# TNF-α Up-regulated the Expression of HuR, a Prognostic Marker for Ovarian Cancer and Hu Syndrome, in BJAB Cells

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## ABSTRACT

**Background:** Hu syndrome, a neurological disorder, is characterized by the remote effect of small cell lung cancer on the neural degeneration. The suspicious effectors for this disease are anti-Hu autoantibodies or Hu-related CD8+ T lymphocytes. Interestingly, the same effectors have been suggested to act against tumor growth and this phenomenon may represent natural tumor immunity. For these diagnostic and therapeutic reasons, the demand for antibodies against Hu protein is rapidly growing. **Methods:** Polyclonal and monoclonal antibodies were generated using recombinant HuR protein. Western blot analyses were performed to check the specificity of generated antibodies using various recombinant proteins and cell lysates. Extracellular stimuli for HuR expression had been searched and HuR-associated proteins were isolated from polysome lysates and then separated in a 2-dimensional gel. **Results:** Polyclonal and monoclonal antibodies against HuR protein were generated and these antibodies showed HuR specificity. Antibodies were also useful to detect and immunoprecipitate endogenous HuR protein in Jurkat and BJAB. This report also revealed that TNF-α treatment in BJAB up-regulated HuR expression. Lastly, protein profile in HuR-associated mRNA-protein complexes was mapped by 2-dimensional gel electrophoresis. **Conclusion:** This study reported that new antibodies against HuR protein were successfully generated. Currently, project to develop a diagnostic kit is in process. Also, this report showed that TNF-α up-regulated HuR expression in BJAB and protein profile associated with HuR protein was mapped. **(Immune Network 2004;4(3):184-189)**

**Key Words:** Hu syndrome, HuR, antibody, TNF-α, mRNP

## Introduction

The neuronal disease, Hu syndrome, is a para-neoplastic disorder associated with small cell lung cancer, meaning that the mortality of Hu syndrome patients is related with neurological complications rather than the tumor progression (1-4). Anti-Hu auto-antibodies, detected in sera from most of Hu syndrome patients, have been used as specific markers for this disease (5,6). Recently, a Hu antigen was also reported as a prognostic factor for ovarian cancer (7). The mechanism of Hu syndrome is not well understood but pathological roles of anti-Hu autoanti-bodies or Hu-related CD8+ T lymphocytes have been suspected (8,9). Immune responses against Hu proteins expressed in the tumor cells lead to anti-Hu autoantibodies or Hu-related CD8+ T lymphocytes which cross blood-brain barrier and are involved in neuronal disorders. More interestingly, these immune responses act against the tumor growth and Hu antibodies may serve as therapeutic targets (10,11).

Hu proteins are evolutionarily conserved. In mammals, there are four members in Hu family, HuR (HuA), Hel-N1 (HuB), HuC, and HuD (12-14). In Drosophila, Hu proteins belong to embryonic lethal abnormal vision (ELAV) family. Unlike other neuron-specific members of Hu family, HuR is ubiquitously expressed and involved in non-neuronal disorders including ovarian cancer (7). Each of Hu antigens is a RNA-binding protein containing three RNA recognition motifs. An increasing number of evidence has been accumulated to support that Hu proteins are

involved in the regulation of mRNA stability. Short-lived mRNAs such as those of cytokines, proto-oncogenes and growth factors have specific *cis*-elements in their 3' untranslated regions (15-19). So far, AU-rich elements (AREs) are best studied for the control of mRNA turnover. Many extracellular signals work through AREs to regulate the rate of ARE-containing mRNA decay (20). Recent analysis by bioinformatics (21) revealed that up to 8% of human mRNAs contain AREs, implying that ARE-mediated mRNA turnover may be widely used for the control of many unstable mRNAs. HuR protein is of special interest due to its activity to stabilize ARE-containing mRNAs, while other ARE-binding proteins (ARE-BPs) including AUF1, BRF1, KSRP, and TTP promote ARE-mediated mRNA decay (22-28). TNF-α mRNA containing AREs is also a target for HuR protein and the association between them has been studied for years.

Recent technical advance has been made to identify mRNA subsets associated with a protein such as ARE-BP out of endogenous mRNA-protein (mRNP) complexes. This so-called ribonomics eventually allows transcriptomic and proteomic analyses for a subset of mRNP complexes immunoprecipitated with specific antibodies (29,30). This strategy was successfully applied for mRNA target analyses of HuB protein, HuR, and fragile X mental retardation protein (31-33). Successful mRNP analyses were partly limited by rarity of proper antibodies.

In this study, I developed polyclonal and monoclonal antibodies against HuR protein. Generated antibodies showed high specificity to HuR protein and immunoprecipitated endogenous HuR protein from Jurkat and BJAB lysates. Using these antibodies and commercial anti-HuR antibodies, the regulation of HuR expression were examined and protein profile in HuR-mRNA complexes was established.

## Materials and Methods

*Materials.* Unless otherwise specified, all chemicals and plastic wares were purchased from Sigma (St. Louis, MO) and Falcon (Franklin Lakes, NJ), respectively. BJAB and Jurkat cells were cultured in Dulbecco's modified essential medium supplemented with 10% FBS and maintained in 5% $CO_2$.

*Purification of recombinant HuR protein and generations of polyclonal and monoclonal antibodies.* Recombinant HuR protein was purified as described (24). For generation of polyclonal antisera, mice were injected with recombinant HuR protein emulsified in complete Freund's adjuvant and trice boosted with the same antigen mixed with complete Freund's adjuvant. Antisera were collected from eye-bleeding 3 d after last injection. Antisera were used with 1：1000

dilution for Western blot and 1：100 for immunoprecipitation. For generation of monoclonal antibodies, mice injected as for polyclonal antisera production were sacrificed and isolated splenocytes were fused to myeloma cells. Hybridomas from several fusions were initially screened by ELISA, four clones were further characterized by Western blot analyses using purified proteins and cell lysates.

*Preparation of lysates.* Total cell lysates were prepared as described elsewhere (34). Briefly, total cells were lysed in Lysis buffer (50 mM HEPES pH 7.6, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 0.4 mM PMSF, 1μg/ml Antipain, 1μg/ml Leupeptin). Polysome lysates containing endogenous mRNA-protein complexes were prepared and used for immunoprecipitation as previously described (35). Briefly, 100 million cells were used to isolate polysome lysates complexes. Cultured cells were harvested by centrifugation and washed twice with ice-cold phosphate-buffered saline. The cells were then extracted by an equal pellet volume of polysome lysis buffer. Usually, 20～40 mg/ml of polysome lysates was prepared.

*Western blot.* The purified proteins or lysates were quantified by Bradford assay and Coomassie blue staining following SDS-PAGE using known concentrations of bovine serum albumin as standards. The lysates were separated in a 10% SDS-PAGE gel, and then detected after Western blotting by an ECL kit (Amersham, Piscataway, NJ) with antibodies from this study or monoclonal anti-HuR antibody (3A2, Santa Cruz Biotechnology, Santa Cruz, CA).

*Immunoprecipitation.* Immunoprecipitation using polyclonal and monoclonal antibodies were performed as previously described (25). About 1 mg of polysome lysates was used for each mRNP immunoprecipitation as describe elsewhere (35). Briefly, sepharose beads conjugated with Protein-A and -G were washed three times with NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% Nonidet P-40). The washed beads were incubated at room temperature for 3 h with cell or polysome lysates in the absence or presence of monoclonal anti-HuR antibody. Materials retained with the beads were intensively washed with NT2 buffer to remove nonspecific complexes except HuR-bound one.
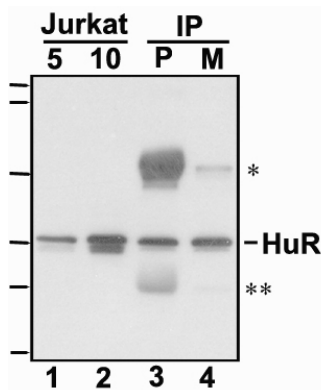
*2-dimensional gel electrophoresis.* To analyze proteins retrieved in HuR-associated mRNP complexes, about 40μg of immunoprecipitated mRNP by HuR antibody was separated by 2-dimensional gel electrophoresis and visualized by silver staining. Purified proteins were first migrated to their isoelectric pH position along the tube gel covering pH 3 to 10. Tube gel was then loaded on top of SDS-acrylamide slab gel and proteins were separated according to their sizes.

## Results

*Generation of polyclonal antibodies against HuR protein.*
Recombinant HuR proteins were expressed as a GST
fusion protein and then purified using glutathione
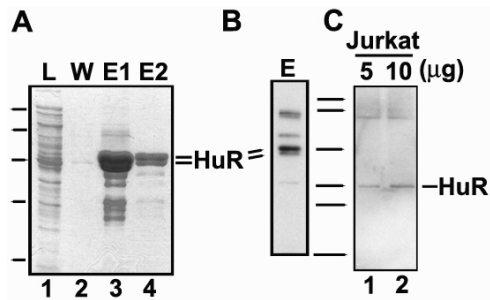


**Figure 1.** Western blot analysis to verify the generation of poly-
clonal antibodies against HuR protein. (A) Purity of purified
GST-HuR was checked by reducing SDS-PAGE analysis and
Coomassie blue staining with following samples: bacterial lysates
(L), washed fraction (W), first eluted sample (E1), and second
eluted sample (E2). (B) A Western blot using polyclonal sera
from mice injected with GST-HuR protein was performed with
eluted sample as a GST-fusion protein (a mixture of E1 and E2
from panel A). (C) In a similar way, another western blot was
performed with 5 and 10μg of Jurkat lysates. The position of
HuR protein were marked to show double major bands in
bacterial lysates and a HuR band in cell lysates. Next to gels,
the locations of molecular weight standards were marked: 97, 66,
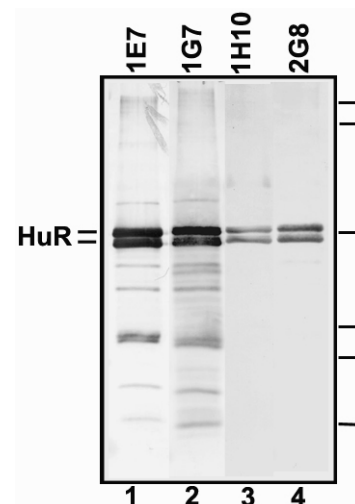45, 31, 21 kDa (A) and 114, 88, 50, 35, 28, 20 kDa (B and C).

beads. It was not clear why GST-HuR existed as
doublet bands even after purification (Fig. 1A, lanes
3-4). But one possible explanation is that the car-
boxy-terminus of this fusion protein is not stable,
yielding multiple forms or sizes. It was more evident
in large quantity of eluted sample (lane 3). Using this
purified GST-HuR, mice were primary injected and
trice boosted. The antisera from injected mice were
collected by eye-bleeding. The polyclonal antisera
clearly detected recombinant HuR protein as shown
in Fig. 1B. More interestingly, the antisera also
detected endogenous HuR protein in Jurkat lysates
which was confirmed by monoclonal anti-HuR
antibody, 3A2 (data not shown).

*Immunoprecipitation of endogenous HuR protein by polyclonal
antisera.* Once the presence of anti-HuR antibodies in
the antisera was confirmed by Western blot analysis,
it was tested to see whether the antisera can immu-
noprecipitate endogenous HuR protein in cell lysates.
Polyclonal antisera immunoprecipitated endogenous
HuR protein from Jurkat lysates as well as mono-
clonal anti-HuR antibody, 3A2, did (Fig. 2). But more
immunoglobulins were needed for antisera as indi-
cated by the intensities of heavy and light chains.
This result implied that the antibodies specific against
HuR proteins existed in antisera but not much as the
purified monoclonal antibodies.

*Generation of hybridoma cell lines producing monoclonal
antibodies against HuR protein.* The results from poly-



**Figure 2.** Western blot analysis to show that polyclonal antisera
immunoprecipitated endogenous HuR protein in Jurkat. Effi-
ciency of immunoprecipitation by polyclonal antisera (P) was
compared to that by monoclonal anti-HuR antibody, 3A2 (M).
Lanes 1 and 2 included 5 and 10μg of Jurkat lysates, respectively.
About 1μg of monoclonal anti-HuR antibodies and 100μl of
polyclonal antisera were used to immunoprecipitate 50μg of
Jurkat lysates and then the precipitated proteins were separated
in a SDS-polyacrylamide gel, followed by Western blot using mo-
noclonal anti-HuR antibody which can detect two bands of
endogenous HuR protein (lanes 3 and 4). Heavy (*) and light
(**) chains of immunoglobulins were marked.



**Figure 3.** Confirmation of anti-HuR antibodies in hybridoma cell
lines. About 100 ng of GST-HuR proteins were used for Western
blot analyses using culture supernatants from four different hybri-
doma cell lines. Antibodies from 1E7 and 1G7 hybridomas de-
tected more strongly the HuR protein when the similar amounts
of the culture supernatants were used for four hybridoma lines.
With higher dilution, supernatants from 1E7 and 1G7 detected
only the HuR proteins (data not shown).
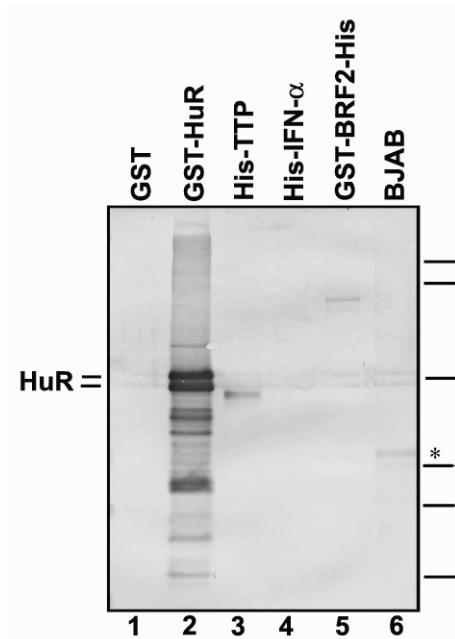
**Figure 4.** Endogenous HuR protein in BJAB cells was detected by Western blot analysis using culture supernatants from a hybridoma cell line, 1G7. About 100 ng of diverse recombinant proteins (lanes 1-5) and 10μg of cell lysates from BJAB (lane 6) were used for Western blot analysis. Antibodies in 1G7 supernatant clearly detected recombinant HuR protein and also detected endogenous HuR protein around the similar size of Jurkat lysates (Fig. 1C). The difference in intensities between recombinant and endogenous HuR proteins may be due to the discrepancy in protein amount. The similar result was also obtained using monoclonal anti-HuR antibodies, 3A2 (data not shown).

clonal antisera well argued for the strong antigenicity of purified recombinant HuR protein and next project to make monoclonal antibodies was set out. After fusion, hybridomas were first screened by ELISA and four hybridoma cells including top 1.5% scores were selected for next screening. At the second ELISA assay, subclones from initial two hybridoma cell lines showed very high scores, about 1.4 and 2.2 above than negative controls. These four cells were expanded and their culture supernatants were used for Western blot analyses as shown in Fig. 3. Complying with the results from ELISA, 1E7 and 1G7 reacted to HuR protein stronger than 1H10 and 2G8.

*Specificity of antibodies from hybridoma 1G7.* In order to verify the specificity of antibodies in 1G7 culture, another Western blot analysis was performed using a series of proteins (Fig. 4). Based on several results, the antibodies from 1G7 were believed to be HuR specific. Any of GST or GST-BRF2-His was not detected as well as GST-HuR, implying that most of these antibodies were not against the fusion tag, GST (lanes 1, 2, and 5). These antibodies were also not against any of nonspecific RNA-binding proteins (lanes 2, 3, and 5) or any non-related proteins (lane 4). More significantly, these antibodies recognized endogenous HuR protein in BJAB (lane 6). These hybridoma cell lines are repeatedly being selected and expanded to stabilize the cell growth for large scale antibody production.

*Functional studies of HuR protein using anti-HuR antibodies.* At present, the monoclonal antibodies developed in this study were in the middle of optimization process
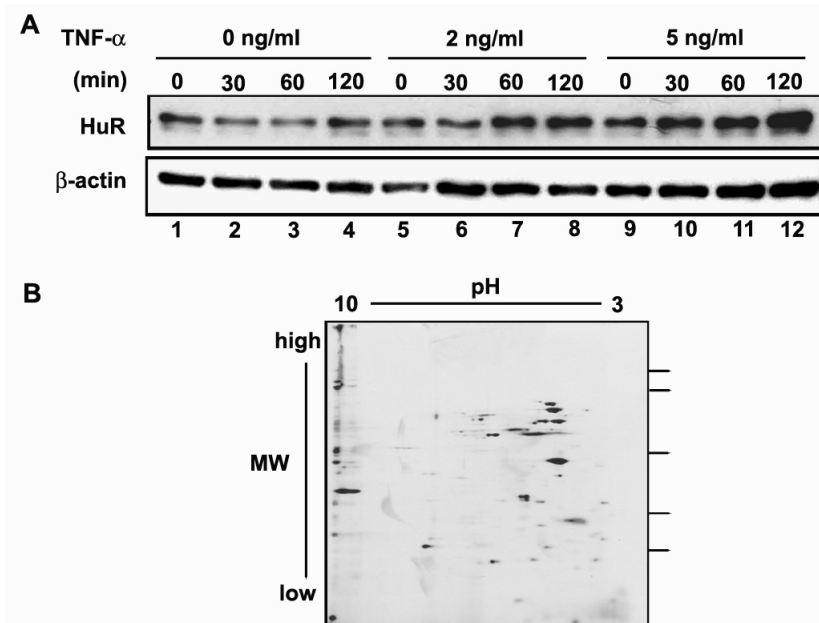


**Figure 5.** Up-regulation of HuR expression by TNF-α treatment in BJAB and 2-dimensional analysis of HuR-associated mRNP. (A) BJAB cells were treated by TNF-α in three different concentration and total cell lysates were prepared after 0, 30, 60, and 120 min later. Cell lysates were then western blotted using anti-HuR antibody or anti-β-actin monoclonal antibodies. (B) Polysome lysates were prepared from BJAB cells and then HuR-associated mRNP complexes were immunoprecipitated by anti-HuR antibodies. About 40μg of HuR-bound mRNP were separated by 2-dimensional electrophoresis and then visualized by silver staining.

for scale-up production and therefore I used commercial antibodies to investigate HuR-related signal pathways. Two aspects of experiments were performed using monoclonal anti-HuR antibody (3A2), signal transduction pathways for HuR expression and protein profiling in HuR-containing mRNP complexes (Fig. 5). About 2 ng/ml of TNF-α treatment in BJAB cells significantly up-regulated HuR expression between 60 min and 120 min. But the up-regulation of HuR was not clear with 5 ng/ml of TNF-α. It might represent side effects from higher amount of TNF-α or transient regulation of HuR expression only in proper strength of stimuli. Further experiments will be necessary to optimize the condition for HuR expression. For another assay, the HuR-mRNA complexes were immunoprecipitated by HuR antibodies from polysome lysates and proteins in these mRNP complexes were separated in 2-dinensonal electrophoresis. After silver staining, many spots were clearly shown. This will be used as basis for searching cellular proteins which interacts with HuR protein. The polyclonal and monoclonal antibodies from this study will further help to analyze the functions of HuR protein in detail.

## Discussion

Until recent reports describing HuR protein as a prognostic marker for ovarian cancer, most of researches about HuR have been focused to define the roles of HuR in ARE-mediated mRNA stabilization (17). Alternative traditional topics about HuR have also been limited to its functions in neural disorders mainly due to early discoveries of Hu autoantigens from patients with neural diseases (1,4). Many productive results have been accumulated from these long-studied topics but much attention has been drawn to recent argument of HuR protein as a prognostic marker for ovarian cancer (7). Another interesting suggestion is therapeutic treatment of anti-HuR antibodies in cancer patients (5,11). These new agenda requires reliable sources of anti-HuR antibody for successful experiments. So, this study was focused to generate specific antibodies against HuR protein.

Indeed, useful antibodies were generated against HuR protein based on following data. First, newly-made antibodies well reacted to recombinant antigen, GST-HuR and also to endogenous HuR in cells such as Jurkat and BJAB (Fig. 1, 2, and 4). The detection of endogenous HuR protein by new antibodies fulfills the minimal requirements for their usage as prognostic or therapeutic purpose. Second, new antibodies showed high specificity. These antibodies reacted to HuR protein but not to other GST-fusion proteins or proteins containing RNA-binding domains (Fig. 4). This argued that new antibodies did not react to the

fusion tag or any nonspecific peptides brought by RNA-binding activities. The availability of new anti-HuR antibodies will help expand the research areas such as antibody-based treatment or diagnostic kit development.

In order to develop basic research areas using anti-HuR antibodies, two experiments were established using anti-HuR antibodies, 3A2. The roles of HuR protein in ARE-mediated mRNA stabilization have been well studied but the signal transduction pathways involved in this process were not fully understood (17,20). It is interesting how HuR expression level is affected by TNF-α treatment because TNF-α is one of early response genes in inflammation and HuR has been suggested to stabilize TNF-α mRNA (36-38). Therefore, these results suggested that TNF-α and HuR may exist in a similar signaling pathway. Indeed, treatment of TNF-α increases the expression level of HuR protein in BJAB. In an alternative study, I tried to set a basis for analyzing HuR-associated proteins in mRNP complexes. To do so, recently developed technique, ribonomics, was applied (35,39). HuR-related mRNP complexes were immunoprecipitated from polysome lysates reflecting the profile of protein expression at the time of extraction. Protein profile was visualized by silver staining followed by 2-dimensional electrophoresis using purified mRNP complexes but further studies will be needed to identify the spots in the gel.

In conclusion, polyclonal and monoclonal antibodies were generated and the specificities of these antibodies were confirmed by Western blot analyses using a series of proteins and cell lysates. This report also established tools to study HuR functions in aspects of signal transduction and associations such as protein-mRNA or protein-protein complexes. Through these studies, it was found that TNF-α up-regulated HuR expression in BJAB within 2 h. HuR-associated mRNP complexes were also immunoprecipitated and the basis for 2-dimensional analysis of proteins in these complexes was established. Transcriptomic and proteomic analyses for these HuR-bound mRNP complexes are in process.

## Acknowledgments

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