longer. This feature allowed us to validate the array results, by quantitative real-time PCR, both in the snap-frozen samples and in the 6 additional FFPE matched tissues.

2.1 *CTNNB1*, *APC* and *IGF-II* genes characterization in HB tissues

Mutations in exon 3 of *CTNNB1* and in *APC* genes are frequent in HB (Koch et al., 1999). We performed a mutational analysis to identify possible mutations in both genes in our tissue samples.
The screening of HB samples showed a point mutation in HBT9 patient causing a A43D substitution and an additional point mutation in HBT13 causing a T40S substitution; no other point mutations nor deletions were observed in exon 3 of *CTNNB1* and its flanking regions. We also performed mutational analysis on *APC* gene and no mutations were detected in exon

15 in our HB samples (table 4).
The *IGF-II* gene transcription is driven by four promoters (P1-P4) that produce distinct transcripts which vary by tissue type and developmental stage (Li et al., 1998). In order to describe *IGF-II* promoter activity we analyzed the methylation status of selected CpG islands located in these promoters. The analysis was conducted in the three frozen matched samples (HBT13/NT13; HBT15/NT15; HBT17/NT17). No differences were detected in the methylation status of P2, P3 and P4 promoters in our samples; in particular both P2 and P3 promoters were found to be methylated in all HB samples. Conversely, P4 CpG island, within promoter 4, was hypomethylated (table 4).

Table 4. Clinical and Pathologic Characteristics of HB Patients.

Case No.	Age (months)/Sex	Histologic Subtype*	*CTNNB1*†	*APC*‡	*IGF-2*§			
					P2A	P2B	P3	P4
HBT1	8/♀	Epithelial (F/E)	WT	WT	ND	ND	ND	ND
HBT3	39/♂	Epithelial (E)	WT	WT	ND	ND	ND	ND
HBT5	17/♂	Epithelial (F/E)	WT	WT	ND	ND	ND	ND
HBT7	34/♀	Epithelial (E)	WT	WT	ND	ND	ND	ND
HBT9	24/♀	Mixed	A43D	WT	ND	ND	ND	ND
HBT11	23/♂	Epithelial (F/E)	WT	WT	ND	ND	ND	ND
HBT13	11/♂	Epithelial (F)	T40S	WT	+/+	+/+	+/+	−/−
HBT15	6/♂	Epithelial (F/E)	WT	WT	+/+	+/+	+/+	−/−
HBT17	26/♂	Epithelial (F)	WT	WT	+/+	+/+	+/+	−/−

Tumour tissues examined in this study were collected at time of surgery or biopsy of primary treated tumours after chemotherapy.
*Predominant epithelial component is given in parentheses: E indicates embryonal differentiation only; F, fetal differentiation only; F/E indicates fetal and embryonal differentiation detectable.
† *CTNNB1* exon 3 characterization by sequencing: WT indicates wild type.
‡ *APC* exon 15 characterization by sequencing: WT indicates wild type.
§IGF-II promoter methylation assay by MSP: ND indicates not determined; +, methylated; −, unmethylated.

2.2 Analysis of NLK, RHOU, TCF7L2 and WNT10A transcripts and of Grb-2 protein levels in HB tissues

In the three snap-frozen HB matched tissues, we examined the expression of NLK, RHOU, TCF7L2 and WNT10A transcripts and the levels of Grb-2 protein, that resulted to be deregulated in liver cancer cell lines compared to normal human hepatocytes. Despite the undetactability of TCF7L2 mRNA levels in tissue samples, we could observe that the pattern of deregulation was similar to that observed in liver cancer cell lines: NLK and RHOU mRNAs were upregulated in tumour counterpart compared to the normal

one, while WNT10A resulted downregulated in tumour counterpart compared to that normal (figure 11A). Similarly Grb-2 protein levels were upregulated in tumour with respect to the normal healthy counterpart (figure 11B). These evidences suggest that these markers are not only restricted to liver cancer cell lines.

2.3 MicroRNA expression profile in human HB

MicroRNA-related studies on cancers are currently based on the different expression profiles of microRNAs in cancer compared to normal tissues. Differential expression of the candidate microRNAs is a possible approach to study the function of microRNAs in cancer pathogenesis. In order to assess the putative role of microRNAs in HB, we performed a microRNA-array analysis, on the three matched frozen samples. Unsupervised clustering (figure 12), performed by using processed data from microRNA-array analysis, showed that tumour samples and adjacent non-malignant tissues were classified into two groups.
In order to identify the differentially expressed microRNAs that clustered tumour versus non-tumour tissues, we calculated mean-log2 ratio of the signal between HBT/NT. Levels of significance and magnitude of change in expression are represented in a volcano plot (figure 13); differentially expressed microRNAs, showing a good level of significance ($P<0.05$) are indicated. The use of 13 microRNAs in the unsupervised clustering analysis can discriminate tumour from non-tumour tissues (data not shown) thus indicating that these microRNAs could be valid diagnostic markers for HB, although, the small number of samples analyzed requires further future analyses.

A

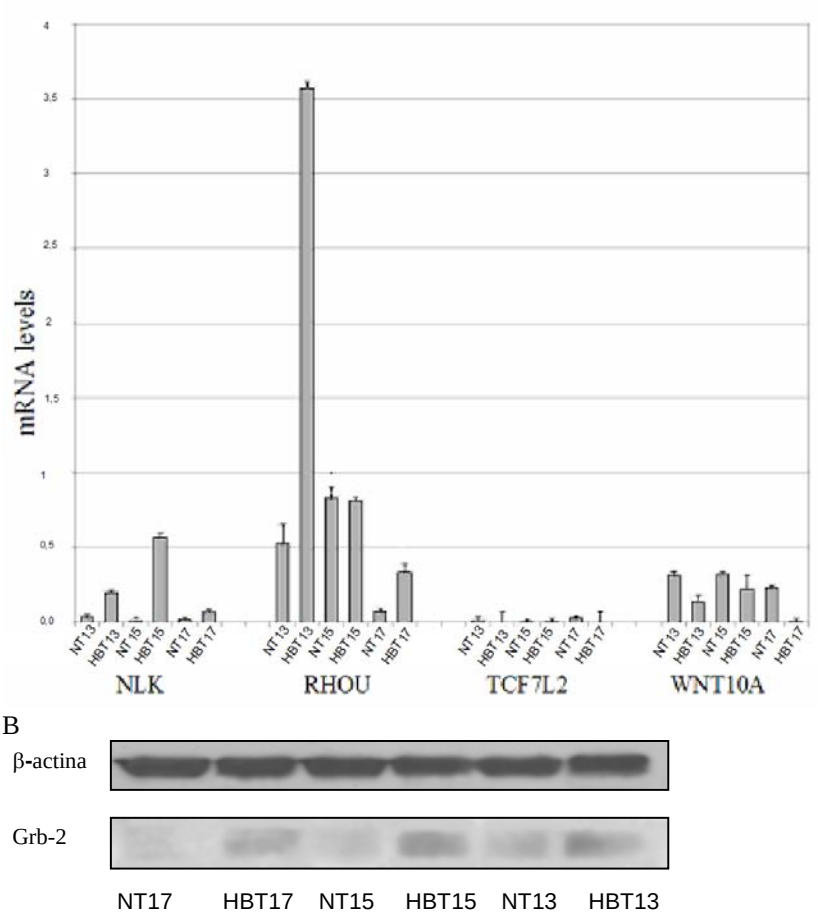


Figure 11. (A) Analysis in the three snap-frozen HB matched tissues of genes deregulated in liver cancer cell lines. The qRT-PCR graphs represent the mean of mRNA expression values in three repeted experiments with their respective standard deviations. (B) Western blot analysis of Grb-2 in the three snap-frozen HB matched tissues. β-actin was used to ascertain equal loading of proteins.

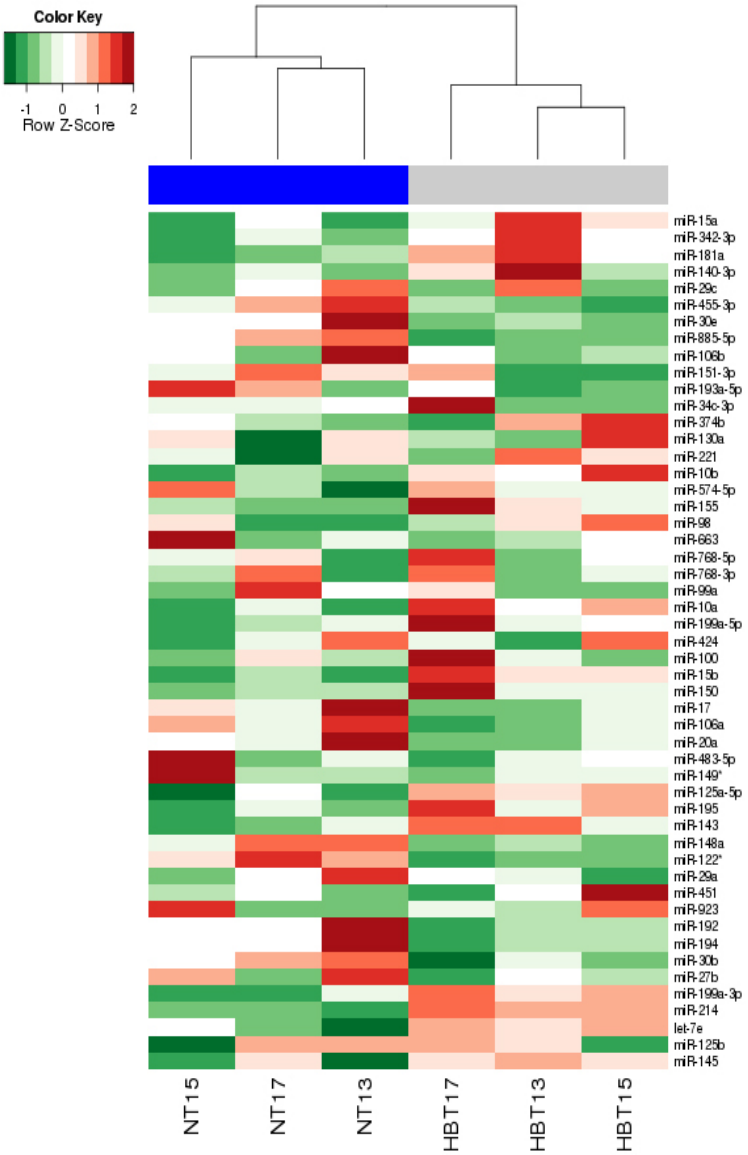

Color Key
-1
0
1
2
Row Z-Score
miR-15a
miR-342-3p
miR-181a
miR-140-3p
miR-29c
miR-455-3p
miR-30e
miR-885-5p
miR-106b
miR-151-3p
miR-193a-5p
miR-34c-3p
miR-374b
miR-130a
miR-221
miR-10b
miR-574-5p
miR-155
miR-98
miR-663
miR-768-5p
miR-768-3p
miR-99a
miR-10a
miR-199a-5p
miR-424
miR-100
miR-15b
miR-150
miR-17
miR-106a
miR-20a
miR-483-5p
miR-149*
miR-125a-5p
miR-195
miR-143
miR-148a
miR-122*
miR-29a
miR-451
miR-923
miR-192
miR-194
miR-30b
miR-27b
miR-199a-3p
miR-214
let-7e
miR-125b
miR-145
NT15
NT17
NT13
HBT17
HBT13
HBT15

Figure 12. Results of the unsupervised hierarchical clustering, in which NT tissues (blue) form a separate cluster from the HB tumours (gray). The heat map shows relative levels of microRNA expression in a green (low relative expression) to red (high relative expression) scale.

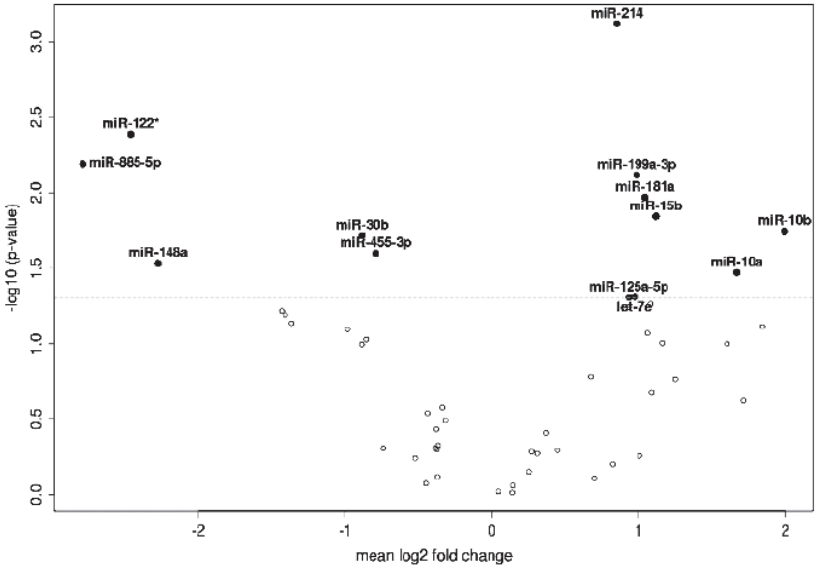


Figure 13.Volcano plot: a useful graphical that plots negative log10 P values (obtained from t test) on the y-axis versus mean log2 fold change on the x-axis, comparing altered microRNA expression in HB patients showing significant alteration of a small number of specific microRNAs. Black dots indicate altered microRNAs in HB versus NT tissues considered to be significant (P < 0.05).

2.4 Five microRNAs differentially expressed in HB showed a different pattern compared to HCC

Our results were compared with recently published data on HCC, which point out that specific microRNAs are involved in the onset and/or progression of the disease (Varnholt, 2008). In all matched samples, we validated, by qRT-PCR the microRNAs showing a different pattern of expression compared to the HCC (table 5). Analysis of selected microRNAs in HB samples (figure 14), revealed that four of them were significantly

upregulated in tumour compared to the non-malignant tissues: miR-150, miR-199a, miR-214 (P<0,01); miR-125a (P<0,05); and one significantly down-regulated: miR-148a (P<0,01). In contrast, miR-106a, miR-145, miR-195 and miR-451 were not significantly dysregulated (data not shown).

Table 5. MicroRNA Showing Different Pattern of Expression in HB Compared with the HCC.

MicroRNA	Locus	Expression in HB Fold Change*	Expression in HCC
Upregulated			
hsa-miR-10	17q2132	1.67	Up
hsa-miR-10b	2q31.1	1.99	Up
hsa-miR-125a-5p	**19q133**	**0.93**	Down
hsa-miR-140-3p	16q22.1	1.25	ND
hsa-miR-143	5q33.1	1.06	Down
hsa-miR-145	**5q331**	**1.16**	Down
hsa-miR-150	**19q133**	**1.71**	Down
hsa-miR-155	21q213	1.84	Up
hsa-miR-15	13q143	0.67	Up
hsa-miR-15b	3q26.1	1.12	ND
hsa-miR-181	1q313/ 9q33	1.04	Down
hsa-miR-195	**17p131**	**0.93**	Down
hsa-miR-199a-3p	**1q243**	**0.99**	Down
hsa-miR-199a-5p	**1q243**	**1.60**	Down
hsa-miR-214	**1q243**	**0.85**	Down
hsa-miR-221	Xp113	1.00	Up
hsa-miR-342-3p	14q322	1.08	ND
hsa-miR-574-5p	4p14	0.83	ND
hsa-miR-923	17q12	0.70	ND
hsa-miR-98	Xp1122	1.09	Up
hsa-Let-7*	19q1333	0.97	Up
Downregulated			
hsa-miR-106	**Xq262**	**–0.98**	Up
hsa-miR-122*	18q2131	–2.46	ND
hsa-miR-148	**7p152**	**–2.28**	Up
hsa-miR-149*	2q373	–0.74	ND
hsa-miR-17	13q313	–0.86	ND
hsa-miR-192	11q13.1	–1.43	Up
hsa-miR-194	1q41/ 11q13.1	–1.41	NC
hsa-miR-20	13q313	–0.89	Up
hsa-miR-30b	8q2422	–0.88	ND
hsa-miR-30	1p342	–1.37	ND
hsa-miR-455-3p	9q32	–0.79	ND
hsa-miR-885-5p	3p253	–2.79	ND

Table 5. MicroRNAs differentially expressed in HBT versus NT samples. MicroRNA analyzed in the study and compared with data from literature (expression in HCC) are in bold face font. *Values are calculated as mean[$\log^2$(HBT)] - mean[$\log_2$(NT)]: Down indicates downregulated in HCC versus NT tissues; NC, no change in the expression pattern; ND, no data are available in the literature; Up, upregulated in HCC versus NT tissues.

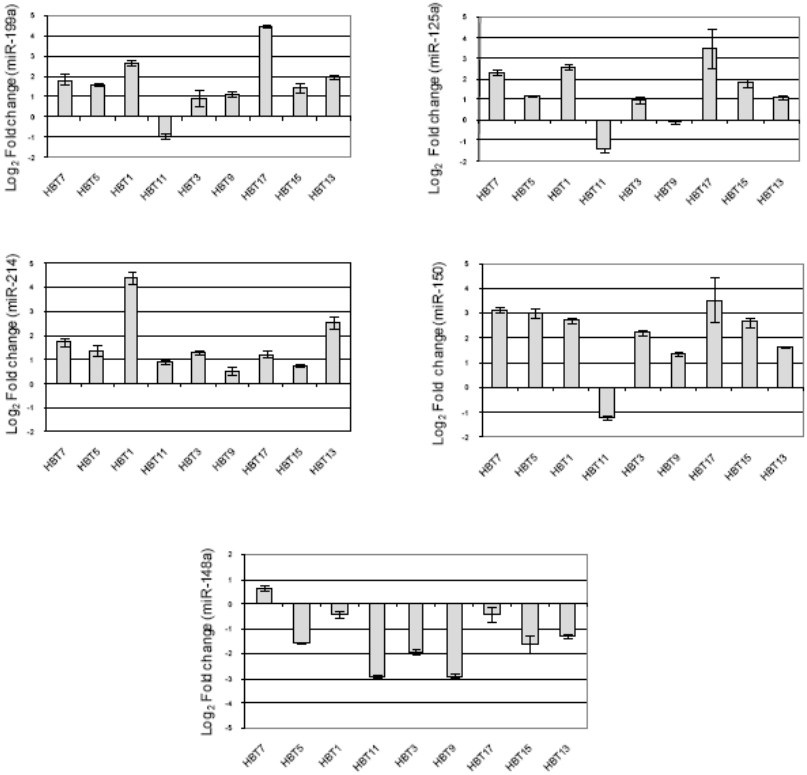


Figure 14. Validation of microRNAs showing different pattern compared to HCC in nine HB patients and in non-neoplastic tissues. Each histogram plots U6 normalized log2 fold change (ratio of tumours to non-tumours). Error bars, SD of three replicas.

2.5 MicroRNA-214 and PTEN

The tumour suppressor PTEN is a negative regulator of the PI3K signalling, and an alteration of this signalling cascade has been shown to occur in several cancers (Simpson and Parsons, 2001). Recent evidences show an active contribution of miR-214 and miR-21 in controlling PTEN protein

level (Meng et al., 2007; Yang et al., 2008). In order to verify the involvement of miR-214 and miR-21 in HB samples we measured their expression levels by qRT-PCR: while miR-21 levels were not significantly changed in all specimens, miR-214 showed an up-regulation in the tumour versus non-tumour tissues (figure 15A). Furthermore, PTEN protein levels were measured and found to be decreased in HBT13/NT13, HBT15/NT15, HBT17/NT17 samples (figure 15B), thus showing an inverse correlation with miR-214. In contrast, PTEN mRNA was measured in all samples and no significant alterations were detected (data not shown).

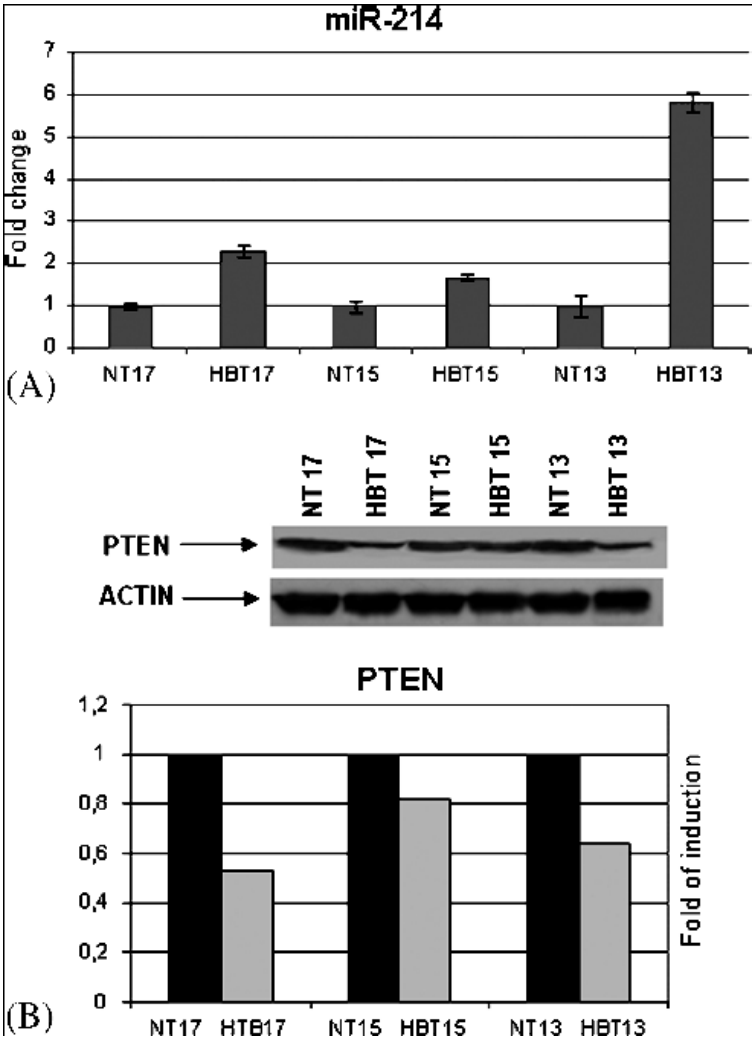


Figure 15. PTEN and miR-214 correlation of three patients from HB frozen samples and relative non-tumour tissues. (A) qRT-PCR. Bars display relative values of miR-214 expression in HB compared with non-tumour tissues (taken to 1). Error bars, SD of three replicas. (B) Western blot analysis of variations in the expression of PTEN protein and relative densitometry analysis. β-Actin was used as loading control.

3. The role of phthalates in hepatocancerogenesis: *in utero* exposure to DEHP

Pregnant mice were orally treated on gestational days (GD) 11-19, identified as a critical window for liver organogenesis and histogenesis (Duncan, 2003). The doses of administration by gavage, selected in order to minimize general toxicity, are two for di-(2-ethylhexyl) phthalate and one for benzofuran (Kavlock et al., 2002; Turusov et al., 2002). F1 animals were examined at weaning and at start of puberty (post-natal date 21 and 35, respectively) representing critical phases of the functional liver maturation (Duncan, 2003): histopathological examination of hepatic tissues, liver intracellular localization of β-catenin (at PND21 and PND35) and expression of AFP (at PND21), as a marker of delayed differentiation (Qin and Tang, 2004), were evaluated.

3.1 General toxicity and reproduction data

DEHP25 (25 mg/kg pro die), DEHP100 (100mg/kg pro die), as well as BF (120 mg/kg pro die) did not produce any signs of maternal toxicity or changes in body weight gain and food consumption. Gestation length was unaltered, all dams delivering on GD18–19. No gross malformations were observed in pups at PND1. No significant effects were observed concerning litter size, sex ratio, post-natal survival and weight gain (table 6). Weight gain and food consumption of F1 on PND21-35 were also unaffected (data not shown)

Table 6. General data on reproductive outcomes.

Parameters	CTRL	DEHP25	DEHP100	BF
Mean litter size (live pups) at birth	13.0 ± 2.8	10.6 ± 2.1	9.2 ± 2.5	10.6 ± 1.7
Male-to-female ratio at birth	0.9 ± 0.3	1.5 ± 0.6	1.3 ± 0.6	1.4 ± 0.7
Mean no. males per litter	5.8 ± 1.7	5.8 ± 0.8	6.1 ± 1.7	4.7 ± 1.5
Mean no. females per litter	6.3 ± 1.0	4.6 ± 1.1	4.0 ± 1.5	4.8 ± 1.8
Mean weight gain birth-to-PND21	8.0 ± 2.2	9.2 ± 1.9	9.0 ± 4.1	8.2 ± 1.8
Mean male weight at PND21	11.1 ± 2.6	12.0 ± 2.0	11.3 ± 3.8	11.1 ± 1.9
Mean female weight PND21	9.8 ± 2.2	10.4 ± 1.7	11.9 ± 2.2	11.5 ± 4.0

Litter data from birth to weaning PND21 on CD-1 mice daily exposed *in utero* on GD11–19 to vehicle only (CTRL), to DEHP 25 and 100 mg/kg bw pro die and BF

120 mg/kg pro die. Data were expressed as mean±SD.

3.2 Histological changes in liver

In dams, no treatment-related effects in liver weight or histology were observed. In F1 at both PND21 and PND35, absolute or relative liver weight was unaltered in all treated groups (data not shown). Histological data are summarized in table 7.
In males at PND21, dose-dependent increases in hepatocyte vacuolization (hepatosteatosis) and presence of hepatocytes with pyknotic nuclei in both DEHP25 and DEHP100 were observed. The presence of hepatocytes with pyknotic nuclei was also increased in BF males to an extent comparable to DEHP25; BF males at PND21 showed also a significant increase of hematopoietic foci. No significant changes in the incidence of these alterations were observable in DEHP- or BF-exposed males at PND35. In PND21 males, glycogen storage, as indicated by the incidence of PAS-negative liver samples showed a significant dose-related decreasing trend in the DEHP-exposed groups; compared to controls, the decrease was significant in DEHP100 as well as BF groups. At PND35, a significant dose-related decreasing trend was evident in DEHP25 and DEHP100; BF showed an effect comparable to DEHP100 (table 7).
In females at PND21, a significant and dose-related increase in the incidence of hepatocyte vacuolization was observed in DEHP25 and DEHP100, with BF showing an effect even more marked than DEHP100. No significant changes were observed in DEHP- or BF exposed females at PND35. A significant, dose-related decrease in glycogen storage was present in DEHP25 and DEHP100 at PND21; BF effect was comparable to DEHP25. No significant effect on glycogen storage was present in any exposed group at PND35 (table 7).

Table 7. Histological data.

Sex	Observations	PND21				
		CTRL	DEHP25	DEHP100	*P*-value for the Mantel–Haenszel χ^2 test	BF
Males	Marked presence of hepatocyte vacuolization/steatosis	0/8	1/9 (11%)	4/9 (44%)	0.0457	0/9
	Presence of hepatocytes with picnotic nuclei	2/8 (25%)	7/9 (78%)	9/9* (100%)	0.0027	8/9* (89%)
	PAS-negative liver tissue	0/5	1/8 (12%)	6/6** (100%)	0.0001	6/7* (86%)
Females	Marked presence of hepatocyte vacuolization/steatosis	0/8	3/7 (43%)	5/9* (56%)	0.0356	5/7** (71%)
	Presence of hepatocytes with picnotic nuclei	4/8 (50%)	1/7 (14%)	3/9 (33%)	0.3511	3/7 (43%)
	PAS-negative liver tissue	2/8 (25%)	5/9 (55%)	8/10* (80%)	0.0118	5/8 (62%)

Sex	Observations	PND35				
		CTRL	DEHP25	DEHP100	*P*-value for the Mantel–Haenszel χ^2 test	BF
Males	Marked presence of hepatocyte vacuolization/steatosis	2/7 (28%)	3/7 (43%)	4/9 (44%)	0.7178	6/8 (75%)
	Presence of hepatocytes with picnotic nuclei	1/7 (14%)	2/7 (28%)	4/9 (44%)	0.3143	3/8 (38%)
	PAS-negative liver tissue	5/8 (63%)	3/7 (43%)	2/10 (20%)	0.0417	2/10 (20%)
Females	Marked presence of hepatocyte vacuolization/steatosis	0/7	3/7 (43%)	2/7 (28%)	0.3583	1/7 (14%)
	Presence of hepatocytes with picnotic nuclei	0/7	1/7 (14%)	0/7	0.5403	2/7 (28%)
	PAS-negative liver tissue	4/7 (57%)	3/7 (43%)	3/7 (43%)	0.4334	0/7

Histological examination of CD-1 mice daily exposed *in utero* on GD11–19 to

vehicle only (CTRL), to DEHP 25 and 100 mg/kg bw pro die and BF 120 mg/kg pro die, and examined at PND21 and PND35. The dose-response trend for DEHP effects was evaluated by the Mantel–Haenszel χ^2 test.
* P≤0.05 for 2-tailed Fisher's exact test.
** P≤0.01 for 2-tailed Fisher's exact test.

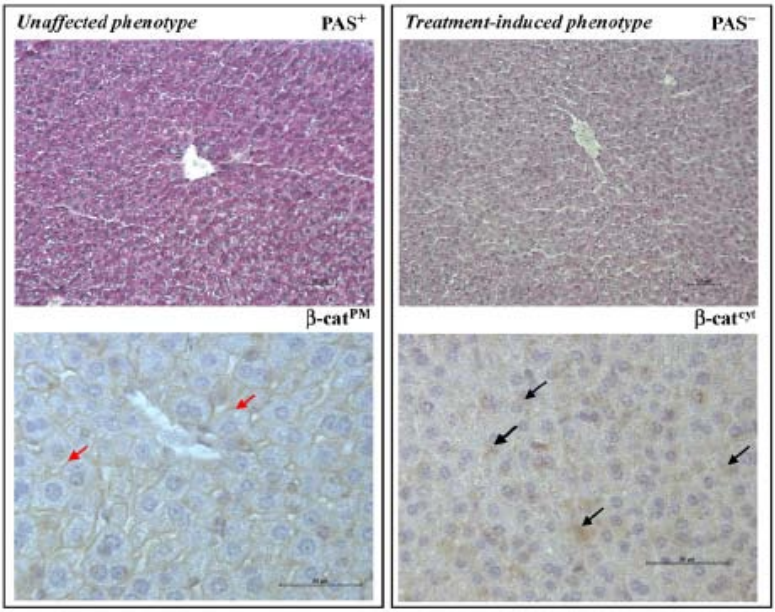
3.3 β-catenin localization in hepatocytes

At PND21, a complete cell membrane β-catenin localization was present in control (CTRL) female group while a significant and dose-dependent increase of cytoplasmic β-catenin localization was observed in DEHP25 and DEHP100 mice (P = 0.0041 Mantel-Haeszel trend test); BF effect was comparable to DEHP100. The incidence of prominent cytoplasmic β-catenin localization was as follows: 80% in DEHP25 (P = 0.0007), 75% in DEHP100 (P = 0.0023), 56% in BF (P = 0.0294) versus 0% in CTRL. In females at PND35 and in males at both PND21 and PND35, such effect was not observable since cytoplasmic β-catenin localization was evident also in controls. However, no BF-treated males at PND21 showed a membrane β-catenin localization (0% in BF versus 75% in CTRL; P = 0.0070).

3.4 Definition of a treatment-induced phenotype

Reduced hepatic glycogen storage and, in females, β-catenin localization appeared as specific features observed in all treatment groups at PND21. Therefore, we investigated whether these features might be consistently associated in a treatment-induced phenotype, identified as "lack of glycogen storage (PAS–) and β-catenin cytoplasmic localization (β-catcyt)" (figure 16A). The phenotype incidence was indeed related to treatment and sex, as shown in figure 16B. No cases of treatment phenotype were observed in controls. The incidence of phenotype in DEHP25 females and BF group was comparable. Noticeably, in DEHP100 males the presence of cytoplasmic β-catenin localization was not different from controls, but it showed an evident association with the PAS negativity; on the contrary, no cases of treatment phenotype were observed in DEHP25 males, indicating a dose-related effect.

(A)



(B)

Sex	CTRL	DEHP25	DEHP100	BF
males	0/4 (0%)	0/7 (0%)	5/5 (100%)	2/6 (33%)
females	0/7 (0%)	3/5 (60%)	4/7 (58%)	4/8 (50%)

Figure 16. Identification of treatment-induced phenotype. (A) Treatment-induced phenotype "PAS-, β-catcyt" (lack of glycogen storage and β-cat cytoplasmic localization) (right panel), and (left panel) unaffected phenotype "PAS+, β-cat^{PM}," (glycogen storage and β-cat plasma membrane localization) of CTRL mice. Photographs were taken from male mice at PND21 with 16× lens (PAS staining) or with 40× lens (β-cat localization). Red arrows identify β-cat membrane localization and black arrows cytoplasmic β-cat localization. (B) Distribution of the treatment-induced phenotype at PND21.

3.5 *AFP* gene expression

The treatment-induced phenotype and, in particular, the reduced glycogen storage suggested a possible delay of post-natal metabolic maturation of hepatocytes. Hence, as a follow-up analysis, the expression of AFP in samples of PND21 mice of both sexes from the three treatment groups

having the treatment-induced phenotype was assessed and compared with samples of CTRL mice of both sexes having the unaffected phenotype. A statistically significant increased activation of *AFP* gene expression was detected in the treatment-induced phenotype mice in comparison to CTRL: our data indicate a dose-dependent increase in the DEHP-exposed groups, whereas BF mice showed an increase comparable to DEHP25 (figure 17).

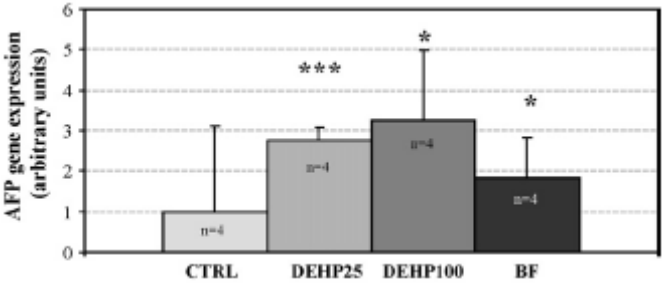


Figure 17. *AFP* gene expression. Analysis of *AFP* gene expression in liver tissues of PND21 mice of both sexes, daily exposed *in utero* on GD11–19 to vehicle only (CTRL), DEHP 25 and 100 mg/kg bw pro die and BF 120 mg/kg pro die. Liver tissue samples of treated mice (DEHP25 n = 4, DEHP100 n=4, BF n = 4) having the treatment-induced phenotype "PAS−, β-catcyt" were compared with CTRL samples (n = 4) with the unaffected phenotype "PAS+, β-cat^{PM}" (see text for details). *AFP* gene expression data are expressed as arbitrary units upon normalization to the housekeeping (GAPDH) gene expression. P-value for Student's t-test: *P≤0.05; ***P≤0.001.

4 The involvement of microRNAs in the DEHP-mediated alterations

4.1 *IGF-II* and miR-483 expression

The expression of other fetal markers, i.e. IGF-II, and the miR localized within its second intron, miR-483, were assessed in control samples of both sexes (PND4, PND14 and PND21): a progressive decrease in their levels is detectable after birth (figure 18A). The expression of miR-483 was performed in both sexes of the three treatment groups having the "PAS−, β-catcyt" phenotype. A 5-fold induction of miR-483 expression was detected in DEHP100-treated mice, while no differences were present in DEHP25, when compared to controls. Our data indicated that the lower dose of DEHP

administration do not affect miR-483 expression; BF mice showed a 1,7-fold induction of miR-483 expression compared to controls (figure 18B). For technical reasons, the q-PCR reaction for *IGF-II* was not performed in the PND21 treatment groups.

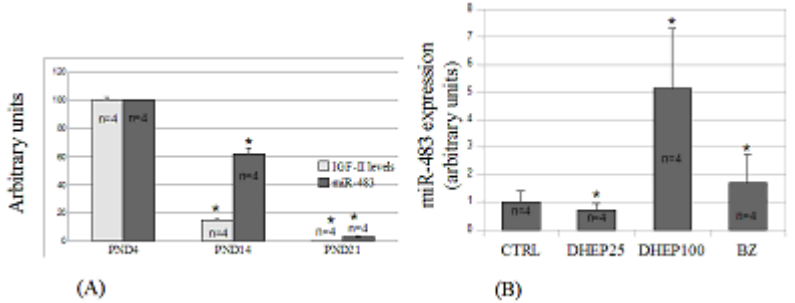


Figure 18. (A) *IGF-II* and miR-483 expression in liver of control samples of both sexes (PND4 n=4, PND14 n=4, PND21 n=4): a progressive decrease in their levels is detectable after birth. (B) miR-483 expression. Analysis of miR-483 expression in liver tissues of PND21 mice of both sexes, daily exposed *in utero* on GD11–19 to vehicle only (CTRL), to DEHP 25 and 100 mg/kg bw pro die and BF 120 mg/kg pro die. Liver tissue samples of treated mice (DEHP25 n = 4, DEHP100 n=4, BF n = 4) having the treatment-induced phenotype "PAS, β-catcyt" were compared with CTRL samples (n = 4) with the unaffected phenotype "PAS+, β-cat^{PM}" (see text for details). *IGF-II* gene expression data are expressed as arbitrary units upon normalization to the housekeeping (*GAPDH*) gene expression; miR-483 data are expressed as arbitrary units upon normalization to U6 RNA. P-value for Student's t-test: *P≤0.05.

4.2 Validation of a miR-483 target

Predictive algorythms on TargetScan Database (www.targetscan.org) showed that miR-483-3p, one of the mature products of the microRNA, could target β-catenin mRNA, both in *Homo Sapiens* and in *Mus Musculus*. The direct interaction of miR-483-3p with 3′UTRs of β-catenin mRNA was investigated in a human model, assuming that this result is conserved also in mice. The predicted wild-type and mutant miR-483-3p target sites of β-catenin mRNA were cloned downstream of the luciferase reporter gene of pGL3-Control vector. The miR-483-3p responsive vector was cotransfected with miR-483-3p into HEK 293 cells, commonly used as a biological system for the validations of microRNA targets. After the transfection with

miR-483-3p oligos, a decrease in luciferase activity was observed in presence of pGL3 containing the wild-type 3' UTR of β-catenin mRNA, compared to that carrying the mutant sequence. The cotransfaction of pGL3 containing the wild-type sequence of β-catenin 3' UTR and a scrambled oligo control did not cause a significant difference in luciferase activity (figure 19B). To further confirm β-catenin mRNA as a target of miR-483-3p, its protein levels were assessed by Western blot analysis on HEK 293 and HepG2 cells transfected with mIRIDIAN-miR-483-3p mimic or anti–miR-483-3p, respectively. Wild type β-catenin (88kDa) expression was reduced in HEK 293, while both wild type and mutant (80kDa) isoforms are induced in HepG2 cells, when compared to controls (figure 19C).

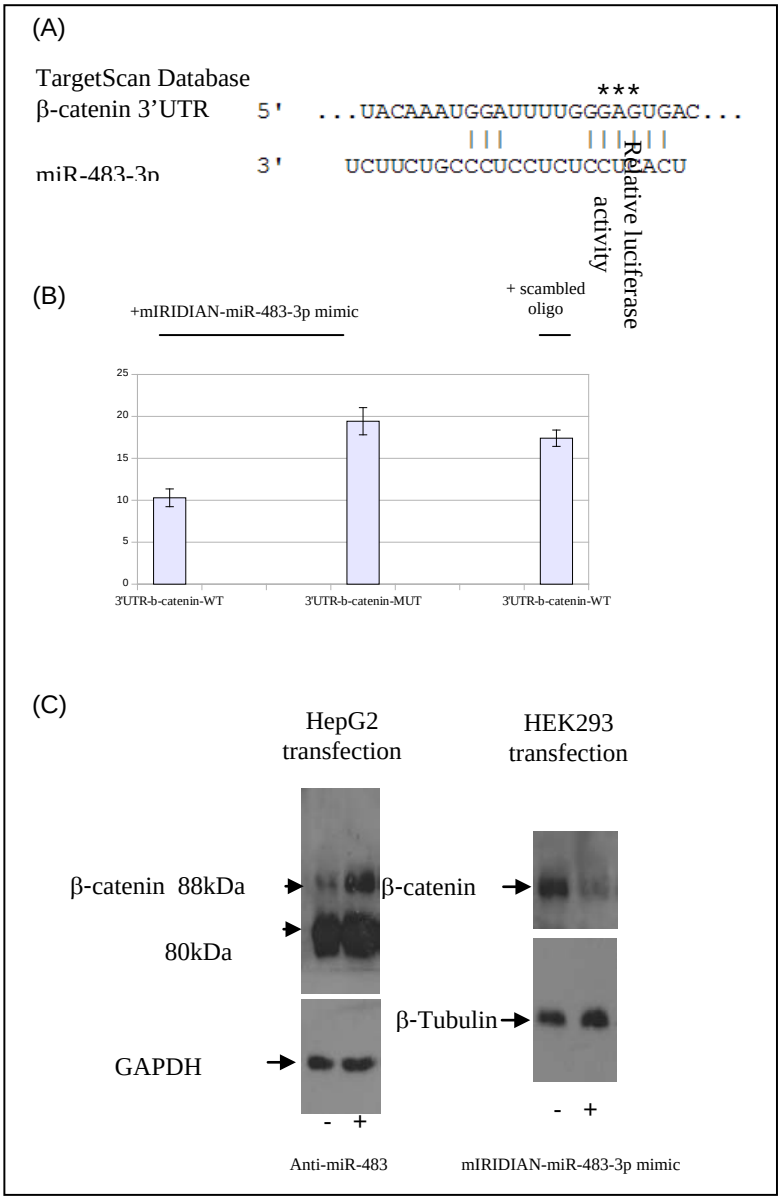

(A)
TargetScan Database
β-catenin 3'UTR
5' ...UACAAAUGGAUUUUGGGAGUGAC...
***
3'
UCUUCUGCCCUCCUCUCCUCACU
miR-483-3p
(B)
+mIRIDIAN-miR-483-3p mimic
+ scambled oligo
Relative luciferase activity
25
20
15
10
5
0
3'UTR-b-catenin-WT
3'UTR-b-catenin-MUT
3'UTR-b-catenin-WT
(C)
HepG2 transfection
HEK293 transfection
β-catenin 88kDa
80kDa
GAPDH
β-catenin
β-Tubulin
- +
- +
Anti-miR-483
mIRIDIAN-miR-483-3p mimic

Figure 19. β-catenin is a target of miR-483-3p. (A) The putative binding site of miR-483-3p in β-catenin 3′UTRs (www.targetscan.org). Asterisks indicate the deleted nucleotides in the mutant target site, used as a negative control in the luciferase assay. (B) β-catenin 3′UTRs regulates luciferase activity dependent on miR-483-3p in HEK 293 (WT, wild-type; MUT, mutant). Firefly luciferase activity was normalized on Renilla luciferase activity of the cotransfected pRL-TK vector. (C) Western blot analysis of β-catenin after transfection with anti-miR-483-3p and mIRIDIAN-miR-483-3p mimic in HepG2 and HEK 293cell line, respectively. Cells were collected after 48 h from oligos transfection.

Discussion

1 Characterization of HuH6, Hep3B, HepG2 and HLE liver cancer cell lines by WNT/β-catenin pathway and protein expression profile

Similarly to other types of tumours, mechanistic studies are commonly performed by using immortalized cell lines established from biopsis, assuming that the unlimited growth does not alter their biological features. In this study we performed a comparative molecular characterization of the most used liver cancer cell lines with the purpose to gain new insights in the pathogenesis of HB and HCC tumours, proving novel biomarkers of childhood liver cancers.

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 HB (HuH6) and HCC (Hep3B, HepG2 and HLE) cell lines, a low-density WNT/β-catenin signalling-focused mRNA microarray was performed. By this approach we found that the expression of FZD7, NLK, RHOU, SOX17, TCF7L2, TLE1, SLC9A3R1 and WNT10A transcripts was 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 the binding of WNT ligands to the transmembrane receptors, Friezzleds (FZDs) and low-density lipoprotein receptor-related proteins (LPR). 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 to have high level in a wide variety of cancer cells, including melanoma, lung (Sagara et al., 1998), esophageal (Tanaka et al., 1998), gastric (Kirikoshi et al., 2001) and colon cancer (Sagara et al., 1998; Vincan et al., 2005; Vincan et al., 2007), as well as in HCC and lymphoblastic leukaemia (Merle et al., 2004; Khan et al., 2007). Our results are in agreement with previously reported data on FZD over-expression: Merle and collaborators (Merle et al., 2004) detected an over-expression of FZD7 in the 90% of HCCs analysed, characterized by a wild-type *CTNNB1* and β-catenin accumulation, as a consequence of an hepatitis B virus chronic infection.

Another important member of the WNT/β-catenin pathway is the 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 was previously observed in human ovarian cancer (Lee BC et al., 2004). RHOU overexpression has been demonstrated also in mammary epithelial cells, where it was demonstrated to trigger cellular transformation (Tao et al., 2001); its induction is independent from β-catenin and involves non canonical WNT pathway (Lee Y et al., 2004). Overall, the contemporary induction of FZD7, RHOU and NLK transcripts, as well as the up-regulation of the 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 that the activation of the non canonical WNT pathway occured 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 (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998). Even if we did not observe *NKD1* up-regulation in the HepG2 cell line by the gene array analysis, this gene resulted to be over-expressed by qRT-PCR 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 (Jiang et al., 2002).

The Groucho/TLE family members binds to all known TCF/LEF, and acts as inhibitor of the WNT/β-catenin signalling pathway in the absence of nuclear β-catenin (Miller et al., 1999; Brantjes et al., 2001; Huelsken and Breherens, 2002; Miller, 2002). The molecular basis of the switch from transcriptional repression to activation in WNT/β-catenin signalling, is not clear, and it is also unknown whether factors other than β-catenin are required to disrupt the interaction between Groucho/TLE and TCF/LEF (Daniels and Weis, 2005). The ability to interact with many different transcription factors suggests that the function of the Groucho/TLEs might be probably dependent on the cell type and the timing of expression during development. A possible role of the TLE proteins as tumour suppressors has been suggested, since TLE1 and TLE4 are expressed at low levels in myeloid cell lines and in a subset of acute myeloid leukaemia (AML)

(Dayyani et al., 2008). Indeed, our data showed 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.
Structurally related to TCF/LEFs, several members of the Sox family of high-mobility-group (HMG) box transcription factors have been found in many cell types (Schepers et al., 2002). Sox17 directly interacts with β-catenin and TCF/LEF proteins, forming a complex with both of them. This finding is significant because one of the prevailing models is that Sox17 may antagonize WNT/β-catenin activity by competing with TCFs for the binding to β-catenin, in this case Sox17 and TCF binding would be mutually exclusive (Zorn et al., 1999; Sinner et al., 2007). Our data showed that Sox17 transcripts 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 (Sinner et al., 2007).
A further result that supports the hypothesis that the WNT 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 canonical signalling pathway since its up-regulation correlates to the re-activaction of massive proliferation in liver oval cells (Itoh et al., 2009). The SLC9A3R1 protein associates with β-catenin, acting as a positive regulator of the canonical WNT/β-catenin signalling pathway (Shibata et al., 2003). 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 (Shibata et al., 2003).
The protein array strategy revealed that Cdc25 phosphatases was down-regulated and Grb-2 was over-expressed in the cell lines investigated, compared to normal human primary hepatocytes. Cdc25C is responsible for the dephosphorylations that activate the cyclin-dependent kinases (CDK) at specific stages of the cell cycle. The expression and activity of Cdc25 is finely regulated by multiple mechanisms including post-translational modifications, and alterations to phosphatases may lead to checkpoint dysfunction and genetic instability as seen during tumourigenesis (Aressy and Ducommun, 2008). In normal conditions Grb-2 protein is activated by the IGF-signalling and leads to the induction of the Raf/MAPK signalling (Foulstone et al., 2005). An increased expression of Grb-2 therefore accounts for an aberrant activation of cell proliferation; in support to this evidence, high levels of this protein have been already correlated with increased oncogenic signalling in breast tumours (Daly et al., 1994) and HCC (Yoon et al., 2001). In addition, the gene expression analysis by

means of qRT-PCR, suggested 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 post-translationally (data not shown). Our data suggest a specific role of WNT non canonical pathways and of Grb-2 protein in the establishment and/or maintenance of the hepatocarcinogenic phenotype.

2 Characterization of HB patients by the study of selected genes, mRNAs, proteins, and analysis of the total content of microRNAs

Research approaches to pediatric rare tumours are strongly linked to the samples' availability, and although markers (i.e., *CTNNB1*, *APC*, *IGF-II*) have been successfully used in the identification and classification of liver cancers (Li et al., 1998; Koch et al., 1999), in our study, the analysis of these genes was not sufficient to characterize the differences between tumour and non-tumour samples. In fact, only two mutations were identified in *CTNNB1* gene and no differences in the methylation status of *IGF-II* promoters were observed between tumour and non-tumour samples. These results could be due to the limited number of samples which often affects the analysis performed on rare tumours. Conversely, the qRT-PCR and immunoblot analyses of transcripts (NLK, RHOU and WNT10A) and proteins (Grb-2 and Cyclin D3) deregulated in liver cancer cells (see paragraph 1 of the Results), showed similar results in tissue matched samples.

The microRNA expression profile was chosen as a further approach to highlight differences between healthy and tumour samples, representing a technique useful also for cancer classification (Calin and Croce, 2006).

In this study, global microRNA profiling identified differentially expressed microRNAs that were commonly deregulated in HB patients. We found that thirty-three out of the fifty-one filtered microRNAs were differentially expressed, and 21 of them being up-regulated, and 12 down-regulated. Moreover, we found that several microRNAs showed a completely different behavior compared to HCC. In fact, while, miR-125a, miR-150, miR-199a and miR-214 were up-regulated in our matched samples, they were down-regulated in HCC; on the contrary miR-148a was down-regulated in HB and up-regulated in HCC ((Varnholt, 2008).

Since microRNAs are small RNA sequences, 20-22 nucleotides long, that can bind to the 3'UTR of specific mRNAs, and regulate their expression by leading to translational repression, mRNA cleavage, and mRNA decay (Calin and Croce, 2006), a further study was aimed to find proteins downregulated by these miRs in hepatoblastoma tumour. MiRecords online database (http://mirecords.biolead.org/) reported that in ovarian cancer cells, miR-214 has been demonstrated to bind the 3' UTR of PTEN mRNA, leading to the down-regulation of its protein levels (Yang et al., 2008). In our three matched frozen samples, a significant reduction of PTEN protein together with an increase of miR-214 levels were found in the tumour counterpart compared to the normal healthy one, suggesting that PTEN protein is negatively regulated by miR-214 also in hepatoblastoma. This regulation doesn't affect its transcripts levels, which do not show significant variations. PTEN is a tumour suppressor and a reduction of its activity is frequently observed in several cancers (Simpson and Parsons, 2001.); this protein is of pivotal importance in the mitogenic signalling, since it's a negative regulator of the PI3K/Akt pathway, which is activated by IGF signalling, in normal conditions (Foulstone et al., 2005). These results show that although no alterations are observable in the pattern of *IGF-II* promoters methylation, the activation of the downstrem signalling cascade is here possibly due to the downregulation of PTEN protein. The effects caused by the activation PI3K/Akt pathway include the deregulation of many key cellular functions, such as glucose metabolism, cell proliferation and survival (Simpson and Parsons, 2001).

In conclusion, our results represent the first evidences to support the importance of microRNA expression profile in HB patients samples. Furthermore, the identification of five microRNAs (miR-125a, miR-148a, miR-150, miR-199a and miR-214) able to discriminate HB from HCC, could constitute valid markers for the classification of pediatric liver tumours.

Future clarification of microRNA actions and functions will substantially improve our understanding of childhood liver carcinogenesis. Development of drugs and molecules that specifically regulate hepatic microRNAs with subsequent normalization of altered target expression, may lead to novel treatments for liver cancers.

3. The role of phthalates in hepatocancerogenesis: *in utero* exposure to DEHP

This study shows, to our best knowledge for the first time, that *in utero* DEHP exposure alters post-natal liver development delaying the programming of glycogen metabolism. Such effects were observed in the absence of maternal hepatotoxicity or overt detrimental effects on litters and post-natal health. A treatment-induced phenotype, characterized by reduced post-natal glycogen storage (PAS$^-$) and increased cytoplasmic localization of β-catenin (β-catcyt), was identified. Other DEHP-related histological changes essentially evidenced hepatocyte vacuolization (hepatosteatosis), suggestive of fatty acid changes, in both sexes at weaning, with a significant dose-relationship; this finding further supports altered liver metabolic programming. Reduced glycogen storage was the only finding persisting in prepubertal animals, hinting to a critical role for this effect, which is in fact a main component of the treatment phenotype identified in our study; moreover, the change was present in prepubertal males only, suggesting an enhanced male vulnerability. The treatment-induced phenotype was accompanied by an improper increased level of *AFP* gene expression. In mice, *AFP* gene expression is usually switched off during the third week of life, when liver accumulates energy as glycogen instead of AFP (Rusyn et al., 2006; Heudorf et al., 2007; Latini et al., 2009) thus, our findings suggest that DEHP, at both dose levels, altered the post-natal AFP-to glycogen switch. Moreover, female controls at weaning still showed the perinatal and transient β-catenin inactivation as a transcription factor, indicated by prominent membrane localization (Xu and Kimelman, 2007); this feature appeared also to be altered by DEHP exposure. Overall, such findings suggest that intrauterine exposure to DEHP may delay the post-natal maturation of hepatocytes. BF induced comparable effects to DEHP, with an incidence of the treatment phenotype similar to the low-dose DEHP group. Both Ps and BF modulate glucose and lipid metabolism via NR-mediated pathways, mainly by PPARs, as evidenced both *in vitro* and *in vivo* in different tissues such as adipocytes, liver and reproductive system (Martinelli et al., 2006; Filzen et al., 2007; Malamas et al., 2007; Boberg et al., 2008; Grun and Blumberg, 2009); thus, available evidence support our findings of BF and DEHP having similar modes of action and targets in the developing liver. The liver developmental effects elicited by DEHP (and by BF as well) were unrelated to any maternal liver alteration and/or overt

prenatal or post-natal toxicity. Several studies show that DEHP effects on adult liver are reversible, including reduced glycogen storage (Gayathri et al., 2004; Rusyn et al., 2006). Taking into account that dams were sacrificed at least 21 days after the end of DEHP *in utero* administration, this can account for the lack of histopathological changes in dams. Our data lend, therefore, further support to the developing organism being more vulnerable to P effects and indicate liver as one potentially specific developmental target. The vulnerability of males to the persistently reduced glycogen storage, points to a differential role for sex-related factors, e.g., steroid-regulated pathways involving NRs cross-talk (Klaunig et al., 2003; Mnif et al., 2007; Eveillard e tal., 2009). To our best knowledge, this is the first study to report reduced liver glycogen storage as a longer-lasting and rather specific effect upon *in utero* DEHP exposure. This effect was reported also in adult rats, associated with changes in serum insulin and blood glucose (Gayathri et al., 2004); however, the alterations were reversible upon ceasing DEHP administration. The reduced glycogen storage may be linked to the increased hepatosteatosis, that was markedly increased at weaning by DEHP, with BF-treated females showing a similar effect; indeed, switching of glycogen synthesis to lipogenesis can occur when the glycogen synthesis pathway is inhibited (Shimano et al, 2007). Interestingly, hepatocyte vacuolization was no more significantly increased at puberty, when different mechanisms start to regulate liver glucose-lipid homeostasis (Spear et al., 2006). Importantly, in rodent livers the metabolic fetal-to-adult switch, replacing AFP with glycogen storage, occurs post-natally: our data indicate that the prenatal exposure to xenobiotics as DEHP and/or BF delays liver maturation by affecting carbohydrate and lipid pathways involved in fetal nutrition (Bjorntorp, 1997; Grun and Blumberg, 2009). Besides reduced glycogen storage, the intracytoplasmatic mislocalization of β-catenin was the other component of the treatment induced phenotype (PAS−, β-catcyt) identified in our study. Glycogen metabolism and β-catenin are closely linked: β-catenin localization is regulated by GSK-3 β a key regulatory enzyme of glycogen storage, further supporting a mechanistic link between the two treatment phenotype markers. It may be worthwhile to discuss whether the observed effects could impact to the proliferation/differentiation switch in the hepatocyte, thus, to the processes involved in liver tumourigenesis. DEHP exposure of human fetuses and newborns is currently raising serious concerns mainly because of potential endocrine-related effects on reproductive development (Latini et al., 2004). Although our study was performed at dose levels much greater than the expected exposure in the general population, peak DEHP intakes may occur

in vulnerable subgroups, e.g., newborns undergoing intensive medical care (Heudorf et al., 2007; Latini et al., 2009).
Some of our study findings show intriguing similarities with HB features, namely: altered β-catenin in hepatocytes and lack of AFP switch-off within the first neonatal trimester are major HB biomarkers; glycogen storage disorders, also associated with liver fatty changes, increase the risk of HB (Ito et al., 1987); finally, HB incidence is gender-related with a marked male predominance (Salvatore et al., 2008). BF showed a significant overlapping with DEHP effects: noteworthy, in ageing mice upon life-time exposure, BF selectively induced HB, originating from stem-like cells (Turusov e t al., 2002).
Human and mouse HBs show some differences in pathogenesis, but both share β-catenin alterations (Yongbaek et al., 2005) including increased cytoplasmic β-catenin accumulation (Park e tal., 2001). Therefore, our data indicate that *in utero* DEHP exposure deserves further consideration as regards the potential long-term consequences on the metabolic maturation and capability of the hepatocytes, including proneness to tumourigenesis.

4 The involvement of microRNAs in the DEHP-mediated alterations

Markers commonly used to evaluate the maturation status of liver are *AFP* and *IGF-II* genes, since they are abundantly expressed in fetal livers, while their concentrations decline after birth, both in human and in mouse (Qin and Tang, 2004; Rodriguez et al., 2007). IGF-II plays a key role in fetal growth and in placental nutrient transfer. MiR-483 was cloned for the first time in human fetal liver (Fu et al., 2005). In human and in mouse, it has been mapped within the second intron of *IGF-II* gene (Fu et al., 2005; www.ensembl.org). Our results show that in the first weeks of life in newborns mice, miR-483 levels progressively decrease, becoming almost unexpressed after 3 weeks of life. The similar trend of expression observed also for *IGF-II* is due to the miR localization and confirms that a common mechanism of transcription is shared. Among its several roles, IGF-II is responsible for the glycogen storage during fetal life, whereas post-natally this function is performed by insulin. At the end of gestation, glycogen storage is triggered via IGF-II to ensure stable levels of glycaemia at birth when the newborn makes the adjustment to extra-uterine life (Lopez et al.,

1999; Hui Tang et al., 2006). Since our results showed that intrauterine exposure to DEHP causes a lack of glycogen storage, the possible involvement of miR-483 in this process was evaluated. Results obtained indicated that miR-483 expression was not switched off at PND21 in both DEHP100 and BF, showing a 5- and 1,7-fold induction compared to controls, respectively. No differences were observed in DEHP25 treated mice, suggesting that low doses of DEHP administration do not affect miR-483 expression. The lack of miR-483 switch-off caused by DEHP100 and BF treatment, thus further confirms a delay in hepatocytes maturation.
A part of our work was aimed in the comprehension of the biological role played by miR-483 in the DEHP-mediated alterations: predictive algorythms on TargetScan Database (www.targetscan.org) showed that miR-483-3p, one of the mature products of the microRNA, could target β-catenin mRNA, both in *Homo Sapiens* and in *Mus Musculus*. Their interaction was validated in HEK 293 cells, by demonstrating that the expression of miR-483-3p downregulates β-catenin protein levels. This evidence suggests that the elevated levels of miR-483 in mice DEHP100 showing the treatment-induced phenotype, could represent an important factor in limiting the proliferation stimulus induced by the high levels of cytoplasmic β-catenin, present in these mice.

The multidisciplinary approach, consisting in *in vivo* and *in vitro* studies, enabled us to identify early and tardive markers of hepatoblastoma. Our studies indicated that the prenatal exposure to xenobiotics as DEHP and/or BZ delays liver maturation by affecting carbohydrate and lipid pathways, involved in fetal nutrition. The incapability to accumulate glycogen, due to the inactivation of GSK-3β function, is counterbalanced by an enhanced synthesis of lipid, as demonstrated by the presence of hepatocyte vacuolization. Furthermore the improper increased levels of *AFP* gene expression, suggested that DEHP alters the post-natal AFP-to-glycogen switch and may delay the post natal maturation of hepatocytes, as suggested also by high levels of miR-483 fetal marker. A biological role for miR-483 in limiting the proliferation activation induced by β-catenin has been also proposed.
The characterization of *in vitro* models (liver cancer cell lines and HB matched tissue samples) has identified new molecular tardive markers of hepatocarcinogenesis, at mRNA- (WNT10A, NLK and RHOU trascripts), protein- (Grb-2 and PTEN), and at microRNA- (miR-214) level. The mRNA-array revealed, in liver cancer cell lines compared to normal human

hepatocytes, the contemporary upregulation of antagonists genes of the canonical WNT pathway, such as *NLK* and *SOX17*, and the downregulation of its agonists genes, such as *TCF7L2*, *TLE1*, *SLC9A3R1* and *WNT10A*. These evidences suggest an inactivation of the canonical WNT signalling; at the same time the overexpression of RHOU transcript indicates the activation of the non canonical WNT signalling. Similar results are expected also for HB tissue samples, but because of the scarsity of biological materials extracted from HB tissue samples, only the expression of *NLK*, *RHOU*, *TCF7L2* and *WNT10A* genes was evaluated. qRT-PCR showed a similar pattern of deregulation in liver cancer cell lines and in HB tissue samples, thus indicating that such markers are not only restricted to liver cancer cell lines. At protein level, the upregulation of Grb-2 both in liver cancer cell lines compared to normal human hepatocytes and in HB tissues compared to the normal healthy samples, lets speculate the activation of the Raf/MAPK signalling pathway. The upregulation of miR-214 in HB tissues compared to the normal healthy samples could affect PTEN protein levels, which resulted to be downregulated in the tumour counterpart. This evidence suggests that the activation of PI3K/Akt could lead to the misregulation of key cellular functions that it controls, such as glucose metabolism, cell proliferation and survival (Foulstone et al., 2005). Although no alterations in *IGF-II* promoter methylation are observable in normal compared to the HB tissue samples, an activation of the downstream proteins of IGF-signalling is suggested as a consequence of the deregulation of Grb-2 and PTEN expression.

Furthermore, even though in *in vivo* and *in vitro* models the patterns of deregulation observed for WNT and IGF-signalling pathways involve different mechanisms, the importance of these pathways and their crosstalks can be considered pivotal in the onset and in the characterization of hepatoblastoma.

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