ARTICLE

The levels of the RNA binding protein Hu antigen R determine the druggability of the neddylation pathway in liver cancer

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Hepatocellular carcinoma (HCC), the most common liver cancer, is an important leading cause of death worldwide. Neddylation is a post-translational modification involved in several processes such as cell growth, viability and development. Importantly, the neddylation pathway is upregulated in liver cancer and specifically enriched in patients with poor prognosis. Hu antigen R (HuR), is a RNA-binding protein that stabilizes target mRNAs involved in hepatocyte proliferation, differentiation and malignant transformation. And notably, HuR levels are highly representative in liver and colon cancer. A ground-breaking knowledge about HCC has been to identify that neddylation plays a role in HCC by regulating the liver oncogenic driver HuR. In addition, the neddylation inhibitor MLN4924 has shown antitumoral effects *in vitro* and *in vivo* in liver cancer, partly through HuR destabilization. Importantly, high levels of HuR made hepatoma cells more resistant to neddylation inhibition while low levels of HuR sensitized cells to the treatment, suggesting that the levels of HuR determine the druggability of the neddylation pathway in HCC. Overall, our findings highlight the impact that neddylation plays in liver cancer and open a completely new area of research, paving the way for novel therapeutical approaches.

Keywords: Neddylation; Hepatocellular carcinoma; HuR; MLN4924

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of death by malignancy [1]. HCC is a very complex and heterogeneous pathology with multiple and distinct signaling pathways converging in the same malignant transformation [2].

Hu antigen R (HuR) is the most ubiquitous member of the mammalian Hu/elav family of RNA-binding proteins (RBPs), that also includes the neuronal members HuB, HuC and HuD [3, 4]. HuR (also known as ELAVL1) has been described to stabilize ARE-containing mRNAs [5, 6] and to modulate their translation, both enhancing and inhibiting it [7]. In the cytoplasm, HuR can promote mRNA stabilization and upregulation or repression of mRNA translation [8, 9].

Many of the HuR-regulated mRNAs participate in the acquisition of cancer traits such as enhanced ability to proliferate, enhanced cell survival, elevation of local angiogenesis, evasion of immune recognition, and invasion and metastasis [9, 10]. Many studies have examined the levels of HuR in different cancer types, finding them misregulated among others in breast, pancreatic, colon and ovarian cancer, constituting an important prognostic factor [8, 9]. HuR has also been found elevated in the liver of cirrhotic patients, together with a prominent increase of the cytosolic localization [11]. Also, HuR has been found highly expressed in the hepatocellular carcinoma (HCC)-derived SAMe-D cell line, in which it stabilizes *HAUSP* mRNA. HAUSP is an ubiquitin specific protease that stabilizes p53 in the cytosol inducing the cell cycle arrest and the apoptotic response [12].

In the liver, HuR plays a role in hepatocyte proliferation, differentiation and hepatocellular carcinoma transformation, participating in the switch from MAT II enzyme (expressed in fetal and proliferating liver and during malignant transformation) to MAT I/III (expressed in normal adult liver)[13]. In this case, HuR stabilizes MAT2A mRNA (that codifies for MAT II) whereas methyl-HuR destabilizes it. Through liver differentiation, the methyl-HuR/HuR ratio increases leading to the decrease of MAT2A mRNA. In the proliferating liver and during malignant transformation the process is the opposite. The modulation of the levels of MAT II and MATI/III regulates the abundance of S-adenosylmethionine, which is critical for liver function [13].

In addition to this regulation, HuR nucleocytoplasmic shuttling is activated by AMPK in response to HGF during hepatocyte proliferation. The blockade of this translocation is able to abrogate cell cycle progression [14], as occurs in the *Gnmt* KO mice after partial hepatectomy, which present an impaired liver regeneration [15].

Moreover, HuR is able to regulate hepatic stellate cells activation and to raise the expression of proinflamatory and chemoattractant genes in a cholestatic liver injury model (bile duct ligation). In this way, HuR increases liver damage, oxidative stress, inflammation, macrophage infiltration and liver fibrosis development, enhancing the risk of HCC development [16]. Finally, both in HCC and colon cancer cells, together with patients, HuR is overexpressed through a novel mechanism based on Mdm2-mediated neddylation [17].

Neddylation promotes timely stabilization of proteins with essential regulatory roles in an extensive variety of biological processes [18]. Through modification of cullins, Nedd8 controls the activity of cullin-ring-ligases and subsequently the stability of multiple substrates. A misregulation in neddylation disturbs protein homeostasis, which is linked to malignant transformation [19]. We have previously identified and characterized multiple functions of neddylation in liver tumorigenesis [17, 20]. Indeed, we have established a significant increase in neddylation levels in human HCC, which is associated with faster tumor progression and with a signature of poor outcome [20]. Additionally, we have found that primary pathways for anticancer drug development including PI3K/Akt, LKB1 and HuR in the liver are new targets for neddylation, undoubtedly providing important insights into the regulatory mechanism of these oncogenic pathways. Importantly, neddylation controls the nuclear localization of HuR, protecting it from degradation and increasing its stability [17].

Neddylation inhibition has been described as a potential treatment for HCC [20, 21]. Indeed, the treatment with the drug MLN4924 and the silencing of Nedd8 and NAE1 showed antitumoral effects inducing apoptosis and a metabolic reprogramming in liver cancer cells and the reduction of tumor progression *in vivo* in the *Phb1* KO HCC mouse model. Importantly, the restoration of LKB1 and Akt expression was able to block the metabolic reprogramming and associated cell apoptosis induced by neddylation inhibition [20]. These data further support that both LKB1 and Akt destabilization partially drive the metabolic phenotype induced by lack of neddylation.

In this work we have studied the role of HuR in the mediation of the apoptotic response induced by neddylation inhibiton in liver cancer cells. Regulating the expression of HuR using specific adenovirus and lentivirus we have been able to modulate the sensitivity to neddylation inhibition in HepG2 and BCLC3 hepatoma cell lines.

These results support a cooperative association between HuR levels and neddylation inhibition and pave the way for novel and more personalized therapeutical approaches for HCC.

Materials and Methods

Cell lines

In vitro experiments were performed using the following human hepatoma cell lines: BCLC3 human hepatoma cell line was previously characterized and provided by Dr. Jordi Bruix and Dr Loreto Boix (BCLC group. Hospital Clinic, Barcelona, Spain), and HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA).

Treatment of cell lines with MLN4924

HepG2 and BCLC3 cell lines were treated with MLN4924 at a dose of 3μM and maintained for 48 and 72 hours. At the indicated times, cells were lysed and proteins were analyzed by Western blotting. MLN4924 was provided by Millennium Pharmaceuticals Inc.

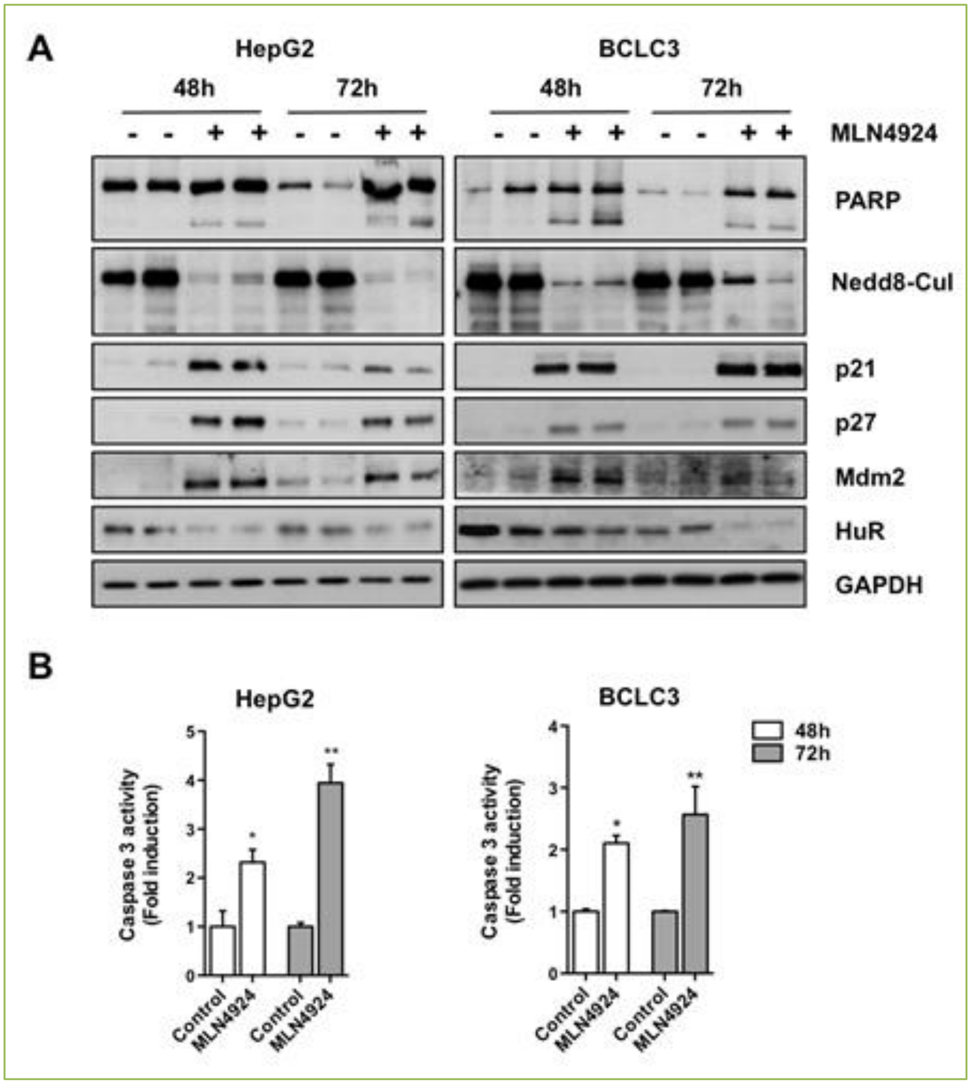

Figure 1. MLN4924 reduces HuR expression and induces apoptosis in hepatoma cells. (A) Western blot analysis using the indicated antibodies and (B) caspase 3 activity assays in total lysates from HepG2 and BCLC3 cell lines after 48 and 72 hours of MLN4924 3μM treatment. *p<0.05; **p<0.01 MLN4924 *vs* control. Data are mean ± SD.

In vitro silencing

BCLC3 and HepG2 cell lines were transfected with 100 nM Nedd8 siRNA (Qiagen) using the Lipofectamine 2000 reagent (Invitrogen). Controls were transfected with an unrelated siRNA (Qiagen). Protein knockdown was confirmed by Western blotting.

Viral infection

For HuR knockdown, HepG2 and BCLC3 cell lines were treated with short-hairpin lentiviral particles against HuR. For HuR overexpression, adenoviral particles, (AdHuR), were added [30]. Importantly, 24 h after the infection the medium was changed.

Protein isolation & western blotting

Extraction of total protein from cultured cells has been described [28]. Four to twenty five μg of protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred onto membranes. A description of the antibodies used is provided in Supplementary Table 1.

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated using Trizol (Invitrogen, UK). One to two μg of total RNA was treated with DNAse (Promega, UK) and reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, UK). Then, qPCR was performed using iQTM SYBR® Green Supermix (Bio-Rad)

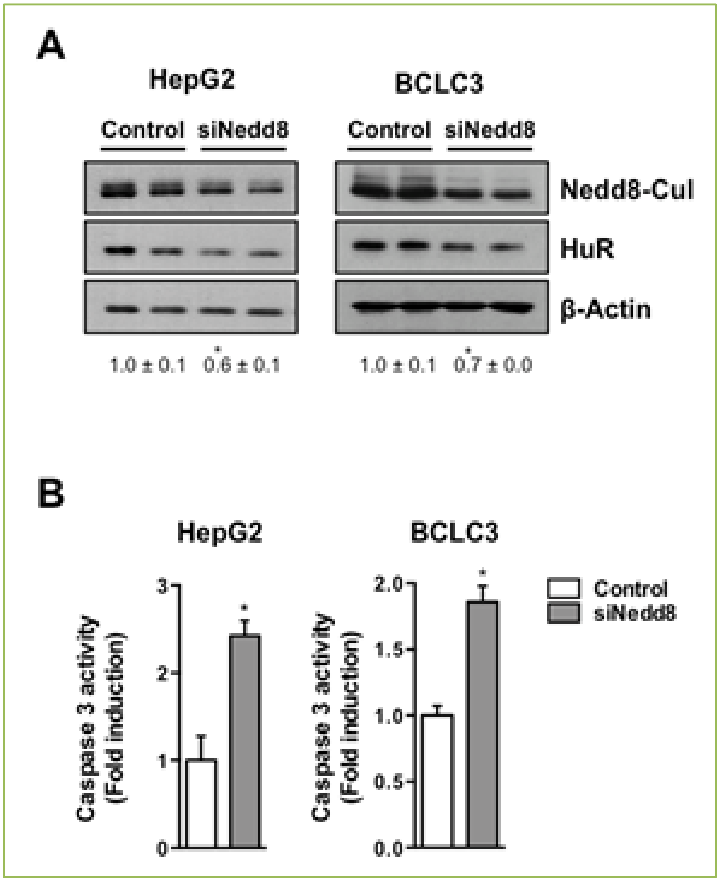

Figure 2. Neddylation pathway specific knockdown induces apoptosis in liver cancer cells. HepG2 and BCLC3 cells were silenced with a specific Nedd8 siRNA for 48 hours. (A) Whole-cell lysates were analyzed by Western blotting using the indicated antibodies and (B) by caspase 3 activity assay. *p<0.05 siNedd8 *vs* control. Data are mean ± SD.

using the CFX ConnectTM RT-PCR Detection System (Bio-Rad). Expression levels were normalized to the average level of α2-macroglobulin mRNA in each sample. The sequence of primers used for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis is described in Supplementary Table 2.

Apoptosis measurement

Caspase 3 activity was quantified on cell protein extracts by proteolytic cleavage of the fluorogenic substrate Ac-Asp-Glu-Val-Asp-AFC (AFC = 7-Amino-4- trifluoromethyl coumarin) (Enzo BML-P409). Fluorescence was quantified with a SpectraMax M2 microplate reader (Molecular Devices, Palo Alto, CA, USA).

Statistical analysis

All experiments were performed in triplicate. Data are expressed as mean ± SD. Statistical significance was estimated with Student´s t test. *p* value<0.05 was considered significant.

Results

Neddylation inhibition triggers an apoptotic response in liver cancer cells

Previous studies reported that MLN4924 induced apoptosis in HepG2 and Huh7 cells [21]. Similarly, we found that NAE1 inhibition suppressed cancer cell growth in HepG2 and BCLC3 cells as demonstrated by the appearance of PARP cleavage and an increase in caspase 3 activity (Fig. 1A-B). Decreased neddylation, measured as accumulation of Nedd8-cullin conjugated levels, was accompanied with an accumulation of well-known CRL/CSF substrates such as p21, p27 and Mdm2 indicating efficient inactivation of NAE1 by MLN4924.

Importantly, neddylation pathway inhibition using a specific Nedd8 siRNA (Fig. 2A) induced the same apoptotic

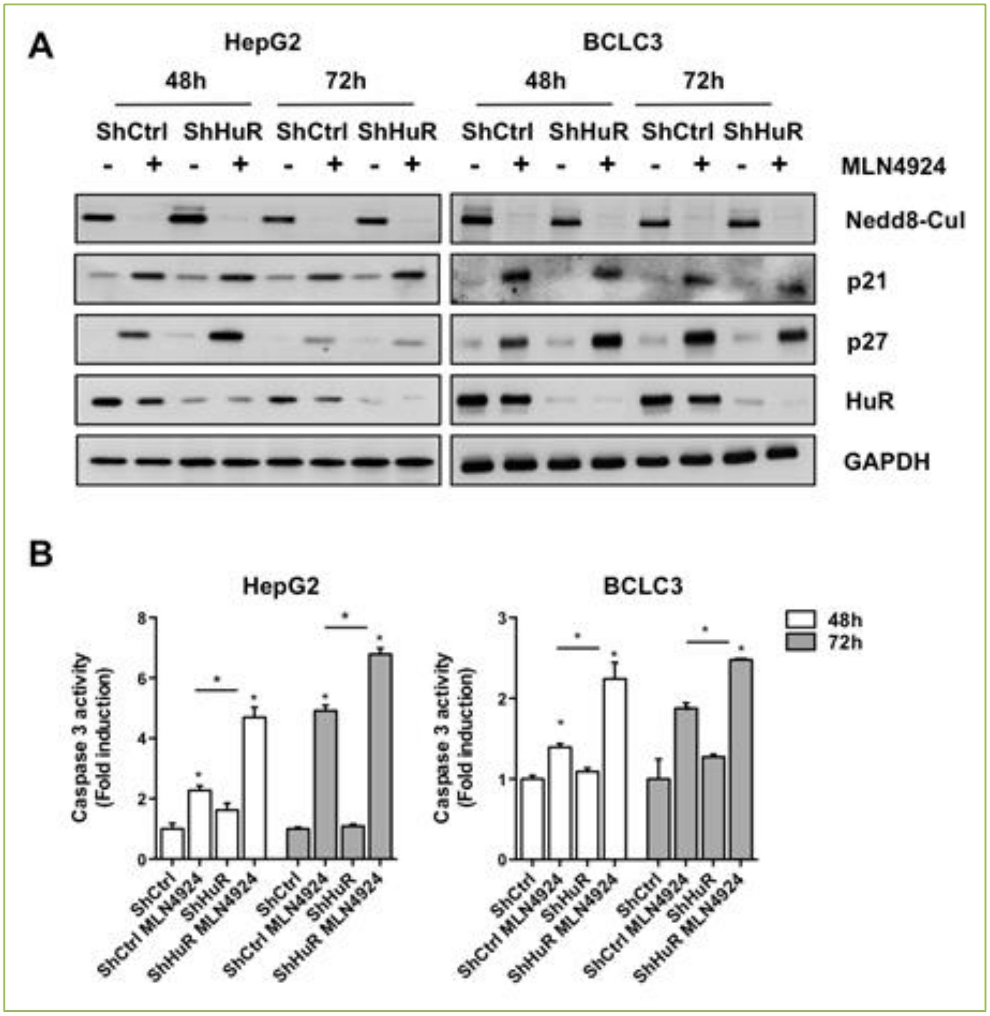

Figure 3. HuR silencing makes cells more sensitive to MLN4924 treatment. HepG2 and BCLC3 cells were infected with a specific HuR lentivirus and treated with MLN4924 (3μM) for the indicated times. (A) Whole-cell lysates were analyzed via Western blotting with the indicated antibodies and (B) by caspase 3 activity assay. *$p<0.05$ MLN4924 *vs* control. Data are mean ± SD.

response in HepG2 and BCLC3 cell lines, as shown by caspase 3 activity (Fig. 2B).

Blocking neddylation reduces HuR levels in liver cancer cells

Considering that MLN4924 exerts its effect by inactivating Nedd8 activating enzyme, we assessed the levels of the recently characterized protein target of neddylation deeply implicated in liver tumors, HuR. We hypothesized that NAE1 inhibitor should induce HuR destabilization and reduction of its total levels. Indeed, a remarkable decrease in HuR was coupled with the apoptotic response detected in each cell line after MLN4924 treatment (Fig. 1A). Importantly, we did not observe any regulation at mRNA level (data not shown).

Along with cell death, Nedd8 knockdown destabilized HuR protein (Fig. 2A), confirming a direct impact of this protein levels in the apoptosis mediated by neddylation blockage in liver tumor cells.

These findings suggest that neddylation inhibition regulates key proteins for tumor survival like HuR, enhancing liver apoptosis. Moreover, these results indicate that elevated HuR levels in tumor cells exert a proliferative and antiapoptotic function and allowed us to hypothesize the role of this RNA binding protein in the MLN4924-induced apoptotic response.

The druggability of the neddylation pathway in liver cancer cells is associated with the HuR-dependent apoptotic response

We have recently described that HuR is a new target for neddylation and plays a central role in modulating a variety of

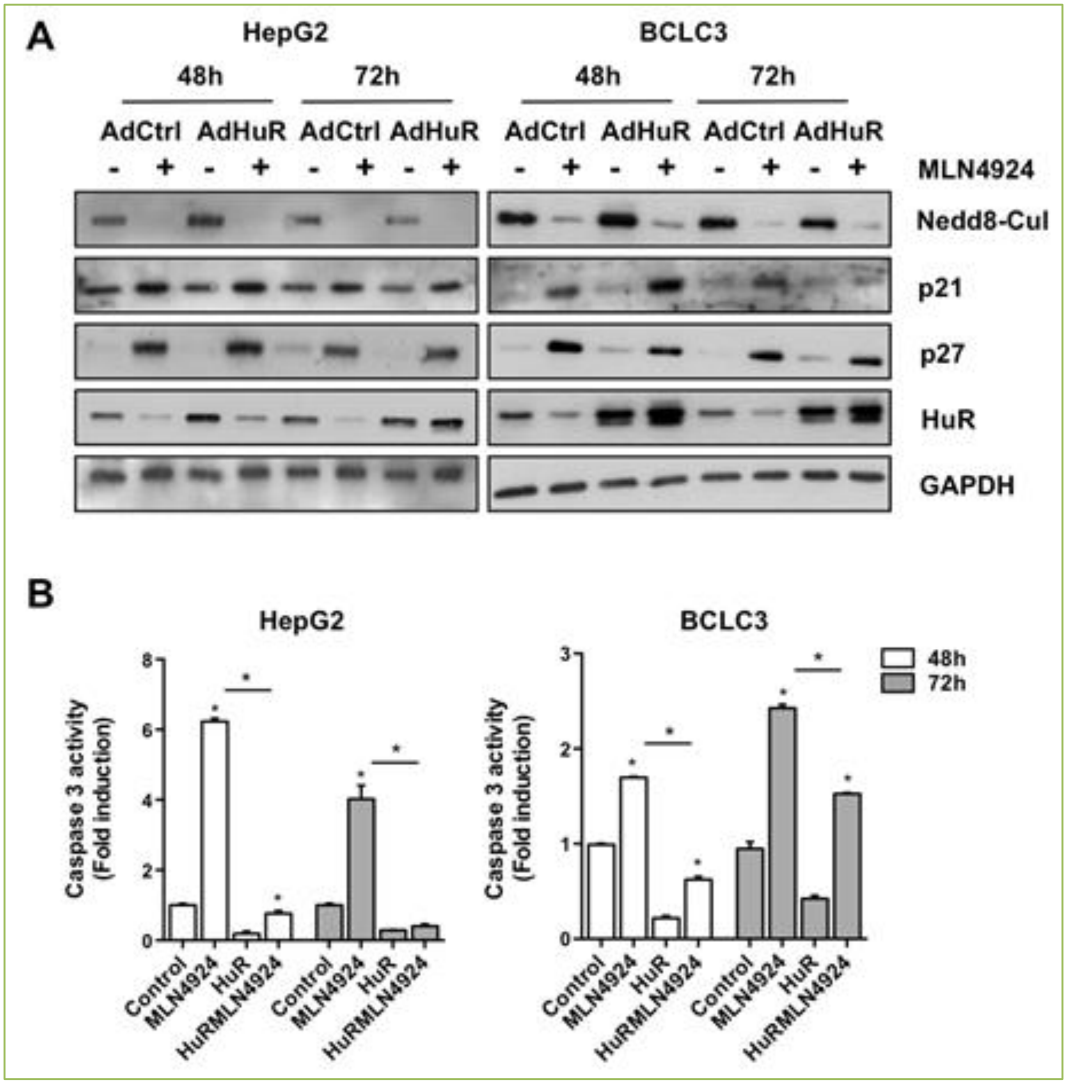

Figure 4. HuR overexpression makes cells more resistant to MLN4924 treatment. HepG2 and BCLC3 cells were infected with a specific HuR adenovirus and cultured with MLN4924 (3μM) for the indicated times. Whole-cell lysates were analyzed via Western blotting with the indicated antibodies (A) and for caspase 3 activity (B). *p>0.05 MLN4924 *vs* control.

complex physiological and pathological processes in the liver [16, 17]. We studied the role of HuR in MLN4924-mediated apoptosis in HepG2 and BCLC3 cells.

First, a lentivirus-based intervention to reduce HuR expression levels (Fig. 3A) affected significantly cell death after MLN4924 treatment for 48 and 72 hours as shown by caspase 3 activity (Fig. 3B). Next, a more accentuated overexpression of HuR was achieved by infection with an adenoviral vector (AdHuR) (Fig. 4A). We tested whether increased HuR levels influenced MLN4924 sensitivity. At 72 and 96 hours after transfection, HuR overexpression resulted in a significant reduction of caspase 3 activity after MLN4924 treatment (Fig. 4B). Low levels of HuR made liver cancer cells more sensitive to the efficiency of the neddylation inhibitor MLN4924 while increasing its expression levels rendered cancer cells more resistant to the apoptotic response induced by the drug.

HuR promotes mRNA stability and/or translation of several anti-apoptotic and proliferative proteins. The positive influence of HuR was due to the stabilization of a subset of mRNAs involved in cell survival. Hence, an increase in Bcl-2, cyclin D1, prothymosin-alpha (PTMA) and X-linked inhibitor of apoptosis protein **(**XIAP) mRNA levels was detected in the hepatoma cells overexpressing HuR (Fig. 5A-B).

Moreover, no variations in CRLs substrates and proliferative pathways were detected after overexpression of

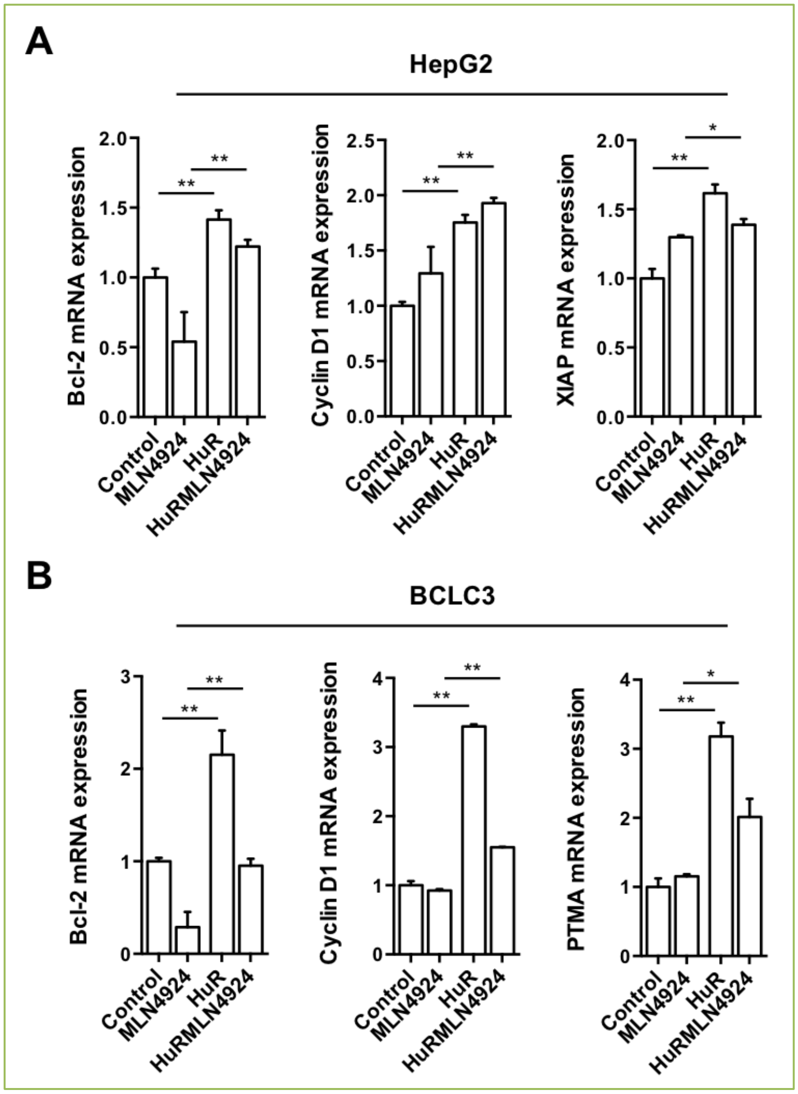

Figure 5. HuR overexpression induces antiapoptotic genes. Graphical representation of the mRNA expression (arbitrary units) of the indicated genes. (A) HepG2 and (B) BCLC3 cells treated with MLN4924 (3μM) for 96h. *p<0.05; **p<0.01 AdHur *vs* control. Data are mean ± SD.

HuR indicating that the prosurvival effect exerted by this RNA binding protein was independent of these mechanisms (Fig. 3A, 4A).

These results support a cooperative association between HuR levels and neddylation inhibition since reducing or increasing its expression results in a sensitivity modulation upon MLN4924 treatment.

Discussion

HCC is the most common liver cancer and the third leading cause of death by malignancy worldwide [1]. It is a poor prognosis cancer, since in most cases is detected at an advanced stage. Moreover, HCC is a very complex and heterogeneous pathology with multiple signaling pathways converging in the same malignant transformation [2]. It is phenotypically and genetically very heterogeneous and resistant to conventional chemotherapy [22]. For these reasons, its incidence is similar to its mortality. The development of the HCC is a multifactorial process, being the alcohol and virus the most common factors. But recently, obesity, diabetes type 2 and NAFLD have been identified as risk factors for HCC development [23].

In spite of its high incidence and mortality HCC still remains poorly understood and no effective treatment has been yet identified. There are currently three drugs being used in patients: Sorafenib [24], a threonin-thyrosin kinase inhibitor, Sunitinib [25], a threonin-thyrosin kinase inhibitor and

Everolimus [26], which acts directly through mTOR. These drugs only give patients four or five more months of life. The only effective treatments nowadays are liver transplantation and resection.

In order to improve HCC clinical treatment it is necessary to unravel new mechanisms that regulate at different levels the variety of pathways implicated in its development.

In this work we propose neddylation, a new post-traslational modification, as a global regulator of HCC progression. Cellular protein homeostasis is essential for multiple physiological systems, and imbalance in this equilibrium can lead to cell death, uncontrolled cellular proliferation and cancer development. The Nedd8 pathway is essential for cell growth through the activation of CRLs and the degradation of their substrates, which are essential in cancer development [18].

Importantly, in human liver samples a significant correlation among global levels of neddylation, NAE1 protein expression and the poorest prognosis of HCC was detected. These data underpin that Nedd8 regulate the homeostasis of proteins essential for liver cancer. Significantly Akt, master kinase for cancer development [27], LKB1 recently associated with liver tumors [12, 28] and HuR, frequently overexpressed in HCC correlating with its malignancy [17], were regulated by neddylation in human hepatoma cells.

Taking into account the central role of HuR in hepatocyte proliferation, differentiation and malignant transformation and its tightly regulated levels, this new mechanism involving the post-translational modification Nedd8 that explains the overexpression of HuR in HCC and colon cancer can offer important advantages for the treatment of liver cancer.

The potential antitumoral activity for the NAE1 inhibitor MLN4924 [29], which is in phase II clinical trials for the treatment of leukaemia, has been shown in human colon, lung and liver tumor xenograft models in immunocompromised mice [20, 21, 29]. Importantly, neddylation inhibition drastically reduced the levels of HuR in liver cancer cells and in a HCC mouse model characterized by high neddylation levels. This protein deregulation was accompanied with an apoptotic response and the regression of the tumor.

Notably, the overexpression of HuR made liver cancer cells more resistant to the death induced by MLN4924. In the same way, HuR silencing increased the susceptibility of cancer cells to the apoptotic response upon neddylation inhibition. These results suggest a role for HuR as an important sensor and regulator of the susceptibility of liver cancer cells to MLN4924 treatment.

Overall, our findings highlight the relevance of increased levels of HuR for hepatocyte proliferation, survival and malignant transformation. Importantly, we show that HuR is upregulated in HCC through neddylation, which is also highly representative in liver cancer and that its levels determine the druggability of the neddylation pathway in HCC. These data open new scenery for new therapeutic and personalized strategies for liver cancer treatment.

Conflicting interests

The authors disclose no financial or personal conflict of interest.

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Abbreviations

AMPK: AMP-activated protein kinase; BCLC: Barcelona Clinic Liver Cancer; CRL: Cullin-ring ligase; GNMT: Glycine N-methyltransferase; HAUSP: Herpesvirus-associated ubiquitin-specific protease; HCC: Hepatocellular carcinoma; HGF: Hepatocyte growth factor; HuR: Human antigen R; LKB1: Liver Kinase B1; Mdm2: Mouse double minute 2 homolog; NAE1: Nedd8 activating enzyme; NAFLD: Non-alcoholic fatty liver disease; Nedd8: Neural precursor cell expressed, developmentally down-regulated 8; PARP: Poly ADP ribose polymerase; Phb1: Prohibitin1; PI3K: Phosphoinositide 3-kinase.

Author contributions

Lucía Barbier Torres: Acquisition of data; analysis and interpretation of data. Critical revision of the manuscript. David Fernández Ramos: Acquisition of data; analysis and interpretation of data. Critical revision of the manuscript. María Luz Martínez Chantar: Study concept and design; analysis and interpretation of data; study supervision; drafting of the manuscript; obtained funding.

References

1. Forner A, Llovet JM, Bruix, J. Hepatocellular carcinoma. Lancet 2012; 379: 1245-1255.

2. Thorgeirsson SS, Grisham JW Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002; 31: 339-346.
3. Deschênes-Furry J, Perrone-Bizzozero N, Jasmin BJ. The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. BioEssays 2006; 28: 822-833.
4. Pascale A, Amadio M, Quattrone A. Defining a neuron: neuronal ELAV proteins. Cell Mol Life Sci 2008; 65: 128-140.
5. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci 2001; 58: 266-277.
6. Hinman MN, Lou H. Diverse molecular functions of Hu proteins. Cell Mol Life Sci 2008; 65: 3168-3181.
7. Abdelmohsen K, Kuwano Y, Kim HH, Gorospe M. Posttranscriptional gene regulation by RNA-binding proteins during oxidative stress: implications for cellular senescence. Biol Chem 2008; 389: 243-255.
8. Srikantan S, Gorospe M. HuR function in disease. Front Biosci (Landmark Ed) 2012; 17: 189-205.
9. Abdelmohsen K, Gorospe M. Posttranscriptional regulation of cancer traits by HuR. Wiley Interdiscip Rev RNA 2010; 1: 214-229.
10. López de Silanes I, Lal A, Gorospe M. HuR: post-transcriptional paths to malignancy. RNA Biol 2005; 2: 11-13.
11. López de Silanes I, Fan J, Yang X, Zonderman AB, Potapova O, Pizer ES, *et al*. Role of the RNA-binding protein HuR in colon carcinogenesis. Oncogene 2003; 22: 7146-7154.
12. Martínez-López N, Varela-Rey M, Fernández-Ramos D, Woodhoo A, Vázquez-Chantada M, Embade N, *et al*. Activation of LKB1-Akt pathway independent of phosphoinositide 3-kinase plays a critical role in the proliferation of hepatocellular carcinoma from nonalcoholic steatohepatitis. Hepatology 2010; 52: 1621-1631.
13. Vázquez-Chantada M, Fernández-Ramos D, Embade N, Martínez-López N, Varela-Rey M, Woodhoo A, *et al*. HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation, and carcinogenesis. Gastroenterology 2010; 138: 1943-1953.
14. Vázquez-Chantada M, Ariz U, Varela-Rey M, Embade N, Martínez-López N, Fernández-Ramos D, *et al*. Evidence for LKB1/AMP-activated protein kinase/ endothelial nitric oxide synthase cascade regulated by hepatocyte growth factor, S-adenosylmethionine, and nitric oxide in hepatocyte proliferation. Hepatology 2009; 49: 608-617.
15. Varela-Rey M, Fernández-Ramos D, Martínez-López N, Embade N, Gómez-Santos L, Beraza N, *et al*. Impaired liver regeneration in mice lacking glycine N-methyltransferase. Hepatology 2009; 50: 443-452.
16. Woodhoo A, Iruarrizaga-Lejarreta M, Beraza N, García-Rodríguez JL, Embade N, Fernández-Ramos D, *et al*. Human antigen R contributes to hepatic stellate cell activation and liver fibrosis. Hepatology 2012; Md 56: 1870-1882.
17. Embade N, Fernández-Ramos D, Varela-Rey M, Beraza N, Sini M, Gutiérrez de Juan V, *et al*. Murine double minute 2 regulates Hu antigen R stability in human liver and colon cancer through NEDDylation. Hepatology 2012; 55: 1237-1248.
18. Xirodimas DP. Novel substrates and functions for the ubiquitin-like molecule NEDD8. Biochem Soc Trans 2008; 36: 802-806.
19. Li L, Wang M, Yu G, Chen P, Li H, Wei D, *et al*. Overactivated neddylation pathway as a therapeutic target in lung cancer. J Natl Cancer Inst 2014; 106: dju083.
20. Barbier-Torres L, Delgado TC, García-Rodríguez JL, Zubiete-Franco I, Fernández-Ramos D, Buqué X, *et al*. Stabilization of LKB1 and Akt by neddylation regulates energy metabolism in liver cancer. Oncotarget 2015; 6: 2509-2523.
21. Luo Z, Yu G, Lee HW, Li L, Wang L, Yang D, *et al*. The Nedd8-activating enzyme inhibitor MLN4924 induces autophagy and apoptosis to suppress liver cancer cell growth. Cancer Res 2012; 72: 3360-3371.
22. Brown KS. Chemotherapy and Other Systemic Therapies for Hepatocellular Carcinoma and Liver Metastases. Semin Interv Radiol 2006; 23: 99-108.
23. Sanyal AJ, Yoon SK, Lencioni R. The etiology of hepatocellular carcinoma and consequences for treatment. Oncologist 2010; 15 Suppl 4: 14-22.
24. Keating GM, Santoro A. Sorafenib: a review of its use in advanced hepatocellular carcinoma. Drugs 2009; 69: 223-240.
25. Zhu AX, Duda DG, Sahani DV, Jain RK. Development of sunitinib in hepatocellular carcinoma: rationale, early clinical experience, and correlative studies. Cancer J 2009; 15: 263-268.
26. Zhu AX, Kudo M, Assenat E, Cattan S, Kang YK, Lim HY, *et al*. Effect of everolimus on survival in advanced hepatocellular carcinoma after failure of sorafenib: the EVOLVE-1 randomized clinical trial. JAMA 2014; 312: 57-67.
27. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007; 129: 1261-1274.
28. Martínez-López N, García-Rodríguez JL, Varela-Rey M, Gutiéerez V, Fernández-Ramos D, Beraza N, *et al*. Hepatoma cells from mice deficient in glycine N-methyltransferase have increased RAS signaling and activation of liver kinase B1. Gastroenterology 2012; 143: 787-798.e1-13.
29. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, *et al*. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 2009; 458: 732-736.
30. Zou T, Rao JN, Liu L, Xiao L, Yu TX, Jiang P, *et al*. Polyamines regulate the stability of JunD mRNA by modulating the competitive binding of its 3’ untranslated region to HuR and AUF1. Mol Cell Biol 2010; 30: 5021-5032.

Supplementary materials

Table 1. Optimal Incubation Conditions, Concentration, and Supplier for Each Specific Antibody Analyzed by Western Blotting

β-actin	Sigma	1/5000	TBS-Tween (0.1%)-milk (5%)
GAPDH	Abcam	1/5000	TBS-Tween (0.1%)-milk (5%)
HuR	Santa Cruz Biotechnology	1/5000	TBS-Tween (0.1%)-milk (5%)
Mdm2	Calbiochem	1/1000	TBS-Tween (0.1%)-milk (5%)
Nedd8	Abcam	1/1000	TBS-Tween (0.1%)-milk (5%)
p21	Santa Cruz Biotechnology	1/1000	TBS-Tween (0.1%)-milk (5%)
p27	Santa Cruz Biotechnology	1/1000	TBS-Tween (0.1%)-milk (5%)
PARP	Cell Signaling Technology	1/1000	TBS-Tween (0.1%)-milk (5%)

Table 2. Sequence of primers used for RT-PCR analysis

GENE NAME	OFFICIAL SYMBOL		SEQUENCE
B-cell lymphoma 2	Bcl2	Forward	5'-CTGCACCTGACGCCCTTCACC-3'
		Reverse	5'-CACATGACCCCACCGAACTCAAAGA-3'
X-linked inhibitor of apoptosis	XIAP	Forward	5'-GGGGTTCAGTTTCAAGGACA-3'
		Reverse	5'-CGCCTTAGCTGCTCTTCAGT-3'
Cyclin D1	Cyclin D1	Forward	5'-GCGCAGACCTTCGTTGCCCT-3'
		Reverse	5'-GCGCAGGCTTGACTCCAGCA-3'
Prothymosin, alpha	PTMA	Forward	5'-CAGCTTTATCGCCAGCGTCC-3'
		Reverse	5'-AGTCCTTGGTGGTGATTTCG-3
Beta-2-microglobulin	B2M	Forward	5'-TCTCTGTCTGGATGATGACGTGAG-3'
		Reverse	5'-TAGCTGTGCTCGCGCTACT-3