Role of HuR in Neurofibromas and Malignant Peripheral Nerve Sheat Tumors

Marta Palomo-Irigoyen*, Marta Iruarrizaga-Lejarreta, Marta Varela Rey and Ashwin Woodhoo.
CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Derio, Bizkaia, Spain.

Introduction

Neurofibromatosis type 1 (NF1) affects 1 in 3,500 individuals worldwide[1] and is caused by somatic inactivation of the *NF1* gene, which encodes for neurofibromin, a tumor suppressor that negatively regulates Ras-GTP levels. Thus, in NF1, high levels of Ras-GTP are found. As a consequence of the elevated levels of Ras-GTP, NF1 patients develop multiple benign neurofibromas on or around the peripheral nerves, either as encapsulated and subcutaneous masses (**dermal neurofbromas**), or as diffuse masses that expand along large segments of the nerves and go deeper inside the nerves (**plexiform neurofibromas**)[2]. Unlike dermal neurofibromas, plexiform neurofibromas can undergo malignant transformation to **Malignant Peripheral Nerve Sheath Tumors (MPNST)**, which are aggressive and highly metastatic soft tissue sarcomas that have very poor prognosis. Half of the MPNST are sporadic in nature and the other half arise in individuals with NF1[3]. Schwann cells are the crucial pathogenic cell type in NFs. In these tumors, Schwann cells are found distant from axons and are admixed with mast cells and fibroblasts in a collagen-rich extracellular matrix. Axon-Schwann cell contact, which regulates key aspects of normal Schwann cell function, is completely disrupted[4].

Gene expression profiling revealed significant differences between normal human Schwann cells, NF1-derived primary benign and malignant cells. **Human antigen R (HuR)** is a ubiquitously expressed RBP that binds to the U- and AU-rich elements in the 3´untranslated region (3´UTR) of its multiple mRNAs targets, generally promoting their stability and translation. HuR plays many important biological functions and altered expression is implicated in the development of various diseases. HuR is aberrantly expressed in several types of cancer and a strong correlation has been found between its expression levels and advancing stages of malignancy[5]. **MicroRNAs (miRNAs)** are small (~22-nt), single-stranded, non-coding RNAs that repress gene expression by promoting mRNA decay and/or suppressing translation. Translation of *HuR* mRNA is repressed by miRNAs by two main means: mRNA degradation and the inhibition of translation initiation. In several cancers, the reduced expression of miRNAs can contribute to the enhanced HuR expression observed[6,7].

In this study, we have examined the role of HuR in Neurofibroma and MPNST development and progression, and the role of miRNAs in regulating HuR expression in cancer.

Materials and methods

HuR expression analysis

(i) Immunohistochemistry (IHC): Paraffin-embedded human tissue array sections (5 µm thick) were deparaffinized and rehydrated. sections were blocked with 0.2% Triton, incubated with mouse anti-HuR primary antibody (1:100) overnight and with anti-mouse Envision system conjugated with horseradish peroxidase 30 minutes.
(ii) Transcriptional regulation: Expression of HuR was analyzed by qPCR and Western blotting.

Monitoring HuR targets *in vivo*

(i) RNA immunoprecipitation and microarray analysis (RIP-CHIP): Appropriate amounts of total RNA from four biological replicates of HuR and mock IPs, as well as two replicates of input mRNA from human frozen tissue and MPNST cell lines were submitted to the Genomics Analysis Platform at CIC bioGUNE for analysis on HUMANHT-12 v4 arrays (Illumina), respectively.

Identifying the functional role of HuR *in vivo* and *in vitro*

(i) Tumor formation capacity *in vivo*: HuR levels were silenced in S462 MPNST cells using lentiviral particles against HuR. 2x10^6 control cells and 2x10^6 HuR-silenced cells were injected into the flanks of each athymic nude mouse belonging to the control group (n=5) or to the HuR-silenced group (n=5), respectively.
(ii) Soft agar assay: Each dish was prepared with a bottom 0.6% agar layer (4 ml of 2X DMEM, 20% FBS and 2% P/S per P60 dish) and a top 0.3% agar layer containing sh control-GFP and sh *HuR*-GFP MPNST cells at a density of 1 × 104 cells per P60 dish. Colonies were stained with 0.1% crystal violet in 20% methanol for 10 minutes at RT and dishes were rinsed in dH2O.
(iii) Foci formation assay: Control and *HuR*-silenced MPNST cells were seeded in triplicates for each timepoint (Days 0, 2, 4, 6 and 8) at a density of 100 cells per M12 well and incubated overnight at 37oC, 5% CO2. Cells were fixed in formalin at those timepoints. Fixed cells were washed with 1X PBS and stained with 0.1% crystal violet in 20% methanol for 10 minutes at RT. Plates were rinsed in dH2O.

microRNAs expression and HuR regulation analysis

(i) RT-PCR: RNAs were extracted from tumours and cells and were diluted to a final concentration of 2ng/µl. 10ng of total RNA from each sample were reversed transcribed, using 40 cycles of 95ºC, 10 minutes; 95ºC, 15 seconds and 60ºC, 1 minute. Data was normalized to the expression of small nucleolar RNA (snoRNA) U6.
(ii) miR-29c and miR-342 were overexpressed in 90-8, T265p21 and STS26T cell lines (100.000 cells per M6 well) by transfecting them with miRNA mimics to a final concentration of 100 nM for 72 hours and expression of HuR was analyzed by qPCR and Western blotting.

Results

1. HUR EXPRESSION

There is a correlation between HuR levels and degree of malignancy in human samples (Figure 1).

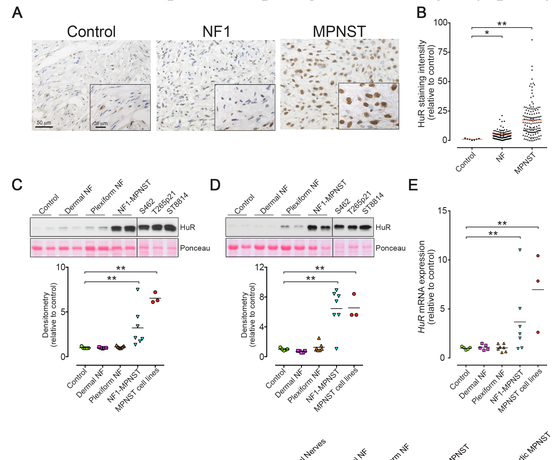

Figure 1 (A) Representative pictures of normal human nerve, dermal NF and MPNST sections showing higher HuR expression in cancer tissues (DAB; *brown*).) **(B)** HuR^{+ve} intensity was measured in normal human nerves (n=7), NFs (n=105) and MPNST (n=34) using the Frida software and box plot of measurements are shown, *, **, p<0.01 (relative to control nerves). HuR expression significantly increases with malignancy as shown by **(E)** qPCR and **(C), (D)** Western blotting of total protein extracts from control (n=5), dNF (n=5), pNF (n=7), NF1-MPNST (n=8), sporadic MPNST (n=7) human tissue samples and MPNST cell lines (n=4).

2. GENOME WIDE ANALYSIS OF HUR TARGETS

A correlation between HuR levels and degree of malignancy in human samples was found (Figure 2).

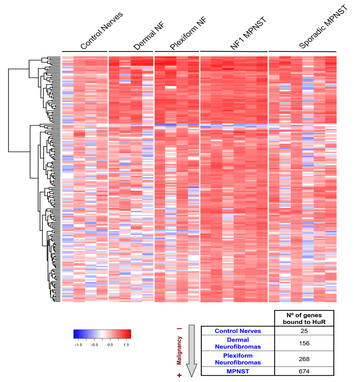

Figure 2. RIP-CHIP analysis identfied several HuR targets *in vivo* in control (n=5), dNF (n=5), pNF (n=7), NF1-MPNST (n=8) and sporadic MPNST (n=7) human tissue samples. Heat map showing relative enrichment of mRNA targets of HuR compared with control IgGs. The color scale indicates the degree of enrichment (blue-red scale).

The number of HuR targets increases as malignancy progresses. The number of mRNAs bound to HuR for each condition is indicated in the table: 25 in control nerves, 156 in dNFs, 268 in pNFs and 674 in MPNST.

3. FUNCTIONAL ROLE OF HUR *IN VIVO*

HuR silencing inhibits tumor growth in athymic nude mice and essential physiological aspects of tumorigenesis in MPNST cell lines (Figure 3).

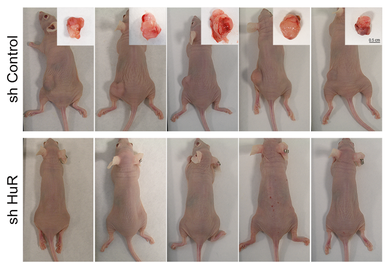

Figure 3. (A) Representative pictures showing tumor formation capacity of control and HuR-silenced S462 cells, three months after injections. HuR levels were silenced in S462 MPNST cells using lentiviral particles against HuR. The cells were injected into the flanks of athymic nude mice. Three months after injections, control S462 cells gave rise to tumors of varying sizes, while no tumors arose from HuR-silenced S462 cells. This result implicates HuR as a possible therapeutic target for these tumors.

4. FUNCTIONAL ROLE OF HUR *IN VITRO*

HuR controls essential physiological aspects of tumorigenesis in MPNST cell lines (Figure 4).

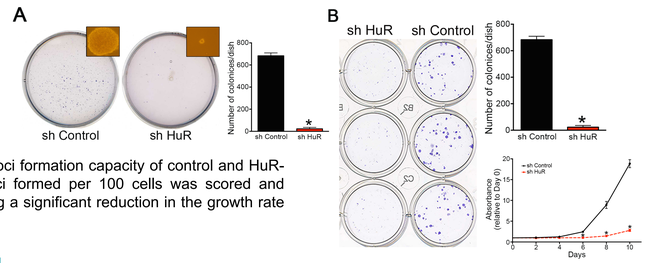

Figure 4. A) Anchorage-independent growth. Soft agar assay showing a reduction in the number of colonies formed by HuR-silenced cells compared to control cells, ***, p<0.01. The number of colonies formed per 10000 cells was scored. **B)** Representative picture showing foci formation capacity of control and HuR-silenced cells. The number of foci formed per 100 cells was scored and growth curve is shown representing a significant reduction in the growth rate of HuR-silenced cells, ***, p<0.01.

5. MicroRNAs EXPRESSION

Progressive downregulation of microRNAs levels from control to MPNST (Figure 5).

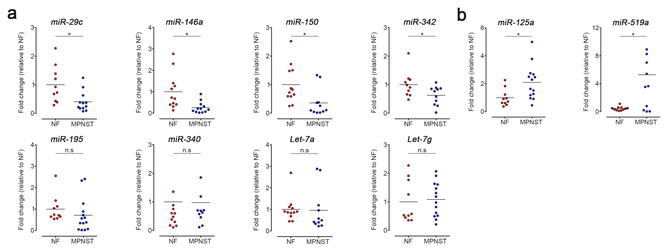

Figure 5. The levels of 9 selected microRNAs were examined in control nerves (n=5), dNFs (n=5), pNFs (n=7), NF1-MPNST (n=8), sporadic MPNST (n=7) human tissue samples and MPNST cell lines (n=4) by qPCR. miRNAs levels progressively decreased through the different stages towards malignancy.

6. HUR REGULATION BY microRNAS

miR-146a & miR-342 overexpression in MPNST cell lines significantly reduce HuR levels (Figure 6).

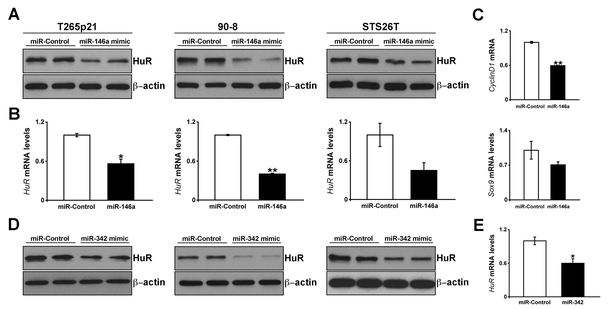

Figure 6. To examine the effect of miR-146a and miR-342 on HuR levels, MPNST cell lines were transfected with miR-control, miR-146a mimic and miR-342 mimic for 72 hours. HuR levels were analyzed. Both **A)** Western blotting of total protein extracts and **B)** qPCR, show a significant reduction in HuR levels in samples overexpressing miR-146a compared to control samples. **C)** Representative graphs showing downregulated levels of cancer-related HuR mRNA targets in the presence of miR-146a compared to control. **D)** Western blot of total protein extracts showing a reduction in HuR protein levels after miR-342 overexpression. **E)** Representative graph showing a reduction in HuR mRNA levels after miR-342 overexpression.

Conclusions

- There is a strong correlation between HuR expression and degree of malignancy in NF and MPNST human samples.
- HuR silencing *in vitro* in T265p21 cell line shows that HuR could potentially have a role in controlling gene expression in NF and MPNST, regulating essential aspects of tumourigenesis such as growth and invasion.
- HuR silencing inhibits tumor formation in athymic nude mice *in vivo.*
- There are several microRNAs that are downregulated in MPNST that could be playing a role in the aberrant expression of HuR in these samples. Among them, we have identified miR-146a and miR-342 as negative regulators of HuR.

References

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