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Original Paper

HuD, a Neuronal-Specific RNA-Binding Protein, is a Potential Regulator of *MYCN* Expression in Human Neuroblastoma Cells

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HuD is one of a family of neural antigens recognised by the sera of patients with antibody-associated paraneoplastic encephalomyelitis. Localised exclusively to neurons, these proteins are among the earliest markers of the developing nervous system. Sequence analysis suggests that HuD is an RNA-binding protein. Hu protein levels were determined for the three cell types characterising human neuroblastoma cell lines: sympathoadrenal neuroblasts (N), substrate-adherent Schwann/glial/melanoblastic precursors (S) and stem cells (I) which can give rise to both N and S cells. Western blot analysis showed similar levels of protein in three N-type cell lines; S cells have no detectable Hu protein. Northern blot analysis indicated that N cells express all three Hu genes, *HuD*, *HuC* and *Hel-N1*. N cells, mostly from *MYCN*-amplified cell lines, have consistently higher steady-state levels of *MYCN* mRNA than S cell counterparts. Nuclear run-on and mRNA half-life experiments revealed no differences in transcription rate or mRNA stability between N and S cells from the LA-N-1 cell line, implicating differences in post-transcriptional regulation. HuD is postulated to be instrumental in splicing/processing and/or stabilisation of mRNAs involved in cell growth and neuronal differentiation. As determined by gel-mobility shift assays, HuD fusion protein binds to the 3′UTR of human *MYCN* mRNA. Analysis of HuD deletion mutants has demonstrated that the first and second RNA-recognition motifs (RRMs) are required for binding. Whether HuD regulates *MYCN* expression and thereby influences tumour aggressiveness is of major interest. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

In neuroblastoma, the *MYCN* proto-oncogene is amplified and overexpressed in approximately 30% of advanced stage tumours and in most cell lines. Although probably not a causative factor in the genesis of this cancer, amplification of the gene is indicative of a rapid disease course and a poor prognosis. Understanding the factors regulating expression of *MYCN* in neuroblastoma is thus of importance clinically as well as of interest scientifically.

Cell lines established from neuroblastoma tumours generally comprise several phenotypically distinct cell types, reflecting the neural crest origin of this cancer [1]: malignant neuroblastic N-type cells, with properties of immature sympathoadrenal neurons; substrate-adherent S-type cells, with properties of precursors of neural crest non-neuronal cell lineages (e.g., immature Schwann cells and melanoblasts); and morphologically intermediate I-type cells recently identified as malignant neural crest stem cells [2]. We have demonstrated the spontaneous interconversion of N and S cells as well as the capacity of I cells to give rise to both N and S cell types [2]. Unlike N and I cells, S cells have a substantially lower or abrogated malignant potential as shown by assays of tumorigenicity and/or anchorage-independent growth capacity [3]. Studies probing the relationship between *MYCN* overexpression and neuroblastoma cell phenotype

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have revealed that *MYCN*-amplified N-type cells have 4–8-fold higher steady-state levels of cytoplasmic *MYCN* mRNA and 2–36-fold higher levels of protein than their non-malignant S cell counterparts [3]. Thus, cell lineage is an important determinant of *MYCN* expression and malignancy in human neuroblastoma cells. However, the mechanism(s) of *MYCN* gene downregulation in S cells, whether transcriptional or post-transcriptional, is not known.

Considerable evidence indicates that expression of the *MYC* gene and, by extension, of the *MYCN* gene as well, is regulated at many levels [4]. There is a growing body of data concerning regulation of gene function by protein–protein interactions, e.g., the modulation of Myc function by interactions of Myc, Max and the potential tumour suppressor Mad proteins (Mad, Mxi1, Mad3 and Mad4) [4]. However, little is known about proteins that may interact with *MYCN* RNA and alter its stability or cellular location. Based on findings that HuD, a neuronal-specific RNA-binding protein, is abundant in N- and I-type cells and not detected in S cells [5, 6] and on the brief report that HuD protein binds *in vitro* to the 3′ untranslated region (UTR) of *MYCN* mRNA [7], we are examining the possibility that HuD or another Hu protein is a regulator of *MYCN* expression.

HuD is a member of a family of neuronal-specific RNA-binding proteins encoded by three (or more) distinct genes. The three gene products, HuD, HuC and Hel-N1, also termed Hu antigens, have partially homology with ELAV, a protein which contains three RNA-binding domains and is required for the normal development and maintenance of the *Drosophila* nervous system [8]. In the human, Hu antigens are implicated in a neurological syndrome, paraneoplastic encephalomyelitis and sensory neuropathy, occurring primarily in patients with small cell lung cancer (SCLC). It was found that an antibody to Hu antigens generated by these patients reacted with neurons and SCLC cells and that Hu expression correlated with the neuroendocrine phenotype of the tumour [9]. A similar syndrome has been identified in several children with neuroblastoma [10].

The present series of experiments was undertaken to determine (i) which Hu antigens are expressed in human neuroblastoma cell variants, (ii) whether any or all of the Hu proteins bind to the 3′ UTR of *MYCN* and with which RNA-binding domain(s) and (iii) how the expression of HuD, HuC and/or Hel-N1 correlates with *MYCN* mRNA and protein expression. Current findings substantiate our hypothesis that genetic determinants of cell lineage, in particular those conferring a neuroblastic phenotype, are important factors influencing *MYCN* overexpression and thereby malignancy, in human neuroblastoma.

MATERIALS AND METHODS

Cell culture

Cell lines were cultured in a 1:1 mixture of Eagle's Minimum Essential Medium and Ham's Nutrient Mixture F12 supplemented with 15% heat-inactivated fetal bovine serum as previously described [1]. No antibiotics were used.

Northern blot analysis

Cytoplasmic RNAs isolated as previously described [11] or total cellular RNAs isolated by the RNeasy method (Qiagen) were separated by electrophoresis through 1% agarose gels and analysed by standard techniques. Probes—either PCR products of *HuD*, *HuC* and *Hel-N1* or a γ-actin cDNA—were radiolabelled by the random primer technique [12]. Blots were hybridised at high stringency and mRNA levels quantified by densitometry.

Bacterial expression of Hu proteins

The pET-21b(+)-HuD construct, containing the full-length open reading frame of *HuD*, and the pET-21b(+)-HuD deletion mutants Δ1b, Δ2, Δ3 and Δ4 have been described previously [13]. HuC and Hel-N1 were generated by PCR from clones containing their open reading frames. Expression of the protein was induced with IPTG.

Gel mobility shift analysis

The 3′UTR of *MYCN* was generated by PCR and cloned into pCRII plasmid (Invitrogen). ^{32}P-labelled *MYCN* 3′UTR probe, generated by *in vitro* transcription, was incubated with bacterial cell lysate at room temperature for 10 min. RNA–protein complexes were resolved by electrophoresis through a 3.2% non-denaturing polyacrylamide gel.

Nuclear run-on experiments

Cells in exponential growth phase were lysed and nuclear transcription was assayed by standard techniques [14]. The extent of hybridisation of the radiolabelled RNA to the plasmid containing the 3′ UTR of human *MYCN* was quantitated by densitometry and normalised to that for γ-actin.

RESULTS

Hu antigen expression in N and S cells

Consistent with their neural crest origin, human neuroblastoma tumours and cell lines are Hu-immunoreactive [6, 10] What is not known, however, is which of the neuroblastoma cell variants are Hu-immunoreactive and which of the Hu proteins is expressed (HuD, HuC and/or Hel-N1). Therefore, we examined Hu antigen expression in human neuroblastoma N and S cell variants from three different human neuroblastoma cell lines by immunoblotting with polyclonal antiserum from SCLC patients (Table 1). N cells are highly Hu-immunoreactive, whereas no or very little Hu antigen is detected in S cells. There is no obvious correlation with *MYCN* amplification level; abundant protein is present in N cells from SK-N-SH which has only a single copy of *MYCN* and from cell lines with high level *MYCN* gene amplification (SMS-KCN and LA-N-1).

The polyclonal human antiserum used for these blots reacts with all three known Hu family members. Our initial RT–PCR studies with mRNA from N and S pairs suggested that only HuD was present in N-type cells [6]. However,

Table 1. *Differential expression of Hu protein and HuD, HuC and Hel-N1 mRNA in human neuroblastoma N and S pairs*

Cell line	Phenotype	Hu protein*	mRNA levels† for HuD	HuC	Hel-N1
SK-N-SH	N	37.6	33.7	3.4	34.0
	S	0	0.9	1.1	10.0
SMS-KCN	N	30.2	23.3	1.2	16.5
	S	1.7	1.7	1.1	1.4
LA-N-1	N	35.5	22.8	8.5	50.0
	S	0	2.9	1.8	3.5

*Relative amount of protein, normalised to actin. †Relative amount of mRNA, normalised to γ-actin loading control.

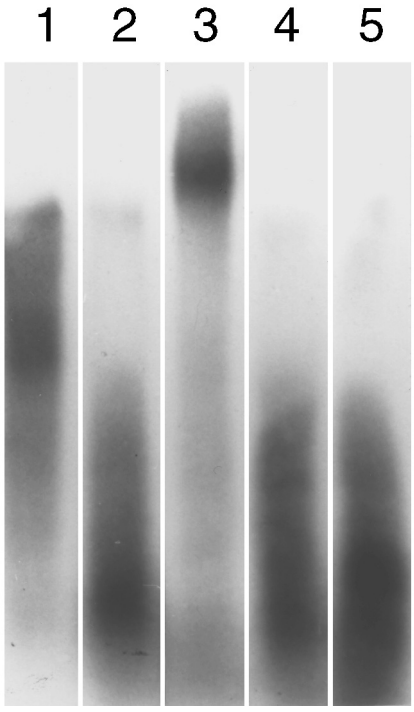

Figure 1. Gel mobility shift assay of radiolabelled *MYCN* 3′UTR probe with bacterial lysates containing equivalent amounts of Hu fusion proteins. Lanes contain: 1, probe alone or probe plus: 2, vector; 3, HuD; 4, HuC; or 5, Hel-N1.

recent Northern blot analyses with probes specific for each Hu family member show clearly that all three Hu genes are expressed in N-type cells. The existence of multiple splice forms for the Hu protein mRNAs and differences in probe length and specificity make comparisons of the relative amount of mRNA encoding each Hu antigen in any one cell line tenuous at best. However, *Hel-N1* and *HuD* appear to be expressed at moderate to high levels, whereas *HuC* is expressed at a much lower level (Table 1). The non-neuronal S-type cells contain little or no *HuD* or *HuC* mRNA and show only low-level expression of *Hel-N1*.

HuD binding to MYC*N mRNA*

RNA-binding proteins are thought to regulate mRNA stability and/or transport by binding to AU-rich elements in the 3′UTR of mRNAs [15, 16]. Studies in our laboratory were undertaken to investigate the ability of each of the Hu family members to bind to the 3′UTR of *MYCN* mRNA. Gel mobility shift assays were performed with a radiolabelled 398 nucleotide portion of the human *MYCN* 3′UTR incubated with human HuD, HuC or Hel-N1 fusion proteins. As shown in Figure 1, only HuD was able to retard migration of the *MYCN* probe. By contrast, all three Hu proteins bind and retard the migration of a *MYC* 3′UTR probe (data not shown).

Table 2. Effect of RRM deletion on HuD binding to the MYC*N 3′UTR*

Deletion mutant	RRMs present	Gel shift with *MYCN* 3′UTR
Δ1b	I, II, S	+
Δ2	I, II	+
Δ3	I	–
Δ4	II, S, III	–

Table 3. MYCN gene copy number, transcription rate, cytoplasmic mRNA level and protein content in N and S cell variants

Cell line	Phenotype	Gene copy number	Transcription rate*	Cytoplasmic mRNA level†	Protein content
LA1-55n	N	160	1.85	107	73
LA1-5s	S	118	2.02	28	25
N/S ratio		1.4	0.9	3.8	2.9

*Relative rate of transcription compared to γ-actin control. †Relative levels, normalised to γ-actin control.

HuD domains required for binding

Based upon nucleotide sequence homology with the *Drosophila* ELAV and sex-lethal genes, Hu proteins possess three putative RNA-recognition motifs (RRMs). To determine which of the HuD RNA-binding domains is required for binding to *MYCN*, fusion proteins from four HuD deletion mutants lacking one or two RRMs and/or the intervening stringer (S) region [13] were used in gel mobility shift assays. The deletion mutants and the ability of the resulting proteins to bind *MYCN* are summarised in Table 2. Results indicate that both the first and second RRMs of HuD are necessary for binding.

MYC*N in N and S cells*

Our previous studies with human neuroblastoma N and S cell variants have demonstrated differential regulation of *MYCN* expression occurring at the transcriptional and/or post-transcriptional level. As an initial step in identifying the transcriptional or post-transcriptional mechanism(s) involved, we measured parameters affecting *MYCN* mRNA levels in N (LA1-55n) and S (LA1-5s) sublines cloned from LA-N-1. These two variants have only a 1.4-fold difference in gene copy number (Table 3). By contrast, levels of cytoplasmic *MYCN* mRNA are 3.8-fold greater in N versus S cells, consistent with the 2.9-fold difference in MYCN protein content. To determine whether the disparity in mRNA levels results from differences at the transcriptional level, nuclear run-on experiments were performed in which the rate of *MYCN* transcription was compared to that of γ-actin. Representative data from one of three experiments (Table 3) show that there is little or no difference in the relative rate of *MYCN* transcription between LA1-55n and LA1-5s cells. Therefore, the quantitative difference in *MYCN* mRNA between these N and S cells likely arises at the post-transcriptional level.

DISCUSSION

In the present study, we explored the possibility that *MYCN* expression in human neuroblastoma cells may be modulated at the post-transcriptional level by interaction with HuD, a neuronal-specific RNA-binding protein.

Marusich and Weston [17] have shown that cells of the avian neural crest stain positively for Hu antigen(s) prior to overt morphological differentiation along a neuronal cell lineage. By contrast, Hu-positive cells were never seen among the numerous non-neurogenic crest cells. Likewise, in cultured neuroblastoma cell lines, N-type (sympathoadrenal progenitor) but not S-type (non-neuronal precursor) cells are

Hu-immunoreactive. Thus, these proteins may, in some way, initiate and/or maintain neuronal differentiation during development.

RNA-binding proteins are thought to regulate mRNA splicing and stability by binding to AU-rich elements in the 3′UTR of mRNAs [15, 16]. Several previous studies have shown that Hu family members bind to AU-rich elements in the 3′UTR of mRNAs involved in cell growth and differentiation, e.g., *MYC*, *FOS*, Id and GM-CSF [15]. With regard to the *MYC* family of putative transcription factors, studies from the Furneaux [16] and Keene [15] laboratories have demonstrated that both HuD and Hel-N1 bind to the 3′UTR of *MYC* mRNA. However, Hel-N1 does not bind to *MYCN*. In a preliminary report, Chagnovich and Cohn [7] showed that a 40 kDa Hu-immunoreactive protein from N cells of the human neuroblastoma NBL-W cell line, as well as purified HuD protein, does bind to the 3′UTR of *MYCN*. Our findings—that all three Hu proteins bind *MYC* but that only HuD binds *MYCN*—are consistent with and extend these observations.

Similar to the *Drosophila* ELAV protein, Hu proteins are thought to possess three putative RNA-binding domains. Levine and associates [15] demonstrated that only the third domain of Hel-N1 was required for its binding to the *MYC* 3′UTR. Our results of studies with the HuD deletion mutants indicate that the first and second domains are involved in binding to the *MYCN* mRNA 3′UTR.

The cellular expression of *MYC* and other family members is highly regulated at the transcriptional, post-transcriptional, translational, and post-translational levels. In cells with chromosomally integrated amplified *MYCN* genes and therefore relatively stable gene copy numbers, S cells have markedly lower cytoplasmic mRNA and protein steady-state levels compared to their N cell counterparts [3]. What might be the source of this disparity? One possibility is altered transcription of the gene, a possibility excluded for the LA-N-1 subline pair, at least, by the finding of similar transcription rates in nuclear run-on experiments. A second possibility is that *MYCN* mRNA is more rapidly degraded in the cytoplasm of S cells. Chagnovich and Cohn [7] have reported markedly different *MYCN* mRNA half-lives for the N and S pair from NBL-W. However, preliminary experiments in our laboratory have shown that cellular *MYCN* mRNA half-lives in LA1-55n and LA1-5s cells are approximately the same (data not shown).

A third possibility is that *MYCN* mRNA is differentially processed and/or sequestered in the nucleus of S and N cells. Members of the Hu family of RNA-binding proteins may serve to process and/or stabilise/destabilise a variety of neuronal-specific mRNAs or to mediate their transport from the nucleus. The ability of HuD, present in human neuroblastoma N-type cells, to bind *MYCN* mRNA *in vitro* is suggestive of this type of interaction. If HuD is instrumental in the processing/stabilisation of *MYCN* mRNA, its absence in S-type cells could thus serve to dramatically lower mRNA levels in the cytoplasm. Further knowledge of this potentially important mechanism of *MYCN* regulation in human neuroblastoma cells may reveal another target for therapeutic attack.

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