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THE ROLE OF NEUROFILAMENT-LIGHT POLIPEPTIDE (NEFL) GENE IN THE ONSET AND PROGRESSION OF HEPATOCELLULAR CARCINOMA

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Presentata da:	Dott.ssa Puggioni Carla Teodora
Coordinatore Dottorato	Prof. Gaetano Di Chiara
Relatore/Tutor	Prof. Amedeo Columbano

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INTRODUCTION

1. HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer mortality in the world (*Ferlay J et al, 2010*). The burden of cancer is increasing in economically developing countries as a result of population aging and adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and unhealthy dietary habits. Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver cancer (*PaRaskevi and DePinho, 2006*). HCC is seldom detected at an early stage and once detected treatment faces a poor prognosis in most cases (*Singh P et al, 2012*).

1.1 EPIDEMIOLOGY

Hepatocellular carcinoma (HCC) belongs to the group of epithelial cancers and represents, with a frequency of about 85%, the most common primary liver cancer (*McKillop IH et al, 2006*). The HCC incidence may be estimated between 500.000-1.000.000 new cases for year, globally (*Gomaa AL et al, 2008*), and is characterized by a wide geographic variation; it ranges from less than 10 cases/100,000 in the USA and Western Europe to 50-150 cases/100,000 in areas of Africa and Asia (*Blum HE and Spangenberg HC, 2007*).

This enormous discrepancy can be explained by the different distribution of some of the most important risk factors of HCC, like hepatitis B virus (HBV) infection or exposure to Aflatoxin B1- contaminated food, which are predominant in developing countries (*Llovet*

JM et al, 2003). Incidence of HCC is not only characterized by regional differences, but also by sex dependence, as the incidence in men is about twice as high as in women.

Although the incidence of HCC rises with increasing age, reaching its peak in those aged above 65 years and more commonly in men (*Rudolph KL. et al, 2000*), over the past two decades there has been a shift in incidence towards a younger age group. The number of HCC-related deaths appears to be stabilizing, or even decreasing in some Asian population studies (*McGlynn KA et al, 2001*), a result of improved surveillance and treatment regimens together with aggressive HBV vaccination programmers. In contrast, there has been a reported increase in mortality rates in the western world, for example in the United States, where middle aged men in particular are most affected (*El-Serag HB and Rudolph KL, 2007*).

1.2 RISK FACTORS

Liver cirrhosis is the most common condition in the majority of cases of HCC and HBV-related chronic hepatitis and the main cause of HCC in the world (*Parkin DM et al, 2001*). Usually HCC occurs on an established background of chronic liver disease and it rarely develops in a healthy liver. A number of underlying risk factors are recognized in the development of HCC; some are well established whilst the role of others remains controversial.

1.2.1 Hepatitis B virus (HBV)

HBV is the commonest risk factor for the development of HCC, a fact particularly relevant to HBV endemic areas, where vertical transmission is responsible for the majority of HBV acquisition (*El-Serag HB and Rudolph KL, 2007*). In such patients HCC can develop before the onset of cirrhosis as a result of the direct oncogenic effects of HBV.

With the increasing global implementation of HBV immunization, it is anticipated that the incidence of HCC will decline as a consequence of the reduced burden of HBV infection (*Kane MA, 2003*). Several factors are implicated in the pathogenesis of HBV related-HCC, including the chronic liver damage by HBV with necro-inflammation and consequent regeneration of hepatocytes. The other is the direct oncogenic property of HBV which integrates itself into the hepatocyte DNA, producing cis- or trans activation of cellular oncogenes (*Liu CJ and Kao JH, 2007*).

1.2.2 Hepatitis C virus (HCV)

Chronic HCV infection is a major risk factor for HCC development in Western European and North American countries (*Montaldo G. et al, 2002*). Usually, HCC develops after 10-20 years of HCV infection and the increased risk is largely restricted to patients with cirrhosis or advanced fibrosis; the significance of HCV genotype as a risk factor for HCC is a subject of debate but, in a recent meta-analysis, HCV genotype 1b was implicated as key factor in HCC development, particularly in the context of early stage liver disease (*El-Serag HB and Mason AC, 2000*). Follow-up studies have shown that patients with combined HBV and HCV infection have a higher risk of developing HCC than those with HBV- or HCV alone (*Montaldo G. et al, 2002; Sato S. et al, 1994*).

1.2.3 Aflatoxin B1 (AFB1)

Aflatoxin B1 is a mycotoxin produced by the *Aspergillus flavus* and *Aspergillus parasiticus fungi.* Areas of high AFB1 exposure correspond to a high prevalence of HCC, and the Agency for Research on Cancer classifies it as carcinogenic compound (*IARC, 1987*). Aflatoxin B1 is metabolized to an active intermediate, AFB1-exo-8,9-epoxide, which can bind DNA and cause damage, and has been linked with mutations consisting in a G to T transversion at the third position of codon 249 of the *p53* gene (249ser)*(Garner RC et al,*

1972), a common finding in HCC *[Bressac B et al, 1991; Turner PC et al, 2002].* This mutation may be valuable as a biomarker, since it is also detectable in circulating cell-free DNA from the plasma, and some studies suggest that the detection of serum (249ser) months before the clinical diagnosis of HCC may permit early diagnosis and facilitate targeted treatment. Some reports suggest that the (249ser) mutation is more common in HBV-positive tumors than in HBV-negative tumors. Indeed, it has been observed that areas with a high prevalence of HCC and high aflatoxin intake also correspond to areas with endemic HBV infection (*Montaldo G et al, 2002; Groopman JD et al, 1996*).

1.2.4 Alcohol

Prolonged heavy alcohol intake leading to cirrhosis represents one of the common and well established risk factors for HCC (*Morgan TR et al, 2004*). Recent reports demonstrate that ingestion of more than 80gr/day of alcohol for more than 10 years increases the risk o developing cancer about 5 times. On the other hand, alcohol acts as cofactor with other risk factors in developing countries. Positive synergism exists between alcohol intake, HBV and HCV infections in escalating HCC risk.

The mechanisms by which alcohol causes HCC are incompletely understood, but it is hypothesized that oxidative stress, altered retinoic acid metabolism, DNA methylation, genetic susceptibility and decreased immune surveillance may play a role (*Morgan TR et al, 2004*).

1.2.5 Nonalcoholic fatty liver disease (NAFLD)

NAFLD is the most common liver disorder in western countries and Japan probably as a result of the rise in obesity and diabetes mellitus. It occurs in the absence of alcohol intake, although liver histology appears consistent with alcoholic hepatitis.

The most serious form of NAFLD is the nonalcoholic steatohepatitis (NASH) that represents the hepatic manifestation of several metabolic disorders (*Falck-Ytter Y et al, 2001; Angulo P et al, 1999*). NASH represents the advanced disease progression stage in the spectrum of NAFLD and as much as 20% of NASH may progress to cirrhosis as well as end stage complications such as HCC. NASH-associated HCC was reported to be linked with liver cirrhosis in the majority of studies (*Bugianesi E. et al, 2007*). The relationship between NASH and HCC is supported by the notion that HCC develops in patients with obesity and diabetes, both of them positively associated with NASH (*Sanyal AJ et al, 2009*).

1.2.6 Metabolic disorders

Obesity and diabetes are the most common metabolic disorders associated with an increased risk of HCC and several cancers *(Calle EE at, 2003; Bianchini F et al, 2002; Giovannucci E et al, 2010; EL-Serag HB et al, 2006)*. In a cohort of 900.000 american adults, the risk of dying from liver cancer has been reported to be 4.5 times higher in men with a body mass index (BMI) ≥ 35kg/m^2 or above compared to the reference group with a normal BMI (18.5 to 24.9 kg/m^2) (*Polesel j et al, 2009*). A recent meta-analysis concluded that the summary relative risk of liver cancer was 117% for overweight subjects and 189% for obese individuals *(Larsson SC and Wolk A, 2007).* Substantial evidence indicates that also diabetes promotes development and progression of HCC *(Giovannucci E et al, 2010; El-Serag HB et al, 2006)*. It has been demonstrated that diabetes confers a three-fold increased risk of HCC (*Davila JA et al, 2005*), and also that synergistic interactions exist between diabetes and other HCC risk factors (*Baffy et al, 2012; Hassan MM et al, 2002*).

1.3 DIAGNOSIS OF HEPATOCELLULAR CARCINOMA

The European Association for Study of Liver disease (EASL) has proposed recommendations for the diagnosis of HCC, which are crucial for both early detection and the implementation of appropriate treatment. A number of different treatment modalities, dependent on the disease stage and offering varying prognoses, are recognized as possible options for the management of HCC. The concept of combining therapies has also been considered to improve survival. A known cirrhotic patient presenting a liver lesion on ultrasound >2cm in diameter has a greater than 95% chance of having HCC [*Frazer C, 1999*]. An Alpha Fetoprotein (AFP) level >200ng/ml, as well as radiological features consistent with HCC (e.g. hypervascularity) obtained on two dynamic imaging modalities such as Computerised Tomography (CT) and Magnetic Resonance Imaging (MRI), provides the diagnosis of HCC and negates the need for liver biopsy [*Bruix J. et al, 2001*]. Conversely, if the AFP is <200ng/mL and the characteristic vascular profile is not visualized on imaging, then liver biopsy is indicated to confirm the diagnosis.

For lesions of 1-2 cm in diameter a guided liver biopsy should be performed, regardless of their vascular profile [*Bruix J. et al, 2002*]. However, it must be taken into account the technical difficulty of biopsying such small lesions and the discrepancy that occurs between pathologists in discriminating between dysplasia and well differentiated HCC. Thus it is arguable that lesions measuring between 1-2 cm should first be imaged with two dynamic studies and if the findings are not characteristic of HCC, only then proceed to biopsy [*Bruix J. Et al, 2005*]. Liver lesions smaller than 1cm in diameter are far less likely to be malignant in nature, especially on a background of cirrhosis and even less so if they fail to take up contrast on dynamic imaging [*Iwasaki M. et al, 1998*].

However, the potential for malignant transformation of even tiny nodules over time still remains [*Fracanzani AL. Et al, 2001; Takayama T. et al, 1990*] and it is therefore prudent to continue ultrasound follow-up every 3-6 months in order to prevent HCC development [*Bruix J. Et al, 2005*]. A lack of increase in size over a period greater than 1-2 years permits return to the routine surveillance programme [*Bruix J. Et al, 2005*]. Comparing different tumour markers for early HCC diagnosis, AFP had a sensitivity of 66% and specificity of 81%, at a new cut off of 10.9 ng/mL [*Marrero JA, et al, 2009*]. The serum biomarkers Protein Induced by Vitamin K Absence or Antagonist-II (PIVKA-II) and glypican-3 (GPC3) have recently been assayed in patients with HCC (*Beale G et al, 2008*). The clinical or symptomatic phase of HCC is characterized by the occurrence of symptoms caused by the tumor burden. In patients with chronic liver disease, HCC usually becomes symptomatic when it reaches a size of 4.5-8cm (*Yuen MF et al, 2000; Trevisani F. et al, 2002*).

1.4 GENETIC AND EPIGENETIC EVENTS IN HEPATOCARCINOGENESIS

Hepatocarcinogenesis is a very complex multistep process whereby HCC development correlate the presence of chronic liver damage, and only rarely occurs in individuals with healthy liver. Cirrhosis is the predisposing condition to the development of HCC, which is diagnosed after about 20-40 years of latency. The aim of current research studies is to understand the molecular basis of hepatocarcinogenesis, namely the genetic and epigenetic changes that occur during the stages of initiation, promotion and progression of the pathology (*Aravalli RN, 2008; El Serag HB and Rudolph KL, 2007*). In a setting of chronic inflammation, the organ microenvironment experiences a variety of molecular changes (*Grisham JV, 2001; Bosh FX et al, 1999; Buendia MA, 2000*).

In liver, cytokines and reactive oxygen and nitrogen species produced by inflammatory cells have been shown to mediate liver damage and induce the liver's regenerative response. This predisposes the proliferating cell to a variety of changes at the genomic and transcriptional levels, increases the risk of genetic mutations in hepatocytes and promotes survival and expansion of initiated cells (*Levrero M, 2006; Maeda S, 2010; He G and Karin M, 2011*). Additionally, reactive oxygen species (ROS) and nitrogen oxygen species (NOS), generated by both "initiated" and infiammatory cells, could accelerate hepatocarcinogenesis through several mechanisms such as the induction of oxidative DNA damage, aberrant DNA methylation, and ultimately hepatocyte injury (*He G and Karin M, 2011*).

Large-scale quantitative comparisons of HCC with non-tumoral tissue by the use of comparative genomic hybridization (CGH) arrays and loss of heterozygosity (LOH) analysis have revealed the occurrence of chromosomal and microsatellite instability in HCC subtypes (*Wilkens L et al, 2000; Chen YJ et al, 2000*).

The most frequently deleted chromosomes arms are 1p, 4q, 6q, 8p, 9p, 13q, 16p, 16q and 17p and regional gains are most often observed in 1q, 6p, 8q and 17q (*Thorgeirsson SS and Grisham JW, 2002; Homayounfar K et al, 2009*), which, in general correspond to autosome arms that contain allelic deletions identified by LOH: 1p, 1q, 4q, 5q, 6q, 8p, 9p, 13q, 16p, 16q and 17p [*Thorgeirsson SS and Grisham JW, 2002; Homayounfar K et al, 2009; Laurent-Puig P et al, 2001*]. Regardless of tumor size, individual HCCs can present multiple allelic deletions and chromosomal gains and losses, which can accumulate during successive cell proliferation events and result in a heterogeneous mixture of genomic aberrations.

The heterogeneity of tumors can help to identify tumor origin and, due to the sensitivity of CGH and single nucleotide polymorphism (SNP) arrays, genomic alterations can be used as fingerprints to identify whether a tumor is a recurrent event or a second primary tumor [*Wilkens L et al, 2000; Chen YJ et al, 2000*]. The frequent loss of chromosome regions observed by LOH and SNP arrays has revealed the concomitant loss or mutation of tumor suppressor genes such as TP53 (p53), retinoblastoma (RB1) [*Edamoto Y et al, 2003; Murakami Y et al, 1991*], CDKN2A (p16INK4A) [*Laurent-Puig P et al, 2001; Liew CT et al, 1999*] and insulin- like growth factor-2 receptor (IGF-2R) [*De Souza AT et al, 1995; Oka Y et al, 2002*] which are strongly associated with signaling pathways involved in carcinogenesis.

The TP53 gene encodes the p53 protein which plays a pivotal role in the DNA-damage response network, including cell cycle arrest, apoptosis, DNA repair and cellular senescence. Therefore, it is not surprising that TP53 loss-of-function mutations or allelic deletions in chromosome 17p are commonly associated with human carcinogenesis [*Hussain SP and Harris CC, 2006*]. AFB1 is a mutagen of TP53, causing G:C to T:A *transversions* at the third base in codon 249 (converting arginine to serine) and the rate of TP53 249ser mutation may be accelerated in the presence of viral infection [*Aguilar F et al,*

1993; Kirk GD et al, 2005]. HBV encodes a viral protein, HBx, which can specifically bind to p53 and suppress p53-induced apoptosis. Strong associations have been observed between TP53 249ser mutation levels and HCC risk, especially with respect to primary tumor development and also to the interval between surgical resection and recurrence [*Wang XW et al, 1994*]. Structural genomic mutations and epigenetic changes may lead to altered gene expression patterns that significantly affect the signal transduction pathways in HCC and the resulting variability in pathway activation may be related to the cellular origin of HCC (*Aravalli RN, 2008*).

The Wnt/β-catenin pathway is commonly known for its fundamental role in embryogenesis, which aids the cell in differentiation, proliferation and apoptosis (*Cox RT and Peifer M, 1998*). In the absence of Wnt signaling, cytoplasmic-β-catenin complexes with the tumor suppressors adenomatosis polyposis coli (APC) and Axin1, as well as the glycogen synthase kinase-3b (GSK-3b). In this complex, GSK-3 β phosphorylates β-catenin, targeting it for ubiquitination and subsequent degradation.

In the case that Wnt signaling receptors are engaged, conformational changes in the Axin complex cause the release of β-catenin, which then localizes to the nucleus and activates the transcription of target genes: Myc, cyclin D1 and COX2 [*Chiba T et al, 2007; Clevers H 2006; Kikuchi A, 2000*]. In HCC, transcriptomic and proteomic studies have indicated an increase in Wnt signaling, possibly as a result of an accumulation of Axin1 mutations at sites that bind β-catenin and/or CTNNB1 mutations along sites marked for phosphorylation by GSK-3b [*Yamashita T et al, 2009; Cavard C et al, 2008*]. It was hypothesized that an increase in signaling from the Wnt pathway is necessary to maintain "stemness" in HCC, i.e. to sustain a subset of cells (cancer stem cells, CSC), that are responsible for the maintenance and growth of the tumor [*Yamashita T et al, 2009*].

Transforming growth factor-β (TGF- β) is an inflammatory cytokine implicated in an array of functions such as cell growth, differentiation, migration, apoptosis, adhesion, survival and immunity [*Derynck R et al, 2001*]. IGF-2R (insulin- like growth factor-2 receptor), a tumor suppressor gene, promotes the degradation of mitogen IGF-2 and also the simultaneous activation of TGF-β signaling, thereby halting cell proliferation and inhibiting carcinogenesis [*Dennis PA and Rifkin DB, 1991*]. Inflammation and subsequent genomic mutations in IGF-2R result in IGF-2 over-expression and a reduction in the inhibitory effects of TGF-b signaling, a feature commonly observed early in the development of HCC [*El-Serag HB and Rudolph KL, 2007*].

Myc is a potent oncogene, which appears to be constitutively up-regulated in many human cancers, representing a phenomenon of "oncogene addiction." Though about 30% of HCC cases show an up-regulation of Myc because of the Wnt/β-catenin pathway activation; its increased expression in HCC is also attributable to the activation of its locus through chromosome amplification (*Wilkens L et al, 2004*). One possible mechanism by which Myc contributes to hepatocarcinogenesis is through the induction of telomerase, which also appears to be active during HCC development, thereby bypassing cellular senescence (*Wu KJ et al, 1999*). Moreover, the up-regulation of Myc in a variety of tumors has also been associated with deregulated microRNA (miRNA) expression in many human malignancies, which have a significant impact on tumorigenesis and progression. On the other hand, the inactivation of Myc in HCC causes a subpopulation of cells to differentiate while the rest remain dormant, giving rise to a phenotypically diverse tumor population. [*Kumar M et al, 2011; Reya T et al, 2001*].

PI3K/PTEN/Akt. Akt is involved in a number of biological process. The activation of the Akt pathway is mediated by either an activated tyrosine kinase receptor, or more rarely the

constitutive activation of PI3K or the loss of Phosphatase and Tensin Homolog (PTEN). PTEN is a tumor suppressor gene and the PTEN protein functions as a negative regulator of Akt. The loss of PTEN expression via a loss of heterozygosity in chromosome 10q along with an activation of Akt has been reported in 40%-60% of HCC cases [*Hu TH et al, 2003; Blanco-Aparicio C et al, 2007*]. Since Akt is involved in a number of biological processes, such as cell survival, cell growth, apoptosis and differentiation, its deregulation has been implicated in many human cancers (*Hu TH et al, 2003).*

Epigenetics modifications are involved in the regulation of many cellular processes including embryonic development, chromatin structure, X-chromosome inactivation, genomic imprinting and chromosome stability (*Kierszenbaum AL, 2002; Okamoto I et al, 2004; Robertson KD, 2005*). The epigenetic modifications include: DNA methylation at cytosine residues in CpG dinucleotides, histone tail methylation, acetylation, phosphorylation, ubiquitynation, sumoylation that result in alteration of chromatin structure (*Pons D and Jukema JW, 2008*). Though methylation is imperative for normal development and differentiation, aberrant hypomethylation at gene promoters in HCC and many human cancers can lead to the expression of oncogenes, or, similarly, hypermethylation can lead to the silencing of tumor-suppressor genes [*Feltus FA et al, 2003; Miyoshi HH et al, 2004*]. In the last decade there has been increasing evidence to support the occurrence of aberrant DNA methylation patterns in human HCC [*Thorgeirsson SS . et al, 2002*].

In HCC, an increased expression of DNA methyltransferases (DNMTs), enzymes which catalyze cytosine methylation occurs early in the development of tumorigenesis. The frequency of aberrant DNA methylation increases from precancerous lesions to dysplastic nodules and finally HCC, supporting their important role in tumor progression [*Wong CM. and Ng IO, 2008*]. For instance, the tumor suppressor genes RB1 [*Sakai T, et al, 1991*] and

CDKN2A [*Liew CT, et al, 1999*] have been shown to be hypermethylated in HCC, leading to uncontrolled cell proliferation. Likewise, PTEN promoter methylation has also been reported in HCC, which allows the activation of the PI3K/PTEN/Akt pathway [*Wang L et al, 2007*]. Epigenetic changes in HCC have also been reported at the miRNA level. Apart from their potential as a diagnostic tool, further understanding of methylation patterns in HCC may provide them useful in predicting recurrence and survival, as well [*Zhang YJ, et al, 2007*].

miRNAs in HCC. It has been established that specific miRNAs modulate various cellular processes in the liver and several studies revealed that the expression of miRNAs is deregulated in human HCC in comparison with matched non-neoplastic tissue and that their aberrant expression correlates with severity and poor prognosis of HCC *[Murakami Y et al, 2006; Gramantieri L et al, 2008; Ura S et al, 2009; Calin GA et al, 2004; Huang XH et al, 2009]*. It was also found that the dysregulation of miRNAs not only is involved in tumor progression, but it is also associated with the role of risk factors directly involved in tumor development, as demonstrated by the discovery that HBV and HCV induce different sets of miRNAs during infection *[Nordenstedt H et al, 2010; Ura S et al, 2009].* Although changes in the expression of microRNAs between tumor specimens and the normal corresponding tissue have been investigated in HCC as well, the obtained results are often discordant and do not allow the identification of the miRNAs critical for development and progression of HCC. Furthermore, among the microRNAs whose expression has changed, several are probably altered not as cause but as consequence of the tumorigenic status. Nevertheless, several microRNAs were identified as aberrantly expressed by more than one study, these microRNAs were most likely involved in liver tumorigenesis. Consistent deregulation of miR-122, miR-199, miR-221, and miR-21 appears to be particularly

important in HCC; among these miRNAs both miR-122 and miR-199a are among the miRs most highly expressed in normal liver *[Hou J et al, 2011]*.

miR-122 is a hepato-specific miRNA, accounting for more than 70% of the total liver miRNA population and it acts as a key regulator of fatty acid and cholesterol metabolism *[Jopling C, 2012]* and as a regulator of the differentiation of adult hepatocytes via repression of genes not specific to the liver *[Xu H et al, 2010; Esau C et al, 2006; Krutzfeldt J et al, 2006]*. The loss of its expression was observed in more than 70% of HCC and one of the mechanisms through which it plays an active role in tumorigenesis appears to be the activation of cyclin G1 *[Gramantieri L et al, 2007].* miR-221 has received much attention for its suggested tumor-promoting activity. It is up-regulated in 70%-80% of HCC samples and HCC cells overexpressing miR-221 show increased growth, proliferation, migration, and invasion capability *[Garofalo M et al, 2009; Pineau P et al, 2010]*. miR-21 has been shown to be overexpressed in HCC as well as in other several human malignancies, including breast, colon, lung, pancreas, prostate, and stomach cancer *[Volinia S et al, 2006*]. Discovery of aberrantly expressed miRNAs in HCC has helped to reveal novel mechanisms of liver tumorigenesis. Furthermore, since the profiling of miRNA expression levels in HCC could be associated with bio-pathological and clinical features, miRNA expression can be a potential useful tool for HCC classification and for improving prognostic stratification, in particular in early HCC, where the availability of potentially curative treatments requires a more sophisticated diagnostic approach.

2. SOLT-FARBER MODEL OF LIVER CARCINOGENESIS

Cumulative clinical and experimental evidence over several decades suggests that carcinogenesis is a multistage process which may involve a series of sequential cellular alterations. In tissues, evidence of this ongoing process may present as new cell populations with altered organizational, structural, and biochemical properties. Unfortunately, knowledge about molecular events in early stage HCC development is limited because of clinical difficulties in the histopathologic distinction between non-malignant nodular lesions (low grade and high grade dysplastic nodules) from early HCC. Animal models facilitate the study of different stages of hepatocarcinogenesis in that discrete lesions at different stage of progression can be identified and analyzed, thus helping to detect molecular alterations already present at early pre-neoplastic stages. The sequential analysis of the early steps in carcinogenesis in liver is based on a principle formulated as early as 1938 by *Alexander Haddow*, who observed that cancer cells can arise under conditions (e.g., carcinogen exposure) which inhibit the growth or otherwise impair the life of normal cells (*Fould L, 1954; Fould L, 1964*). This phenomenon of "selective cytotoxicity" also appears to result in preferential growth of carcinogen altered cells which have not yet acquired all the attributes of malignancy (*Farber E, 1973*). One of the most widely used experimental models for the characterization of the hepatocarcinogenesis process is the Resistant-Hepatocyte Model, also known as Solt-Farber Model, which allows to carry out the analysis of the different sequential steps leading to HCC development, using the rat as animal model (*Solt DB et al, 1977*). In this model, initiation is achieved through a single necrogenic administration of a chemical carcinogen, diethylnitrosamine (DENA), followed by a promoting regimen consisting of a 2 weeks-diet supplemented with the selective inhibitor of hepatocyte proliferation,

2-acetylaminofluorene (2-AAF), coupled with a powerful growth stimulus, such as 67% partial hepatectomy (PH). This protocol allows expansion of DENA-initiated cells that rapidly proliferate to develop into nodules (*Sold D and Farber E, 1977*), identified by their immunohistochemical positivity for the placental form of the enzyme glutathione-S-transferase (GST-P). The subsequent fate of the nodules is variable. In the following weeks, nodules become macroscopically visible and occupy most part of the liver. Later on, a significant number of pre-neoplastic lesions undergoes a process of maturation and remodeling, merges with the surrounding original liver, gradually loses the staining for GST-P, and reacquires a differentiated phenotype (*Enomoto K et al, 1982*). Conversely, a minority of nodules persists, with some of them giving raise to adenomas, and after 10-14 months after DENA, to HCC. Recently, it was found that GST-P+ pre-neoplastic nodules can be further divided into Cytokeratin 19 (KRT-19) positive or negative lesions, depending on the expression of KRT-19, an intermediate filament protein, normally expressed in adult liver by the bile duct epithelial cells, but not by normal hepatocytes *(Andersen JB and al, 2010)*. Previous studies conducted in our laboratory showed that all the HCCs arising in the Solt-Farber model are KRT-19+, although only a minority of pre-neoplastic lesions developed at early stages of the process are positive for this marker. Furthermore, HCCs and KRT-19+ nodules share a common gene expression profile, suggesting that in the Solt-Farber model HCCs are derived from this sub-population of KRT-19+ pre-neoplastic lesions. The translational value of the Resistant Hepatocyte model has already been demonstrated with comparative genomic studies, and showed that the HCCs generated with the Solt-Farber model have a gene expression signature very similar to a specific human HCC subtype that express KRT-19 (characterized by worst prognosis) *(Andersen JB et al, 2010).*

More recently, studies performed on the R-H model have also shown that the expression signature of genes and microRNAs is quite similar between HCC and early preneoplastic lesions; this finding suggests that some of these genes/microRNAs alterations might represent critical events in HCC development (*Petrelli A et al, 2014*).

3. INTERMEDIATE FILAMENT OF CYTOSKELETON

Intermediate filaments (IFs) represent the largest cytoskeletal gene family comprising >70 genes expressed in tissue specific manner. Together with the actin microfilaments and the microtubules, intermediate filaments (IFs) are the components of the cytoskeleton of eukaryotic cells, that is involved in the maintenance of cell shape, locomotion, intracellular organization, and transport *(Bershadsky and Vasiliev 1988; Ku et al. 1999)*. Individual IF proteins consist of a conserved central coiled-coil α-helical rod domain (interrupted by linkers) which is flanked by N-terminal (head) and C-terminal (tail) domains. The N- and C-terminal domains contribute to the structural heterogeneity and are major sites of posttranslational modifications with phosphorylation being the best characterized one *(Omary et al. 2006; Godsel et al. 2008; Kim and Coulombe 2007; Herrmann et al. 2007; Goldman et al. 2008)*. This makes them important regulatory domains, since dynamic changes in phosphorylation status are responsible for alterations in IF dynamics, solubility, and organization. In addition to the posttranslational modification, IF function is modified and complemented through interaction with a variety of IF-associated proteins (IFAP). These proteins can be subdivided into several subgroups, which reflect multiple IF functions. For example, IFs interact with a variety of anchoring proteins thereby forming transcellular networks which contribute to proper tissue architecture (*Strnad et al, 2008).* IFAPs also include several cytolinker proteins (i.e. proteins connecting different cytoskeletal filaments and other intracellular components), which provide the structural framework for coordinated cytoskeletal function *(Green et al. 2005; Omary et al. 2006)*.

In contrast to the actin and tubulin system, IFs emerged later in the evolution and are important supportive elements of the cell rather than their essential components. Therefore, IF variants are observed in various human diseases, which reflect their tissue specific distribution, whereas only few actin and tubulin variants have been described so far, likely due to their embryolethality. More than 30 diseases are caused by/associated with IF mutations (see Table 1; *Strnad et al, 2008*).

Type	Name/Localization	Disease location	Remarks
I ($n = 28$)	K9-28 (epithelia)	K10,14,16,17-skin	Acidic keratins
	K31-40 (hair/nail)	K12-cornea	(pI < 5.7)
		K13-stratified mucosa	Type I/II obligate 1:1 polymers
		K16,17-nail	
		K18[a]-liver	
II ($n = 26$)	K1-8, K71-80 (epithelia)	K1,2e,5,9-skin	Basic keratins
	K81-86 (hair/nail)	K3-cornea	(pI ≥ 6.0)
		K4-stratified mucosa	Type I/II obligate 1:1
		K6a,6b-nail	polymers
		K8[a]-liver	
		K75[a],81,83,85,86-hair	
III	Desmin (muscle)	Muscle, heart	Desmin, vimentin and GFAP are found in stellate cells
	Vimentin (mesenchymal)		
	Peripherin (neurons)	Brain[a], spinal cord[a]	
	GFAP (astrocytes/glia)	Brain	
IV	NF-L (neurons)	Brain[a], spinal cord	α-internexin forms polymers with NFs
	NF-M (neurons)	Brain[a], spinal cord[a]	
	NF-H (neurons)	Brain, spinal cord[a]	Synemin β is also called desmuslin.
	α-internexin (CNS neurons)		Nestin-stem cell marker
	Synemins (muscle)		
	Syncoilin (muscle)		
	Nestin		
V	Lamin A/C (ubiquitous)	Heart, muscle, fat, premature aging, complex defects	The only nuclear IFs, longer rod domain
	Lamin B1/2 (ubiquitous)		
Orphan	Phakinin (lens)	Lens	Beaded filaments in lens epithelia
	Filensin (lens)	Lens	

Table 1 Intermediate filament proteins, [a] Not a causative association, variants represent a risk factor. *(Strnad et al, 2008)*.

3.1 INTEMEDIATE FILAMENT OF THE LIVER

The liver consists of different cell types with characteristic IF composition. Keratins represent the largest subfamily of IFs consisting of >50 unique gene product members *(Schweizer et al. 2006; Kim and Coulombe 2007; Godsel et al. 2008)* which include 37 epithelial and 17 hair keratin members in humans *(Schweizer et al. 2006).* Epithelial keratins can be subdivided in types I (acidic) and II (basic) corresponding to keratins 9–20 (K9-K28) and keratins 1–8 plus keratins 71–80 (K1–K8; K71–K80), respectively *(Coulombe and Omary 2002; Schweizer et al. 2006)*. Keratins are found as obligatory type I and type II heteropolymers (i.e., consisting of at least one type I and one type II keratin) and a homozygous disruption of a keratin results in degradation of its keratin partner at the protein level *(Ku and Omary 2000; Omary et al. 2004)*. Similarly to IFs, keratins are expressed in a tissue specific manner, with different pairs being the major cellular IFs in different cell populations (*Moll et al. 1982; Ku et al. 1999; Coulombe and Omary 2002*).

Adult hepatocytes are unique among epithelial cells in that they express exclusively K-8 and K-18, whereas other glandular epithelia exhibit a more complex keratin expression pattern (*Omary et al. 2002; Ku et al. 2007*). The hepatocytic keratin IF network is dense, particularly around bile canaliculi and at the cell periphery, and acts as cytoskeletal backbone to the functionally more dynamic and contractile actin-micro-filament system (*Strnad et al, 2008).* Biliary epithelial cells differ from hepatocytes by additional expression of keratin 7 and 19. Keratins in cholangiocytes, but not hepatocytes, exhibit polarized and compartment-specific expression pattern (*Zatloukal et al. 2004*). The biological significance of such expression and distribution is incompletely understood, but it may be related to cell polarity and secretory processes.

Among nonepithelial cells, stellate cells express variable amounts of glial fibrillary acidic protein (GFAP), desmin, vimentin, and nestin dependent on their activation status, localization, and other parameters (see Table 2; *Strnad et al, 2008* modified from *Omary et al. 2002*).

Cell type	IF composition
Hepatocyte	K8/K18[a]
Oval cells	K7/K8/K18/K19
Cholangiocyte	K7/K8/K18/K19
Kupffer cell	Vimentin
Stellate cell	GFAP, Vimentin, Desmin, Nestin[b]
Endothelial cell	Vimentin

Table 2 IFs of liver cell populations (*P. Strnad et al, 2008* modified from *Omary et al. 2002).*
[a] During embryogenesis, hepatocytes also express variable levels of K19 (*Vassy et al. 1997).*
[b] Stellate cells represent a highly heterogeneous population with variable IF expression dependent on species, activation status of the cell, location within the hepatic lobe and many other parameters. (*Geerts et al, 2001*).

The large body of evidence from animal studies showing the importance of K-8/K-18 for liver homeostasis led to a search for keratin mutations in patients with liver diseases. Several K8/K18 variants were found to associate with the development of cryptogenic liver disease (*Ku et al. 2001*). In subsequent studies, K8/K18 were shown to represent susceptibility genes for the development of end-stage liver disease of multiple etiologies (*Ku et al. 2005*). Moreover, abnormal expression of K-19 in the hepatic parenchyma has been attributed to remodeling of cirrhotic nodules and hepatic progenitor cell (HPC) proliferation (Su Q et al, 2003). In previous studies it has been identified a subclass of human HCC that is enriched for the genes expressed in fetal hepatoblasts (*Lee JS et al, 2006)*, including the progenitor cell markers K-7 and K-19.

The K19-HCC subtype was characterized by the worst clinical prognosis among all HCC subclasses, suggesting that K19 is a prognostic marker for HCC *(Andersen JB et al, 2010; Lee JS et al, 2006; Wu PC et al, 1996).*

3.2 ROLE OF NEUROFILAMENT-LIGHT POLYPEPTIDE (NEFL) IN TUMORIGENESIS

Neuronal Intermediate Filaments (NF) represent a major component of the neuronal cytoskeleton, and are believed to function primarily to regulate axon diameter and play a key role in maintaining the morphology of neurons and in regenerating myelinated axons. NF are composed of polypeptide chains which belong to the same protein family as the intermediate filaments of other tissues such as keratin subunits. NF consist of three subunits: a light (NEF-L), a medium (NEF-M) and a heavy polypeptide (NEF-H), with molecular weights of 68 kDa, 160 kDa and 212 kDa respectively (*Liem RK et al, 1978*). The NEFL gene encodes type IV intermediate filament heteropolymers that functionally maintain the neuronal caliber and play an important role in the intracellular transport of neurotransmitters to axons and dendrites. The targeted disruption of the NEFL gene in mice confirmed the importance of NF-L in IF assembly [*Zhu Q, et al, 1997*]. In absence of NF-L, the NF-M and NF-H subunits are not able to assemble into 10 nm filaments. As a result, mice lacking NF-L have a scarcity of IF structures and exhibit severe axonal hypotrophy. While the targeted disruption of the NEFL gene in mice provided definite proof that neurofilaments are a major determinant of axonal caliber, the specific roles of NF-M and NF-H subunits remain unclear [*Julien JP, 1999; Zhu Q, et al, 1997*]. NF accumulations have been described in several neurological diseases where abnormal IF accumulations can provoke a gradual block of axonal transport.

Various factors may lead to the formation of abnormal IF aggregations, including deregulation of IF gene expression, neurofilament mutations, and post-translational modifications (such as phosphorylation, glycosylation, nitration and protein crosslinking). Notably, NEFL mutations have been associated with Charcot-Marie-Tooth disease type 2E (CMT2E), Parkinson's disease and amyotrophic lateral sclerosis *(Mersiyanova IV et al, 2000*). In addition to the important structural role of intermediate filaments, other cellular functions are being increasingly ascribed to IF. Indeed, it has been recently demonstrated a physiological role of NEFL in regulating mitochondrial morphology, fusion, and motility in neurons; interestingly, disruption such functions occurs in Charcot-Marie-Tooth (CMT) disease type 1F, due to point mutations in the NEF-L gene (*De Jonghe et al., 2001; Jordanova et al., 2003*).

Although studies regarding the role of the NEFL protein have been mostly focused to neurological diseases, such as Charcot–Marie–Tooth's disease (CMT), recent evidence points to a correlation between NEFL expression and cancer development. Indeed, a growing number of studies suggest that NEFL may act as a tumor suppressor in various tumors including breast cancer (*Li XQ et al., 2012*) and head and neck cancer (*Chen B et al., 2012)*. The NEFL gene is located on human chromosome 8p21, a region enriched with tumor suppressor genes. Loss of heterozygosity (LOH) is frequent in this region (*Imbert A. et al, 1996, Kochanski A, 2004*) and is involved in breast (*Li XQ et al, 2012; Kerangueven F et al, 1995; Seitz S et al, 2000; Yaremko ML et al, 1996*), prostate (*Macoska JA et al, 1995*; *Haggman MJ et al, 1997; Kagan J et al, 1995; Schmidt H et al, 2007; Vocke CD et al, 1996*), lung (*Kurimoto F et al, 2001; Lerebours F et al, 1999*), colon (*Lerebours F et al, 1999; Takanishi DM et al, 1997*), and urinary bladder cancers (*Knowles MA, 1993*).

LOH at the NEFL locus is a common genetic alteration in infiltrating and *in situ* breast cancer (*Anbazhagan et al. 1998*). Notably, in breast cancer, LOH at the NEFL locus is associated with the presence of stromal invasion by tumor cells and therefore correlates with invasive capacity (*Yaremko ML et al, 1996*). Interestingly, although NEFL in normal tissues is expressed in neurons with strict histological specificity, it was recently demonstrated that ectopic NEF-L mRNA expression can be detected in various malignancies including breast cancer (*Li XQ et al., 2012*). Notably, in breast cancer NEFL is down-regulated in lymph node metastases compared to the primary tumors (*Li XQ et al, 2012*). Moreover, a low NEFL mRNA expression level was found to be a prognostic factor to predict disease-free survival of early-stage breast cancer patients (*Li XQ et al, 2012*). These data indicate that the ectopic occurrence and change in NEFL mRNA expression levels play an important role in tumor development and metastatic process in breast cancer, although the exact role of NEFL expression in cancer and its prognostic power for breast cancer patients remains unclear.

In head and neck cancer (HNC), LOH at the NEFL locus has been shown to decrease survival time in patients with advanced tumor stage (*Coon et al., 2004*). Furthermore, in head and neck cancer cell lines, NEFL mRNA expression has been shown to positively correlate with cancer cell apoptosis, and negatively correlate with cancer cell growth and invasion capacity (*Huang Z, 2014*). Another study showed that, in head and neck cancer cell lines, downregulation of NEFL mRNA was due to hypermethylation of the NEFL gene promoter and correlated with increased resistance to cisplatin (*Chen B et al. 2012*). Furthermore, in patients treated with cisplatin-based chemotherapy, hypermethylation of the NEFL gene correlated with resistance to the therapy and diminished overall and disease-free survival (*Chen et al. 2012*). Several studies examined potential pathways

modulated by NEFL. As described in the literature, NEFL interacts with multiple protein targets essential for cell plasticity, proliferation, migration, apoptosis, molecule transport (*Haddad LA. Et al, 2002*) and functional molecular targets of NEFL are found in several critical cancer-associated pathways. Among these, some authors investigated the Tuberous Sclerosis 1 (TSC1) tumor suppressor. TSC1 has been shown to function as a molecular inhibitor of the mTOR oncogenic pathway, reported to be frequently constitutively activated in cisplatin-resistant cancers cell lines (*Mabuchi S. et al, 2009*). NEFL has been shown to bind TSC1 and stabilize the TSC1/2 complex (*Haddad LA . et al, 2002*). Therefore, it was hypothesized that inactivation of NEFL, leading to abnormal activation of the mTOR pathway, confers cisplatin-resistance in head and neck cancer. mTOR is a key downstream protein kinase of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway and has been reported to play a central role in controlling cancer cell growth (*Zoncu R. et al, 2011*). AKT and mTOR function as "switch" proteins to modulate metabolism, the cell cycle, and apoptosis in cancer cells (*Foster DA. Et al, 2009; Castedo M. et al, 2002*). Constitutive activation of mTOR is common in human malignancies. In cultured cortical neurons, NEFL was found to physically bind to the putative tumor suppressor TSC1 (*Haddad LA. Et al, 2002*), a molecular inhibitor of mTOR activity. NEFL has also been shown to physically interact with glutamate receptor, ionotropic, NMDAR1, a subunit of NMDAR, that has recently been shown to be a novel tumor suppressor gene in esophageal carcinoma (*Kim MS, et al, 2006*).

Julien et all *[Julien JP, 1999]* speculated that neurofilaments might have a protective role against the toxic effects induced by SOD1 mutations. Based on this hypothesize, they speculated that the change in NEFL mRNA expression level is involved in the process of adaptive cytoprotection.

When malignant transformation takes place in the presence of physical/chemical carcinogen, tissue cells change their expression profile to adapt to the new microenvironment and to retain the function of normal tissue cells as much as possible [*Kim MS, et al, 2006*].

Despite evidence linking the NEFL gene to the development of head-and-neck and breast cancer, very little is known on the potential involvement of the NEFL gene in other tumors, such as HCC. Indeed, the only evidence linking the NEFL gene with HCC development is provided by genetic studies that report the frequent LOH of the NEFL locus region in human hepatocellular carcinoma (*Becker et al., 1996*). However, whether and which role NEFL may play in HCC development remains totally unknown.

AIM OF THE WORK

It is now becoming increasingly evident that molecular pathogenesis of HCC cannot be understood without a more detailed knowledge of the molecular alterations characterizing its early development. Therefore, one of the priorities in this field of investigation is the identification of key molecular players that drive the hepatocarcinogenesis process starting from its early stages. In this respect the Resistant Hepatocyte (R-H) model of rat carcinogenesis it a valuable one, since i)it allows the analysis of the hepatocarcinogenesis process starting from the very early preneoplastic stages and ii)it has been proven to be of translational value for the human pathology.

As mentioned, very little is known on the involvement of the NEFL gene in the process of hepatocarcinogenesis; therefore whether NEFL plays a role in HCC development is still largely unknown.

Thus, the goal of the present study was to investigate the role of NEFL in the onset and progression of HCC in the R-H rat model of hepatocarcinogenesis, and human HCCs.

MATERIALS AND METHODS

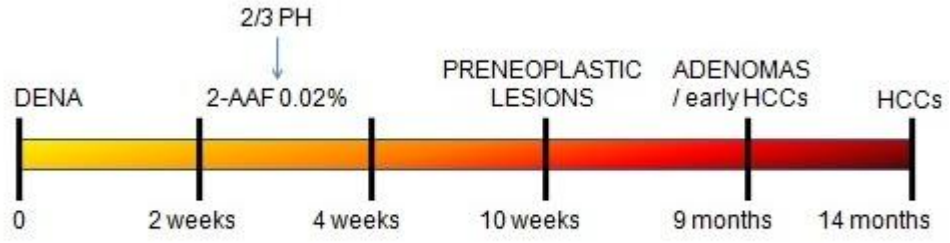
Animals

Male Fisher F-344 rats (Charles River, Milan, Italy) weithing 100-125 gr have been used for this studies. Animals have been fed a standard diet (Mucedola, Milan, Italy) and maintained at 25 °C temperature and 12 hours light/dark dayily cycle, with food and water *ad libitum*. Guidelines for the Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by Ethical Commission of the University of Cagliari and the Italian Ministry of Health.

Experimental Protocol

Rats belonging to the experimental group were treated in accordance with the Resistant Hepatocyte Protocol (R-H model). Animals were injected intraperitoneally (i.p.) with a single dose of diethylnitrosamine (DENA) (Sigma Aldrich, St. Louis, MO,USA), dissolved in saline, at a dose of 150 mg/kg body weight. After a 2-week recovery period, rats were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF) (Sigma Aldrich, St. Louis, MO,USA) for 1 week, followed to a standard two-thirds partial hepatectomy (PH) (*Higgins GM and Anderson RM, 1931*), and kept for an additional week on the 2-AAF-containing diet. The animals were then switched to basal diet all throughout the experiment. Two additional groups were used as controls: one group was subjected to the promoting regime (2-AAF+PH) in the absence of carcinogen, and in a second group both the initiating and the promoting regimes have been omitted. Each experimental group was divided into two subgroups; the first of them was sacrificed at ten weeks from DENA administration, at a time when GSTP-positive pre-neoplastic lesions are present; the second one has been

sacrificed at fourteen months to evaluate the presence of fully developed HCCs. HCCs were selected on the basis of the criteria proposed in *"histologic typing of livel tumors of the rats" (Stewart HLW,1980).*



For assessment of NEFL expression during liver regeneration, rats were subjected to a standard 2/3 Ph according to the method described by Higgins and Anderson (*Higgins GM and Anderson RM, 1931*), and sacrificed after 24 hours after PH. Livers collected at the time of surgery were used as controls.

Treatment with 5'-bromo-deoxyuridine (BrdU)

In order to assess the proliferative activity of hepatocytes, rats subjected to the analysis of KRT-19- and KRT-19+ preneoplastic lesions and animals subjected to PH were treated with 5'-bromo-deoxyuridine (BrdU) (Sigma Chemical Co., St Louis, MO, USA). For assessment of proliferative activity in pre-neoplastic lesions, BrdU was dissolved in drinking water (1mg/mL) and given *ad libitum* for 7 days before sacrifice. For assessment of hepatocyte proliferation during liver regeneration associated to PH, BrdU was administered intra-peritoneally (i.p.) 2 hours prior to sacrifice at a dose of 50 mg) 100gr body weight.

Histology, Immunoistochemistry, Immunofluorescence and Western blot analyses

Tissue preparation

Immediately after sacrifice, livers were cut into several pieces and subjected to different analyses. For immunoistochemitry analysis, liver sections were fixed in 10% formalin, embedded in paraffin and stored at RT. Other sections were frozen in cold isopentane or quickly frozen by immersion in liquid nitrogen and stored at -80 °C.

Hematoxylin and Eosin staining

Four micrometer-thick (4 um) paraffin-embedded livers sections were deparaffinized in xylene and then hydrated in a decreasing series of alcohol. Sections were then stained with Carazzi Hematoxylin for 15 min and in 1% acidified alcoholic eosin for 30 seconds, then dehydrated in ascending series of alcohol.

GST-P and KRT-19 Immunoistochemistry

Six micrometer-thick (6 um) isopentane-frozen liver sections were fixed in acetone at -20 °C for 20 minutes. Endogenous peroxidases were blocked with Peroxidase Block Reagent (Dako, Milan, Italy) for 10 minutes. Blocking of aspecific sites was performed in normal goat serum 1:10 in PBS buffer for 1 hour at RT. Anti-GSTP antibody (rabbit polyclonal antibody MBL, Germany) was applied overnight at 4 °C at 1:1000 diluition. Sections were then incubated with anti-rabbit HRP secondary antibody at 1:200 diluition for 1 hour at RT. Sections were then stained by a brief incubation with 3-3’ diaminobenzidine tetrahydrochloride hydrate (DAB) (Dako Envision, Denmark). KRT-19 protein was detected by applying anti-KRT-19 primary antibody (mouse monoclonal antibody Novacastra, Leica Biosystem, Milan) diluted 1:50 for 2 hours at RT and by incubation sections with anti-mouse HRP secondary antibody 1:200 in PBS at RT for 1 hour. Staining was revealed by a

solution containing the chromogenic DAB. Counter staining was performed with Harris Hematoxylin Solution (Sigma-Aldrich, St Louis, MO, USA). Finally, sections were dehydrated in ascending series of alcohol and mounted with coverslip.

Immunofluorescence

Six micrometer-thick (6 um) isopentane-frozen liver sections were fixed with methanol for 10 min. Allow sections to fix for 10 min at room temperature. Rinse slides three times in PBS-T for 5 min each. Block specimen in blocking buffer (1X PBS/ normal goat serum) for 40 min. tissue were then stained overnight at 4°C with the following primary antibody diluited in PBS: anti-NEFL antibody (rabbit monoclonal antibody, Cell Signaling), anti-KRT-19 primary antibody (mouse monoclonal antibody Novacastra, Leica Biosystem, Milan), Anti-GSTP antibody (rabbit polyclonal antibody MBL, Germany). Sections were washed with PBS and incubate for 2 h with the respective secondary antibody conjugated to Alexa Fluor^{R} 594 goat anti–mouse IgG (H+L), Alexa Fluor^{R} 488 goat anti–rabbit IgG (H+L)(Life Technologies). Slides were then rinsed in PBS and coverslip slides with DAPI (4-6-DIAMIDINO-2-PHENYLIN, Life Technologies).

Protein isolation

Rat liver samples were homogenized in RIPA Buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxxycholate, 0.1% SDS, 140 mM NaCl) containing protease and phosphatase inhibitors (Sigma-Aldrich, St Louis, MO, USA), incubated for 2 h at 4 °C with shaking and centrifuged at 1200 rpm for 20 min at 4 °C. the supernatant was collected and stored at -80 °C. protein concentration of whole-cell lysates was evaluated with BCA Protein Assay kit (Pierce Biotecnology, Rockford, USA) and equal amount of total protein were used to perform western blotting experiments.

Western blotting

100 ug of protein were resolved in NuPAGE Tris-Acetate Mini Gel 7% (Life Technologies, Carlsbad, USA) and transferred to nitrocellulose membrane. The membranes were blocked for 1 h at RT in 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) diluted in TBS-T buffer (NaCl 5M, Tris-HCl 1M pH 7.4, 0.1% Tween-20), washed three times in TBS-T for 10 min, and incubated overnight at 4 °C with anti-NEFL antibody (rabbit monoclonal antibody, Cell Signaling) 1:500 in 5% BSA. After washing 3 times for 10 min in PBS, membranes were incubated for 1h at RT with anti-rabbit peroxidase-conjugated IgG secondary antibody at 1:1000 dilution (Santa Cruz Biotecnology, CA, USA) and the washed 3 times for 10 min in PBS. Protein were detected using Supersignal West Pico Chemiluminescent Substrate (Pierce Biotecnoly, Rockford, IL, USA). Subsequently, membranes were stripped and incubated 1 h with anti-β-actin antibody diluted 1:500 (Monoclonal anti-Actin, Clone AC-40, Sigma-Aldrich, ST. Louis, MO, USA), washed 3 times with PBS and incubated for 1 h at RT with HRP-conjugated anti-mouse secondary antibody at 1:1500 dilution, washed 3 times and then subjected to the final detection step. Levels of NEFL proteins were normalized to expression of the housekeeping gene β-actin was used as loading control.

Laser-capture Micro-dissection (LMD)

Pre-neoplastic lesions of animals sacrificed ten weeks after initiation were identified by immunoistochemical staining for GST-P and KRT-19 proteins. Fourteen-um-thick serial frozen sections were attached to 2-um PEN-membrane slides (*Leica,* Bannockburn, IL). Immediately before performing dissection, each section was rapidly stained with a 2.45

minutes H&E staining. This step was performed in order to identified the localization of the lesions of our interest, which were previously identified on serial sections subjected to GST-P and KRT-19 IHC. Micro-dissection was performed using a Leica laser microdissection apparatus (*Leica,* LMD6000). RNA was extracted from micro-dissected samples using the PicoPure RNA Isolation kit and mirVana miRNA Isolation kit (mirVana) according to manufacturer's instructions.

RNA EXTRACTION

RNA extraction from pre-neoplastic lesions: total RNA was extracted from preneoplastic lesions and from respective control livers with mirVana miRNA Isolation kit (mirVana, Ambion, Life Tecnologies, Monza) according to manufacturer's instructions. Briefly, dissected lesions were dissolved in 300 µl of Lysis Buffer (LB) and 30 µl of miRNA Homogenate Additive. After 10 min incubation on ice, 300 µl of acid-phenol:chloroform: isoamyl alcohol (125:24:1) were added to samples. Samples were then centrifugated for 5 min at maximum speed to separate the aqueous and organic phases. After recovery of the aqueous phase, 1.25 volumes of 100% ethanol were added and the mixture was transferred to a filter cartridge. Samples were centrifuged and, after three washing steps with Wash Buffer, RNA was eluted in 100 µl of DEPC pre-heated water (95 °C).

RNA purification from rat HCCs and control livers: total RNA from advanced HCCs (14 months after DENA) and control livers was isolated using Trizol^{R} Reagent (Invitrogen, Carlsbad, CA, USA). 1 ml of Trizol was added to 80-100 mg of hepatic tissue and samples were homogenized with a power homogenizer. After a centrifugation step of 10 min at 12000 g at 4 °C, samples were incubated 5 min at RT to permit the complete dissociation

of nucleoprotein complex. 0.2 ml chloroform for each ml Trizol used were added and samples were centrifuged 15 min at 12000 x g. After centrifugation the mixture separates into a lower red phenol-chloroform phase containing proteins, a white interphase containing DNA, and a colorless uppers aqueous phase containing RNA. RNA was then precipitated by addition of 500 µl isopropanol (Fisher Scientific, Thermo Fisher Scientific, France) and subsequently with 1 ml of 100% ethanol. Finally, the pellet RNA was dissolved in RNase-free water (Gibco, Life Technologies, Milan). In order to completely eliminate proteoglycans and polysaccharides (glycogen), the eluted RNA was subjected to a further purification process conducted using 3M sodium acetate pH 5.2. After a second precipitation in 75% ethanol, the RNA pellet was air dried and then resuspended in RNase-free water.

Quantitative and qualitative analysis of nucleic acids

RNA concentration was determined with a Nanodrop spectrophotometer (Thermo Scientific, France) and its quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Milano, Italy). Only RNA samples with a RIN (RNA Integrity Number) equal to seven or higher were included in the study.

Microarray

Liver RNA was extracted and purified from each individual lesion laser microdissected samples (10 control liver samples, 10 pre-neoplastic KRT-19- microdissected lesions, 10 pre-neoplastic KRT-19+ microdissected lesions, 4 microdissected Adenomas, 5 microdissected early HCC and 9 fully malignant HCC, all samples were obtained from F-344 rat subjected to R-H model). 150 ng of RNA were amplified (Illumina TotalPrep RNA

Amplification Kit), labeled and hybridized on Illuminamicroarray (BeadChips, Illumina Inc., San Diego, CA, USA) including 21.791 gene specific oligonucleotide probe.

The Illumina® TotalPrepTM RNA Amplification Kit is a complete system for generating biotinylated, amplified RNA for hybridization with Illumina Sentrix® arrays. The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter using ArrayScript™, a reverse transcriptase (RT) engineered to produce higher yields of first strand cDNA than wild-type enzymes. ArrayScript catalyzes the synthesis of virtually full-length cDNA, which is the best way to ensure production of reproducible microarray samples. The cDNA then undergoes second strand synthesis and cleanup to become a template for in vitro transcription with T7 RNA Polymerase. To maximize cRNA yield, Ambion® MEGAscript® in vitro transcription (IVT) technology along with biotin-UTP (provided in the kit) is used to generate hundreds to thousands of biotinylated, antisense RNA copies of each mRNA in a sample. (In this protocol the antisense amplified RNA is referred to as cRNA, in scientific literature it is also commonly called aRNA.) The labeled cRNA was hybridized with Illumina arrays (RatRef-12 V1 BeadChips).

Microarray data analysis

The intensity files were loaded into the Illumina BeadStudio 3.0.19.0 software (Illumina Inc) and BRB Array Tools (Version 4.2.0) for quality control and gene expression analysis. First, the quantile normalization algorithm was applied on the dataset. Only genes whose expression differed by at least 1.5 fold from the median in at least 20% of the arrays and characterized by a 50th percentile of intensities greater than 300 were retained. The FDR-adjusted p-values were calculated using the Benjamini-Hochberg procedure (*Benjamini Y and Hochberg Y, 1995*). According to these criteria, 1.144 expressed transcripts out of 21.791, showed reproducible up- or downregulation. Custom R scripts based on the

Limma package (*Smyth GK, 2005*) were used to identify genes differentially expressed at $p \leq 0.01$ between early nodules and advanced lesions, based on Log2ratio expression data (median centered). Normal livers were used as reference. Following this analysis, 869 genes showed reproducible up- or down-regulation in at least one comparison. Log2ratio expression data were clustered and visualized in the heat-maps using the GEDAS software (*Fu L and Medico E, 2007*) using Pearson correlation as distance matrix and Complete Linkage as method of calculating distance between clusters.

Functional analysis by means of the Ingenuity IPA Software*:* rat standard gene symbols (RGD ids) were submitted to the Ingenuity IPA analysis pipeline and converted to human gene id, were possible. Analysis of the pathways was based on the number of genes significantly dysregulated (fold difference cutoff ±2.0) with corresponding biological functions, with the restriction of at least 8 genes per function to emphasize the functions with most genes differentially expressed. The significance of each network and the connectivity was estimated in IPA.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To perform the analysis of mRNA and microRNA expression, the RNA has been retrotrascribed to cDNA using two different protocols:

– High Capacity cDNA Reverse Transcription Kit (Applied Biosistem, Life Technologies, Monza, Italy) used for mRNA analysis. 1.5 µg of RNA were diluted in 10 µl of RNase free water to a final concentration of 150 ng/µl. For each sample 2 µl of RT Buffer (10X), 2 µl of Random Primers (10X), 0.8 µl of dNTPs (100 mM), 1 µl of MultiScribe Reverse Transcriptase enzyme, 1 µl of RNase Inhibitor and water until 20 µl were added.

Sample were then incubated at 25 °C for 10 min, at 37 °C for 120 min and 85°C for 5 minutes. Sample were then stored at -20°C until use.

-TaqMan^{R} MicroRNA Reverse Transcription Kit (Applied Biosistem, Life Technologies, Monza, Italy) was used for the retrotranscription of microRNAs. 10 ng of total RNA were diluted to reach a final concentration of 2 ng/µl. For each sample, the retro-transcription mixture was composed of: 4.16 µl of dH_2O, 1.5 µl of Reverse Transcription Buffer (10X), 0.12 µl of dNTPs (100 mM), 0.19 µl of RNase Inhibitor and 1 µl of MultiScribe Reverse Transcriptase. For each miRNA 3 µl of specific primer were used. Subsequently the samples were incubated at 16 °C for 30 min, then at 42 °C for 30 min and at 85 °C for 5 min. samples were then stored at -20 °C until use.

qReal Time PCR

Retro–transcribed cDNAs were used for the assessment of mRNA and microRNA expression levels by real-time PCR. For gene expression analysis, the amplification reaction was performed in a final volume of 10 µl containing: 4 µl of cDNA (2.5 ng/µl), 5 µl of TaqMan Gene Expression Master Mix (Applied Biosistem, Life Technologies, Monza, Italy) and 1 µl of TaqMan probe (Applied Biosistem, Life Technologies, California, USA). The following TaqMan probes were used: -Rn00582365_m1 for the analysis of rat NEFL; -Hs01034882_m1 for the analysis of human NEFL.

For the analysis of microRNAs expression, the RT product was diluted in 52 µl of dH_2O. 4.5 µl of the diluted cDNA was amplified with 14.5 µl of TaqMan Universal Master Mix II no UNG (Applied Biosistem, Life Technologies, Monza, Italy) and 1 µl of a specific TaqMan microRNA Assays (Applied Biosistem, Life Technologies, California, USA). The following TaqMan miRNA probes were used: -002223 for the analysis of miR-30e; -000420 for the

analysis of miR-30d. Parameters used to perform the reaction are: 10 min at 95 °C followed by 40 cycles at 95 °C for 15 sec each and a final step at 60 °C for 1 min. for both mRNA and miRNAs expression, each sample was analyzed in triplicate. The housekeeping gene β-action or GAPDH for gene expression and 4.5S (rat) or U6 snRNA (rat-mouse) and RNU48 (human) for miRNAs expression were used for normalization. Expression levels were evaluated with the $2^{-\Delta\Delta Ct}$ method and represented as relative expression compared to a calibrator control.

Methylation analysis and sequencing

Genomic DNA was extracted from normal liver and HCC tissues isolated from rats subjected to the Resistant-Hepatocyte model with QIAmp DNA mini kit (*Qiagen, Valencia, CA*) following manufacturer's recommendations. Bisulfite conversion was performed using EpiTect Plus DNA Bisulfite Kit (Qiagen). 40 ng of bisulfate-converted DNA was PCR amplified using forward primer 5'-TTGGAGTAAGTAGAATAAGGTTTTG-3' and biotinylated reverse primer 5'-AAAATCTCCTCCAACCCC-3' (10 pmol/each) and a PCR mix containing 1.5 mM $MgCl_2$, O.2 mM dNTPs and 1U of Platinum Taq DNA Polimerase (Invitrogen, Carlsbad, CA). PCR was carried out for a total of 45 cycles (94°C for 30 seconds, 55 °C for 30 seconds and 72°C for 45 seconds) in a PCR system 9700 (Applied Biosystem, Foster City, CA). Pyrosequencing was performed according to the manufacturer's instructions using the following sequencing primer: 5'GTTTTGTATGAGTAGGAG3'.

This analysis allowed evaluation of 10 consecutive CpGs in the sequence:

GATCTGCGACTGGCGGCCGAAGACGCCACTAACGAGAAGCAGGCGCTGCAGGGCGAGCGCG.

Patients

HCC and cirrothic tissues were obtained from 14 consecutive patients undergoing liver resection for HCC. All tissues were obtained by Policlinico S.Orsola-Malpighi, Bologna, Italy (Dr. L. Gramanieri). Tissues were collected at surgery after obtaining an informed consent, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Histopathological grading was scored according to Edmondson and Steiner criteria (*Rozen S. and H.J. Skaletsky, 2000*). No patient received anticancer treatment prior to surgery. The characteristics of patients are detailed in TableI.

Statistical analysis

Time to recurrence (TTR) curves based on NEFL mRNA expression level were computed by Kaplan-Meier product-limit method and compared using a long-rank test. Reported *p-values* are two-sided and were considered significant when lower than 0.05. statistical calculations were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL).

Statistics

Data are expressed as mean ± standard deviation (SD). Analysis of significance was done by t Student's test and by One-Way ANOVA using GraphPad software (La Jolla, California).

RESULTS

NEFL mRNA levels are up-regulated at all stages of rat hepatocarcinogenesis

To address the putative role of NEFL in the development of HCC, we assessed the expression levels of NEFL in the Resistant-Hepatocyte Model, which allows the analysis of the different sequential steps leading to HCC development using the rat as animal model. A microarray transcriptome analysis performed in our laboratory (Fig.1A) revealed that NEFL was one of the 213 genes whose expression levels was dysregulated throughout the carcinogenic process, from the early pre-neoplastic stage to advanced HCCs (*Petrelli A et al, 2014*). NEFL, a gene whose expression is not detectable in control rat liver, was overexpressed all throughout the carcinogenic process (Fig.1B), with much higher mRNA levels in KRT-19 positive pre-neoplastic lesions compared to KRT-19 negative ones (fold change over control 18.87 *vs* 1.29). Interestingly, NEFL mRNA expression levels declined at later stages of hepatocarcinogenesis, showing a trend towards control values in fully malignant HCC (fold change over control 17.19 in early HCCs, 1.68 in advanced HCCs).

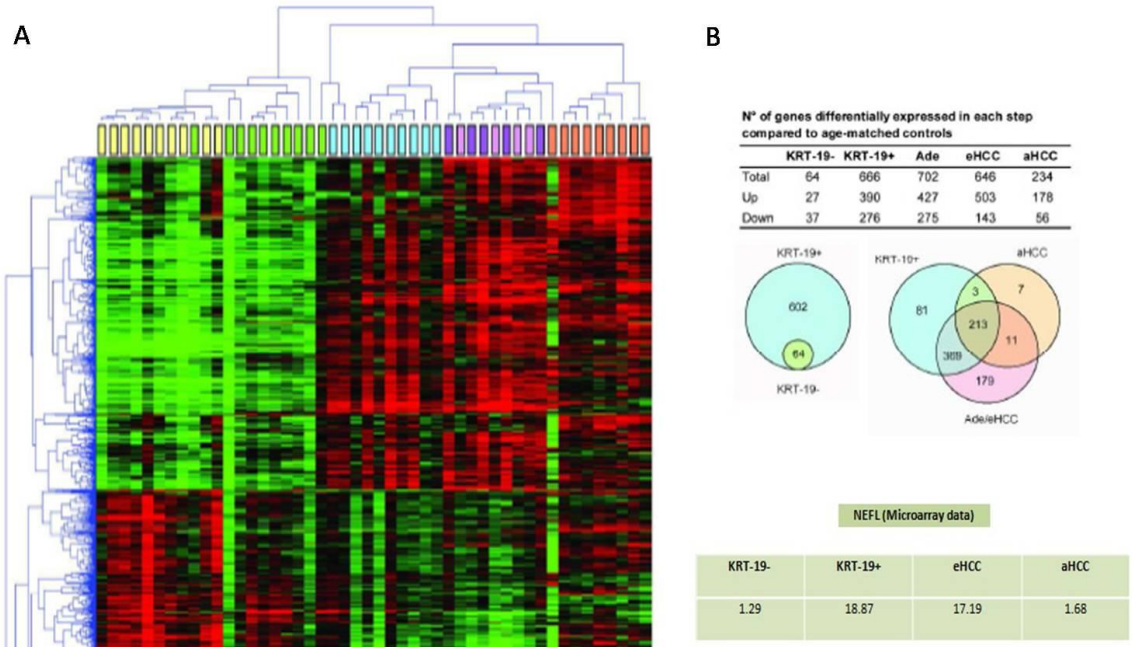


Fig.1 A) Transcriptomic analysis of different hepatocarcinogenesis stages in rats subjected to the R-H protocol. The image represents the hierarchical group of 1144 genes analyzed in control livers, in KRT-19- and KRT-19+, pre-neoplastic lesions in adenomas, early HCCs and in advanced HCCs. Each line represents the expression profile of an mRNA, and each column represents a sample. The red and green colors respectively indicate a high and a low expression of the examined gene. **B**) Venn diagrams show the number of differentially expressed genes in each phase of the carcinogenic process compared to controls. NEFL was strongly expressed in KRT-19+ pre-neoplastic lesions, while its expression decreased towards control values in fully malignant HCC. Expression values for NEFL are reported as fold change over healthy liver control. (From *Petrelli A et al, 2014*).

To validate the cDNA microarray results, we performed qRT-PCR analysis of NEFL, and in agreement with transcriptome data, we observed that while NEFL mRNA was undetectable in normal liver (cycle threshold (CT)> 39), it was overexpressed at all stages of the carcinogenic process, including the pre-neoplastic ones (Fig.2 A,B). in this study, we analyze 20 early pre-neoplastic lesions (10 KRT-19**-** lesions and 10 KRT-19**+** lesions), 5 early HCC (eHCC), 9 advanced HCC (aHCC) and 10 control samples. As shown in fig.2B, analysis of significance by One-Way ANOVA showed a highly significant difference between pre-and neoplastic samples vs. control liver ($p<0.0001$).

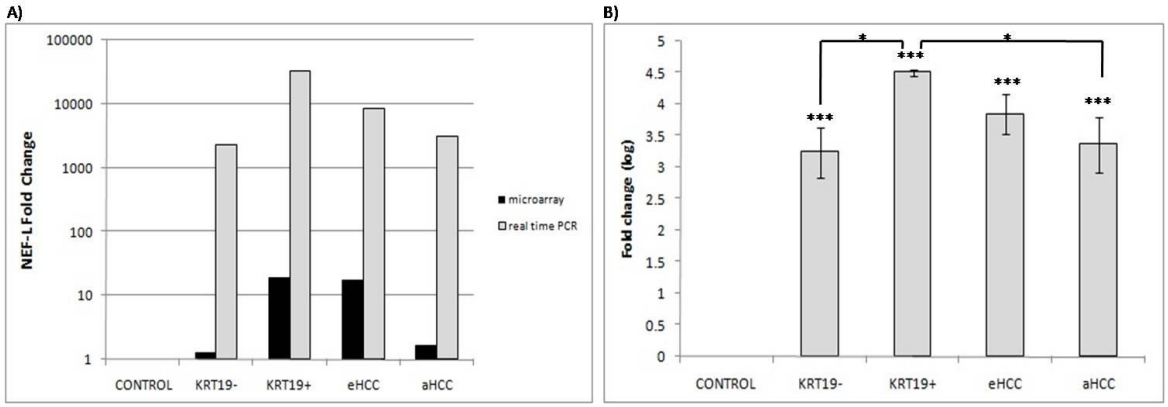


Fig.2 A) Validation of cDNA microarray NEFL gene expression data by qRT-PCR. **B**) qRT-PCR gene expression values from (A) are plotted in semi-log scale. The mRNA expression was assessed in control liver (n=10), KRT-19**-** (n=10), KRT-19+ (n=10), early HCC (n=5) and advanced HCC (n=9) samples. Rat β-actin was used as endogenous control. Data are expressed as mean ± standard deviation (SD) (mean log expression for NEFL in KRT-19**-** *vs* KRT-19**+** *vs* eHCC *vs* aHCC: 3.24±0.40 *vs* 4.50±0.04 *vs* 3.84±0.31 *vs* 3.36±0.43). Gene expression values are reported as fold change over control liver. (*** p< 0.0001 CONTROL *vs* KRT-19**-**, KRT-19+, eHCC, aHCC; * p< 0.01 KRT-19**-** *vs* KRT-19+; * p< 0.01 KRT-19+ *vs* aHCC).

NEFL protein is expressed in KRT-19+ preneoplastic lesions and in early HCCs

Next, we wished to determine whether the increased NEFL mRNA levels observed in pre- and neoplastic stages of hepatocarcinogenesis could also result in increased protein content. To this aim, immunofluorescence was used to detect the presence of NEFL in pre- and neoplastic lesions developed 10 weeks and 10 months after initiation. Lesions were identified by their immunofluorescence positivity for the placental form of Glutathione-S-transferase (GST-P). As shown in Fig.3, while GSTP was undetectable in normal liver (Fig.3A), pre- and neoplastic lesions exhibited an intense fluorescent staining (Fig.3B-D).

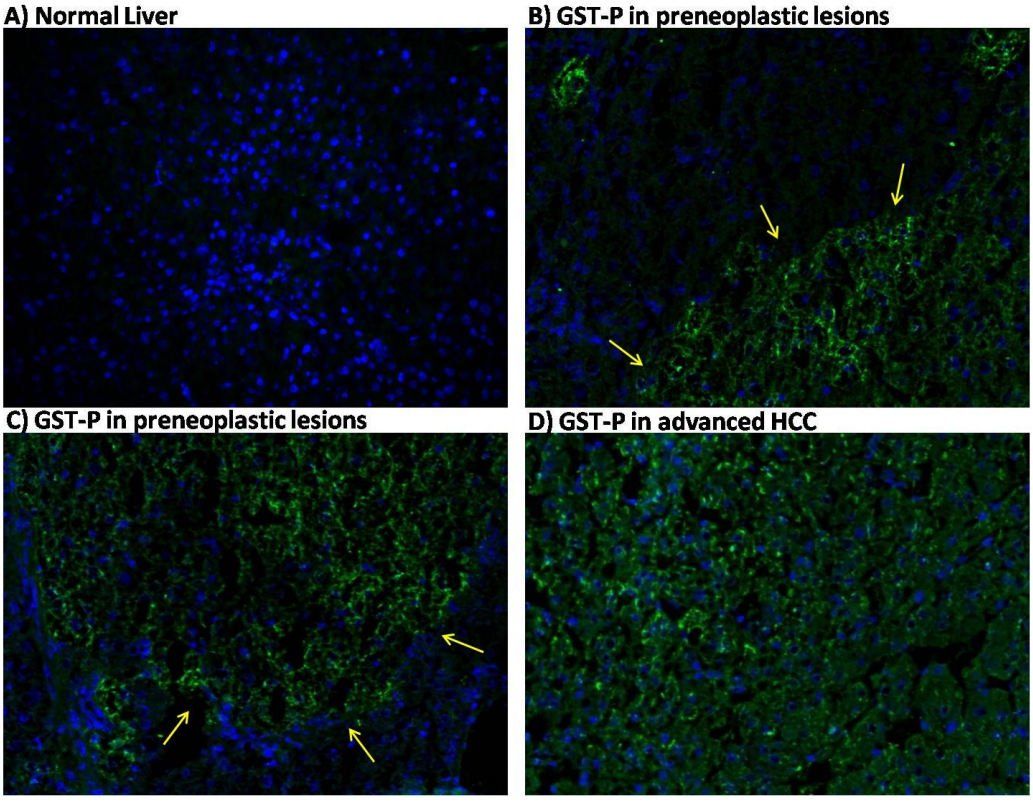


Fig. 3 Immunofluorescence analysis for GST-P in frozen sections of normal liver (A), pre-neoplastic lesions (B,C) and advanced HCC (D). Original magnification 20X. Green staining indicates GST-P expression, whereas blue color corresponds to nuclear staining by DAPI.

When we stained liver sections for NEFL, we found that consistent with mRNA expression data, NEFL protein expression was undetectable in control liver (Fig.4A). On the other hand, an intense NEFL staining was observed in HCCs developed 10 months after initiation (Fig.4 B-D).

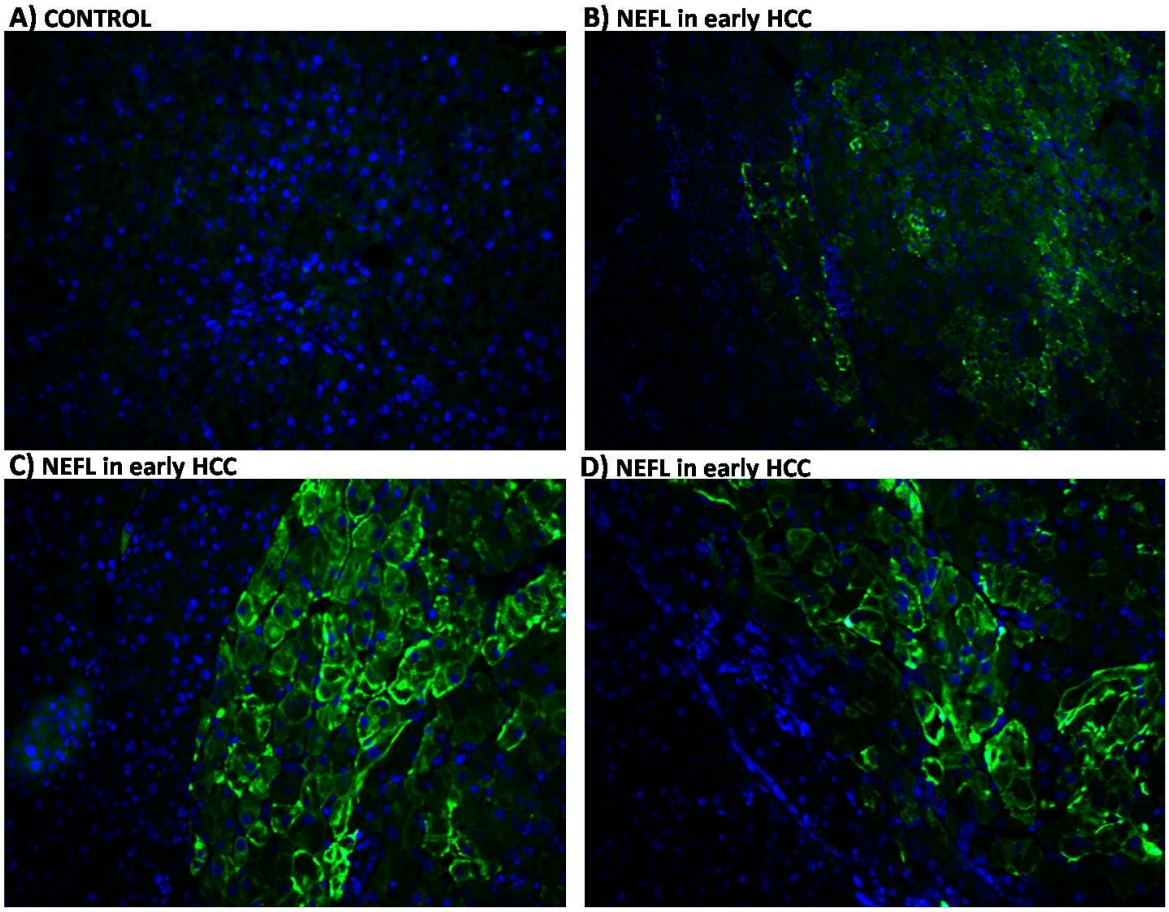


Fig. 4 Immunofluorescence analysis for NEFL protein expression in frozen sections of normal liver and early HCCs (A,C,D original magnification 20x, (B original magnification 10x). NEFL protein expression is indicated in green color; blue color indicates DAPI nuclear staining.

Our previous studies have shown that the vast majority of HCCs arising in the R-H model of hepatocarcinogenesis are KRT-19 positive, despite the fact that KRT-19+ nodules represent a minority of the preneoplastic lesions developed at early stages of the process [*Andersen JB et al 2010*]. This suggests that the subset of KRT-19+ lesions has an intrinsic advantage in the progression to malignancy. Therefore, it is critical to understand whether up-regulation of NEFL occurs in all preneoplastic populations or it discriminates

between KRT-19+ or KRT-19- subpopulations. To this aim, we analyzed NEFL protein expression by immunofluorescence in the preneoplastic KRT-19+ and KRT-19-nodule populations. As shown in Fig.5A, NEFL protein expression was clearly observed by immunofluorescence in KRT-19+ preneoplastic lesions while it was not detectable in KRT-19-nodules (data not shown). As expected, KRT-19 expression was readily detected in early HCCs, which are characterized by positivity for KRT-19 (Fig.5B). Co-localization analysis of the NEFL and KRT-19 proteins at cellular level revealed that only a small number of hepatocytes co-expresses the two proteins (Fig. 5A,B).

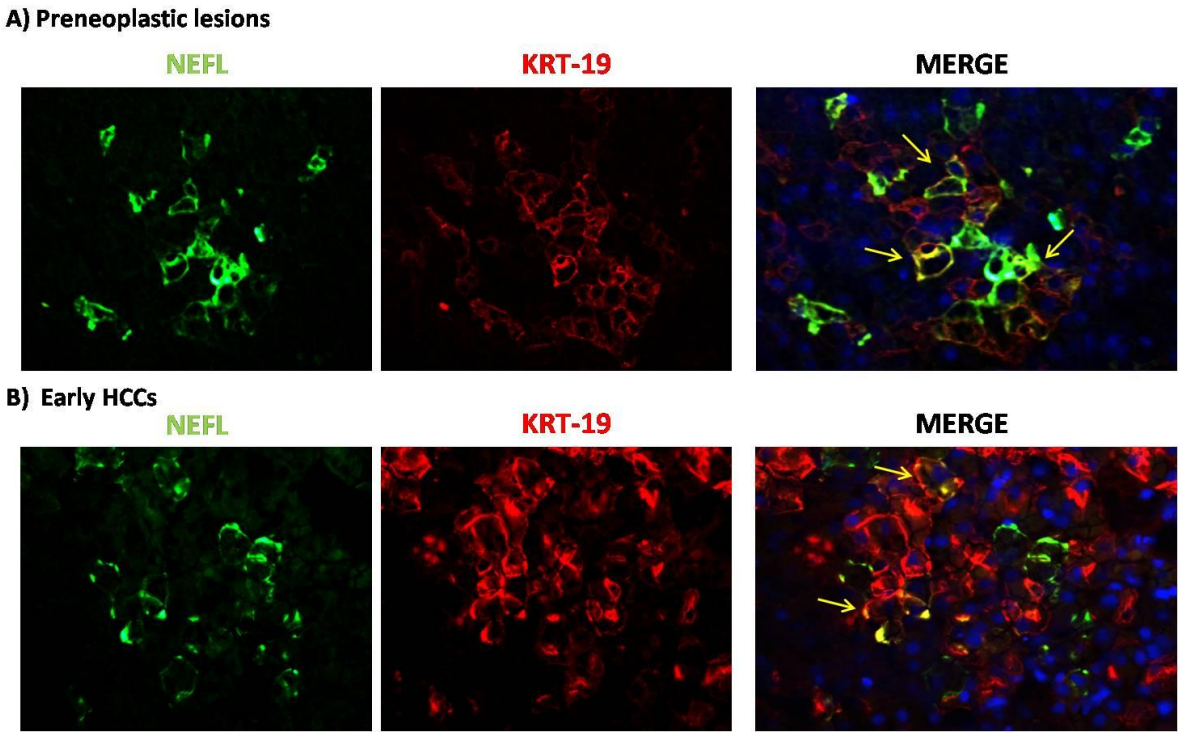


Fig. 5 Immunofluorescence analysis for NEFL and KRT-19 protein expression in frozen sections of **A**) KRT-19+ pre-neoplastic lesions and **B**) early HCCs. (original magnification 20X). Arrows indicate few hepatocytes showing co-expression of NEFL and KRT-19 in both types of lesions.

Overall these results showed that, in agreement with mRNA levels, NEFL protein is expressed only in preneoplastic lesions positive for KRT-19 and in eHCC.

NEFL expression is not present in fetal, neonatal and regenerating liver

Since the presence of this neurofilament protein has never been described in the liver, we wished to investigate whether NEFL expression in the carcinogenic process could be the result of a re-acquisition of proteins expressed in the fetal/neonatal life and lost in adult, differentiated hepatocytes. To this aim, we evaluated the mRNA levels of NEFL in 4 liver samples of 19 days fetuses and 2-days pups compared to 2 normal liver, 3 HCC and 2 normal rat brain samples as positive control. As shown in Fig.6A, almost undetectable levels of mRNA were observed in the liver of 19 days fetuses or 2-days pups; these results indicate that the NEFL gene is not expressed at significant levels in the liver at early developmental stages, and therefore that NEFL expression in the liver carcinogenic process cannot be view as the re-acquisition of a fetal/neonatal phenotype.

It is well known that pre-neoplastic and neoplastic hepatocytes are characterized by a proliferative rate higher than that of normal hepatocytes. Therefore, we reasoned that the up-regulation of NEFL during liver carcinogenesis in the H-R model could merely reflect the increased proliferative activity of pre-neoplastic and neoplastic cells. To verify this hypothesis, we assessed NEFL expression in proliferating normal hepatocytes, i.e. in regenerating liver upon 2/3 partial hepatectomy (PH). To this aim F-344 rats were subjected to surgery and BrdU was administered to label dividing hepatocytes. The results showed that, in spite of the high number of hepatocytes entering into S phase at 24 hours after PH (data are expressed as mean ± SD in 2/3 PH 24h *vs* normal liver: 27.53±0.79 *vs* 1.13±0.56), no significant difference in the expression levels of NEFL was observed in regenerating livers compared to controls (fig.6B).

Taken together, these results demonstrate the NEFL expression in the liver is a specific feature of cancer onset and development.

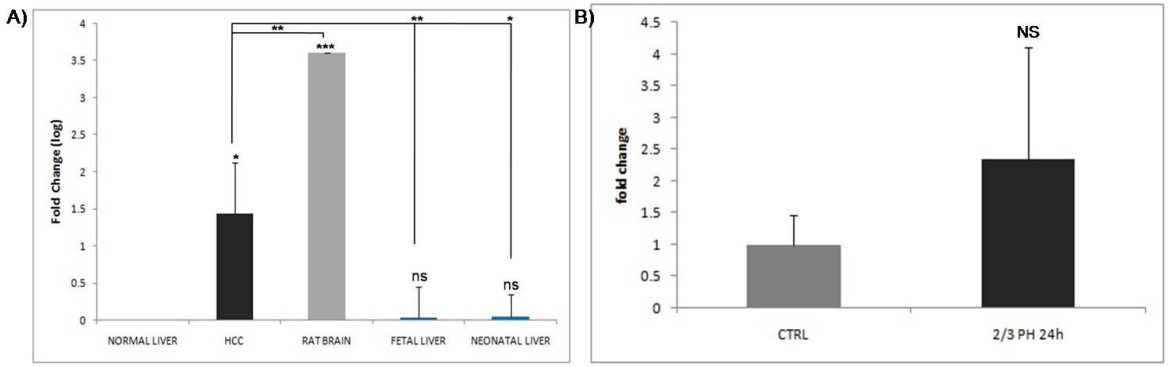


Fig.6 A) Analysis of NEFL gene expression by qRT-PCR in rat liver at different developmental stages and in HCC. NEFL mRNA expression was assessed in normal rat liver (n=2), HCC (n=3), fetal (n=4), neonatal liver samples (n=4) and normal rat brain (n=2) as positive control. Rat β-actin was used as endogenous control. (mean log expression for NEFL in HCC *vs* rat brain *vs* fetal liver *vs* neonatal liver: 1.43±0.69 *vs* 3.61±0.00 *vs* 0.03±0.42 *vs* 0.05±0.30). Analysis of significance was done by One-Way ANOVA ($p<0.0001$). (***$p< 0.0001$ normal liver *vs* rat brain; *$p< 0.01$ normal liver *vs* HCC; **$p< 0.001$ HCC *vs* rat brain, fetal liver; *$p< 0.01$ HCC *vs* neonatal liver). **B**) Analysis of NEFL mRNA expression by qRT-PCR in rat liver at 24 hours after 2/3 PH. The levels of expression are calculated as fold change between control liver (n=2) and liver at 24 hours after 2/3 PH (n=4). Rat β-actin was used as endogenous control. (data are expressed as mean± SD NEFL in 2/3 PH 24h *vs* control: 2.34±1.7 *vs* 1±0.46).

Analysis of CpG Island methylation status of the NEFL gene

Aberrant methylation of the NEFL gene promoter has been described in head and neck cancers (*Chen B et al., 2012*). Therefore we wished to determine whether changes in the methylation status of the CpG islands of the NEFL gene could be responsible for its up-regulation in HCC development. To this aim, we performed pyrosequencing analysis on 5 rat HCCs and 3 liver controls. As shown in Table3, very low levels of methylation of the CpG islands of NEFL were detected in the HCCs analyzed, (average rate of methylation in HCC vs controls was 4.9±1.47 *vs* 3.4±0.20), with no significant differences compared to controls (*P*=0.135). Thus, these results make very unlikely the possibility that up-regulation of NEFL is the consequence of aberrant transcriptional activation due to hypomethylation of the NEFL gene promoter.

	METHYLATION FREQUENCY (%) AT 10 INDIVIDUAL CpG SITES										
	1	2	3	4	5	6	7	8	9	10	Ave.
Control liver 1	3.5	4.5	4.5	4.5	3	3	3.5	4	2.5	1	**3.4**
Control liver 2	3.5	3	3.5	5	2.5	3	3.5	4	3.5	0	**3.2**
Control liver 3	4	3.5	4	5.5	3.5	3	3.5	3.5	3.5	1.5	**3.6**
HCC 1	5	5	4.5	7.5	3.5	7	5.5	5	4	3	**5**
HCC 2	6.5	7.5	4	9	1.5	4	5.5	4	3.5	1.5	**4.7**
HCC 3	7	11	5	12.5	6	5	10	4	5.5	6	**7.2**
HCC 4	5.5	5.5	4.5	9	1.5	4	5.5	4.5	4	1.5	**4.6**
HCC 5	5	5	4	5.5	2	3	2.5	1.5	2	0	**3.1**

Table3: Methylation frequency at specific CpG sites in the promoter of the NEFL gene. 10 CpG sites were scored for methylation by pyrosequencing. 3 age-matched control normal liver and 5 HCCs were analyzed. Average rates of methylation for each sample are indicated in bold character.

Investigation of the role of microRNA in NEFL up-regulation

microRNAs have been shown to play a fundamental role in the control of gene expression (*Calin GA et al, 2006*), and play a major role in cancer development *[Esquela-Kerscher and Siack, 2006; Melo SA, 2011].* Therefore, we investigated whether down-regulation of miRNAs targeting NEFL mRNA could be the cause of NEFL up-regulation in rat HCC development. In silico analysis of predicted microRNA targets indicated that NEFL has a binding site for miR-30 family members. Initially, we assessed the expression levels of two miRNAs predicted to target NEFL, namely miRNA 30d e miRNA 30e. Expression levels were evaluated by qRT-PCR analysis in a set of 9 KRT-19 positive preneoplastic lesions and 4 aged-matched (10 weeks) controls, plus 3 extra normal liver controls at 14 months of age (Fig.7A). qRT-PCR analysis did not show any significant difference in the expression of miRNA 30d e miRNA 30e in the KRT-19+ lesions *vs* respective controls, indicating that the expression of these two miRNAs is not modified during the early phases of the hepatocarcinogenic process. Next, we assessed, by qRT-PCR, the levels of expression of NEFL and miR-30e in parallel in the same set of 12 early HCCs, in order to directly evaluate the relationship between miR-30e levels and NEFL expression in the same sample (fig.7B). The results show that while NEFL is significant up-regulated in the early HCCs respect to control liver (n=3), no change in the NEFL-targeting microRNA miR-30e could be found, suggesting that miR-30e is not involved in the regulation of the expression of NEFL in early HCCs.

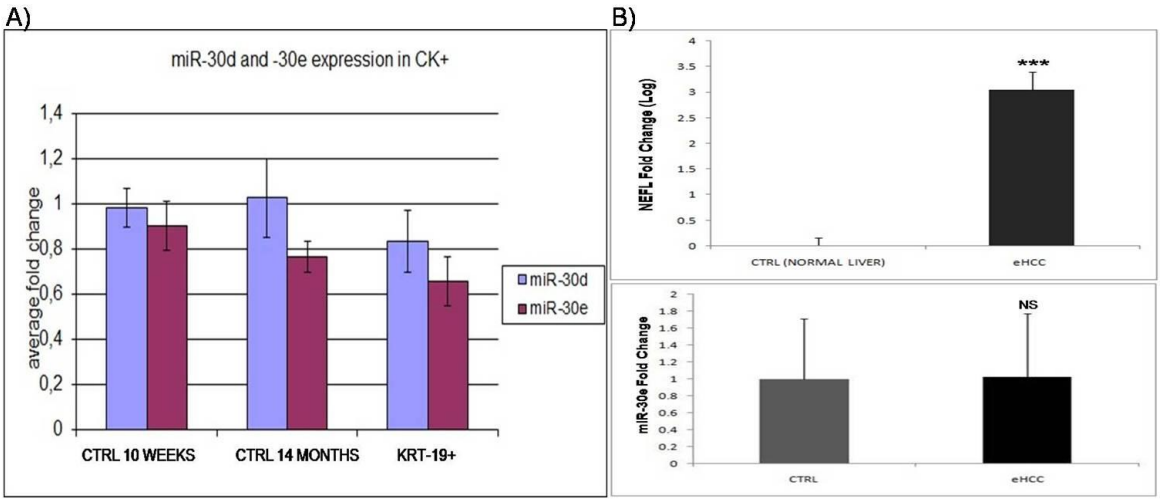


Fig.7 Assessment of miR-30d and miR-30e expression by qRT-PCR at different stages of the hepatocarcinogenesis process. **A**) miR-30d and miR-30e expression was assessed in KRT-19+ preneoplastic microdissected samples, age-matched controls and 14 months normal liver controls. Differences in expression were not statistically significant. Rat β-actin was used as endogenous control. **B**) NEFL (upper panel) and miR-30e (lower panel) expression was assessed by qRT-PCR in parallel in the same early HCCs. The levels of expression were calculated as fold change between 3 control liver and 12 eHCC samples. Rat β-actin was used as endogenous control for NEFL. U6 small nuclear RNA was used as endogenous control for miR-30e.

Analysis of NEFL expression in human HCC

Although the mechanisms responsible for NEFL up-regulation observed in rat pre- and neoplastic lesions remain obscure, the finding that this gene is highly up-regulated in hepatocarcinogenesis may represent a relevant observation endowed with possible prognostic/diagnostic value. Therefore, we sought to determine whether the results obtained in the R-H model could be of translational value for human HCC. To this aim, NEFL mRNA levels were determined by qRT-PCR in 14 human HCCs. NEFL expression levels in HCCs were compared to NEFL expression in matched cirrhotic tissues (CE) (Fig.8A). The results showed that, although statistical significance was not reached due to the low number of samples and the degree of variability, a trend towards an increase of expression of NEFL in HCCs compared to matched non-cancerous cirrhotic tissues (CE) was clearly observed. Notably, similar to rat normal liver, NEFL mRNA levels were undetectable in a liver biopsy of a “healthy” patient (cycle threshold CT>40).

Next, the prognostic value of NEFL expression in human HCCs was evaluated in a cohort of 54 patients (the characteristics of the HCC patients are described in TableI). When patients were divided into NEFL low- or high-expression groups based on the median expression level (Fig.8B), the analysis showed that the time of recurrence following surgery was significantly shortened in the high NEFL expression group compared to that of the low-expression group. Notably, the results were highly statistically significant ($p = 0.031$).

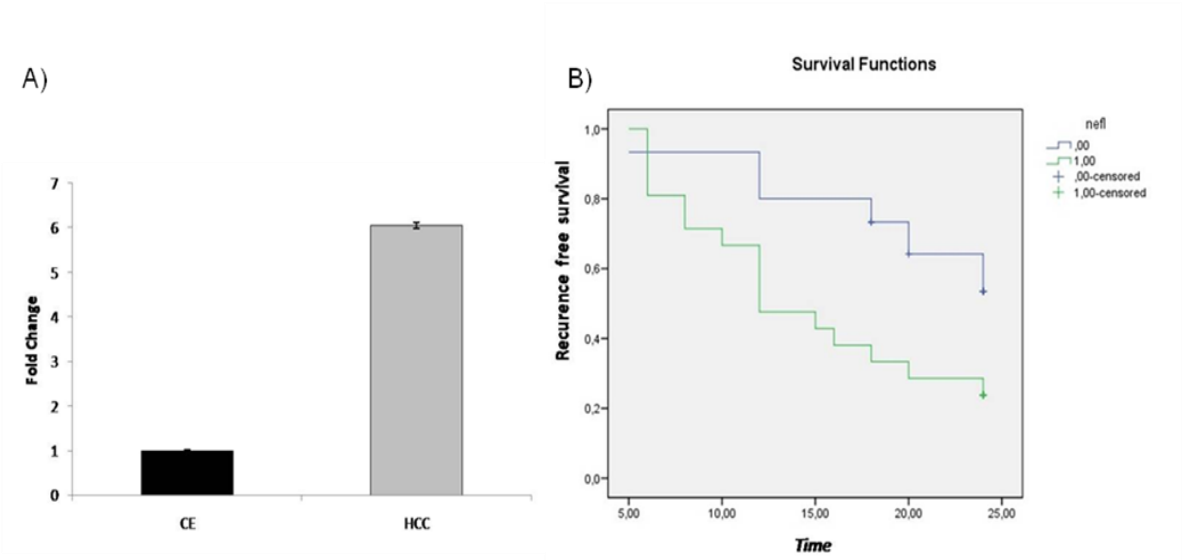


Fig.8 A) Analysis of NEFL gene expression in by qRT-PCR in HCC and matched cirrhotic tissue (CE). Human β-actin was used as endogenous control (*p=0.21*). Analysis of statistical significance was done by t Student's test (p=0.21). (Data are expressed as mean ± standard deviation (SD) (mean expression for NEFL in HCC *vs* CE: 6.05±0.069 *vs* 1±0.01). **B**) Kaplan-Meier plot showing *time of recurrence* relative to patients resected for HCC, divided into two groups based on the expression of NEFL mRNA. The blue curve is relative to patients not overexpressing NEFL, that relapse less. The green curve refers to patients overexpressing NEFL that recur more and in a short time. Log rank (Mantel-Cox) test *p=0.031.*

Table I. Characteristics of HCC patients analysed in this study

Serial N.	Gender	Age	Cause of liver disease (1)	Focality (2)	Size (3)	AFP (4)	Grading (5)
1	M	60	HCV	multi	1.3	252577	G4
2	M	79	HBV	multi	7.0	540	G4
3	M	75	HCV	multi	3	6363	G3
4	M	70	HCV	multi	2.3	46	G3
5	M	59	HCV	uni	5	86	G3
6	M	65	HCV+Ethanol	uni	3.0	20	G3
7	M	78	HCV	uni	3	9	G2
8	M	79	HCV	multi	10	7	G3
9	M	70	HCV	uni	4.0	35	G3
10	M	59	HCV+HBV	uni	7	500	G4
11	M	65	HCV	multi	6.5	167	G3
12	F	65	HCV	uni	3.0	3	G3
13	M	75	HCV	multi	7.0	9	G3
14	M	72	HCV	multi	3.4	18	G3
15	M	76	HBV-Ab	multi	5	10.000	G4
16	M	65	HBV+Ethanol	uni	3.0	5	G2
17	M	74	HCV	multi	3.5	2198	G3
18	M	60	Ethanol	uni	1.8	156	G4
19	M	54	HBV	uni	4	162	G2
20	M	59	HCV	multi	3.0	76	G2
21	M	68	HCV	multi	2.5	5	G2
22	M	65	None	Multi	3	48	G3
23	M	71	HCV	multi	4	96	G3
24	M	74	None	uni	11	78	G3
25	M	69	HCV	uni	10	390	G3
26	F	59	HCV	uni	6	7	G4
27	M	75	None	multi	15	6	G3
28	F	81	Hcv	Multi	10	3000	G4
29	M	59	HCV	Multi	8	56	G4
30	F	70	HCV	Uni	4.8	90	G1
31	M	66	HCV	Uni	5.5	223	G2
32	M	70	HBV	Uni	8.0	162	G3
33	M	69	None	Uni	3.8	8	G2
34	M	53	HCV	Multi	5.0	285	G4
35	M	70	HBV	Multi	4.5	23	G2
36	M	78	HBV	Uni	3	-	G3

37	M	66	HCV	Uni	5	-	G3
38	F	60	HCV	Multi	4,5	445	G3
39	F	73	None	uni	5.5	2	G3
40	M	71	HBV	Multi	4.4	65	G4
41	M	68	HCV	Uni	3.6	7	G2
42	M	60	HBV + HCV	Multi	2.3	34	G2
43	F	80	HCV	Uni	5.0	76	G3
44	F	53	HCV	MULTI	4.0	60	G3
45	M	55	HCV+alcol	Uni	7	3	G3
46	M	77	HBV-Ab	uni	5.0	4	G3
47	F	69	HCV	Uni	4	77	G2
48	M	74	HCV	Uni	6	30	G4
49	F	76	HBV	Uni	2	47	G3
50	F	71	HCV	uni	2.0	276	G3
51	M	54	HCV	Multi	3	19	G3
52	M	63	None	Multi	4,5	6	G2
53	M			Uni	6,5	-	G3
54	F	72	HCV	Uni	3	6	G3

1. Cause of underlying liver disease: HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; Ethanol: History of ethanol abuse; HBV-Ab: presence of the antibodies against HBV; None: negative history for hepatitis virus infection and ethanol abuse.
2. Focality: uni-or multifocality was assessed on the basis of imaging techniques previous to surgery and by means of intra-operative ultrasound.
3. Size of the HCC nodule (in centimeters) used for RNA and protein extraction.
4. AFP: alpha-feto-protein determination was made prior to surgery and is expressed in ng/mL.
5. Grading of the HCC was assessed according to Edmondson and Steiner's criteria.

DISCUSSION

The role of the NEFL gene in cancer development and progression is still poorly understood. Studies regarding the role of the NEFL protein have been mostly focused to neurological diseases, such as Charcot–Marie–Tooth's disease (CMT) (*Mersiyanova IV et al, 2000*). The correlation between NEFL and cancer development is mostly due to the frequent observation of LOH for the NEFL locus in several neoplasms including breast, head and neck, prostate, lung, colon, urinary bladder cancers, and HCC (*Li XQ et al., 2012; Coon et al., 2004; Macoska JA et al., 1995; Kurimoto F et al., 2001; Lerebours F et al., 1999; Knowles MA et al., 1993; Becker et al., 1996*). In some cases, as in breast cancer, LOH at the NEFL locus is associated with increased malignancy, consistent with a tumor-suppressive role for the NEFL gene (*Yaremko ML et al, 1996*).

In normal tissues, NEFL expression is restricted to neurons, where it encodes type IV intermediate filament heteropolymers, which represent a major component of the neuronal cytoskeleton. Intriguingly, ectopic expression of NEFL has been reported for several malignancies, including head and neck and breast cancers (*Chen B et al. 2012, Li XQ et al., 2012*). The aberrant expression of NEFL in cancerous tissues that, in their normal counterpart, do not express this gene, is not fully understood. It may be speculated that it may have an active role in tumor development, or it may merely be part of an adaptive cytoprotective response that follows malignant transformation (*Julien JP, 1999*). It has been shown, in this respect, that tissue cells, when exposed to cumulative physical and chemical carcinogenic factors, change their expression profile to adapt to the new microenvironment (*Kim MS, et al, 2006*).

Our gene expression studies results, obtained by cDNA microarray and qRT-PCR, demonstrate that NEFL is not expressed in normal rat adult liver. This finding was confirmed at the protein level, by immunofluorescence staining. On the other hand, in agreement with evidence collected for other malignancies (*Chen B et al. 2012, Li XQ et al., 2012*), NEFL expression was readily detected during the hepatocarcinogenic process in R-H model. Indeed, cDNA microarray analysis and qRT-PCR analysis showed that the expression of NEFL was strongly up-regulated at all stages of the multistep process of hepatocarcinogenesis, including the very early ones. In particular, NEFL mRNA was strongly up-regulated in KRT-19 positive preneoplastic lesions and in early HCCs.

In agreement with mRNA levels, immunofluorescence studies identified the neurofilament only in the preneoplastic lesions positive for KRT-19 and in early HCCs.

Since the NEFL gene is not expressed in healthy adult liver, we wished to investigate whether NEFL expression during the carcinogenic process in the R-H model could be the result of a re-acquisition of fetal/neonatal life status, a phenomenon commonly associated to cancer development and progression. However, our results indicate that this is not the case, since almost undetectable levels of mRNA were observed in the liver of 19 days fetuses or 2 days pups. Next, we asked whether expression of NEFL in the hepatocarcinogenic process could merely be associated to the increased proliferative activity of preneoplastic and neoplastic hepatocytes, since normal hepatocytes are quiescent in healthy liver, where NEFL gene is silent. However, no NEFL up-regulation was observed in actively dividing hepatocytes in liver regeneration occurring following two/thirds partial hepatectomy (PH), suggesting that NEFL expression in the liver is a specific feature of cancer onset and development.

The mechanisms responsible for the ectopic expression of NEFL through the carcinogenic process in the R-H model are still unknown. Our results demonstrate that dysregulation of the NEFL promoter through CpG island hypomethylation, potentially leading to aberrant NEFL expression, is not involved in the regulation of NEFL expression in the R-H model. Likewise, we ruled out, as a potential mechanism leading to elevated mRNA levels of NEFL during hepatocarcinogenesis, the downregulation of some miRNAs predicted to target NEFL, since their levels are unmodified during HCC onset and progression in the R-H model.

To assess the clinical significance of NEFL expression in human HCC patients, we determined NEFL mRNA levels in 14 HCC patients. The results showed that, similarly to what observed in the rat model, while NEFL expression was undetectable in normal human liver, it was readily detectable in HCCs and in matched cirrhotic liver. This demonstrates that aberrant expression of NEFL takes place at the onset and progression in human hepatocarcinogenesis, translating our findings to human pathology. Interestingly, although not statistically significant, a clear trend towards an up-regulation of NEFL in HCCs when compared to matched cirrhotic tissue was observed. Notably, when patients were divided into two groups, NEFL low- or high-expressors, based on the median expression level, the results showed that *time of recurrence* was significantly shortened in high-NEFL expression compared to low-NEFL expression group, thus demonstrating that NEFL expression levels are a predictive factor for HCC prognosis.

These results are in contrast with findings reported for early breast cancer patients, where a low NEFL mRNA expression level was found to be a negative prognostic factor to predict disease-free survival of early-stage breast cancer patients, supporting a tumor-suppressive role for NEFL (*Li XQ et al, 2012*).

Overall, the role of the NEFL gene in cancer development is unclear. Several reports suggest a potential role as tumor suppressor for this gene in malignancies, such as breast cancer (*Li XQ et al, 2012*) and head and neck cancer (*Huang et al, 2013*). However, our findings in the animal model and more specifically our findings in human HCC patients, where a low expression of NEFL is a positive prognostic factor, clear challenge this view and actually support an oncogenic role for NEFL in liver carcinogenesis.

Further studies are required to identify the mechanism(s) involved in the aberrant expression of NEFL in hepatocarcinogenesis and its role in HCC development. A better knowledge of the role of NEFL in HCC progression might hopefully provide a novel therapeutic target for HCC.

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