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Immunogenicity of RaLA and its tissue-specific expression in hepatocellular carcinoma

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Abstract

In order to understand the immunogenicity of a tumor-associated antigen (TAA), Ras family small GTP binding protein (RaLA) in hepatocellular carcinoma (HCC), autoantibody responses to RaLA were evaluated by enzyme-linked immunosorbent assay (ELISA), Western blotting and indirect immunofluorescence assay in sera from patients with HCC and sera from normal individuals. Immunohistochemistry (IHC) study with tissue array slides was also preformed to analyze protein expression profiles of RaLA in HCC and control tissues. This study demonstrated that RaLA had a relative higher frequency of autoantibody response in HCC (20.1%) compared to liver cirrhosis (3.3%), chronic hepatitis (0%), and normal individuals sera (0%). RaLA also showed a stepwise increased expression from normal liver tissues (26.7%), liver cirrhosis tissues (45.0%) to HCC tissues (63.3%). Sensitivity and specificity of anti-RaLA antibody in detection of HCC was 20.1% and 99.3%, respectively. The data suggested that RaLA might contribute to liver malignant transformation, and could be used as a potential tumor marker in HCC detection.

Keywords

RaLA; Tumor-associated antigen; Hepatocellular carcinoma; Immunoreactivity; Tissue expression

1. Introduction

Although there is rapid increase in the number of serological analysis of recombinant cDNA expression libraries (SEREX) defined antigens, a great majority of these antigens, however, have been found to have no direct relevance to cancer. For example, some of them could be the antigens associated with preexisting non-cancer diseases, some might be related to necrotic products of cancer cells, and some antigens are even the self proteins normally expressed in human tissues. Existing evidences have demonstrated that a significant portion of the currently identified tumor antigens by SEREX are autoantigens of this kind (1). To address the issue regarding the immunogenicity of the identified antigens, our aim is to focus on the strategies

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to narrow down the relevant tumor-related antigens and evaluate them to be used as serological diagnostic markers or therapeutic targets of immuno-therapy. Particularly, serological screening from normal individuals, cancer patients and patients with nonneoplastic diseases is of critical importance. Using recombinant protein as target antigen, screening human sera in a large scale by immunoassay such as ELISA or Western blot, can provide such indications of the serological reaction pattern of sera from patients with or without cancer (2).

Ral, a member of the Ras super-family of small GTPases, is encoded by two genes, RalA or RalB. RalA had tissue-specific pattern of expression in developing organs. Tissue recombination experiments showed that Ral expression was increased by interaction in the lung, indicating that RalA expression is functionally regulated (3). RalA cDNA was identified in a previous study with HepG2 cDNA expression library screening (4). In the subsequent study, cDNA encoding RalA was subcloned into an expression vector, and the recombinant protein was further expressed and purified. Using recombinant RalA as a target antigen, the immunoreactivity of RalA was evaluated by ELISA and Western blot in sera from patients with hepatocellular carcinoma (HCC), liver cirrhosis (LC), and chronic hepatitis (CH) as well as normal human sera. Indirect immunofluorescence assay with cell line and immunohistochemistry (IHC) study with tissue array slides were performed to analyze the protein expression profiles of RalA in HCC tissues.

2. Materials and methods

2.1. Sera and antibodies

Sera from 129 patients with HCC, 30 patients with CH, 30 patients with LC, and 82 normal human sera were obtained from the serum bank of the Tumor Cell Engineering Laboratory of Xia'men University (Fujian Province, P.R. China). All HCC patients were diagnosed according to the criteria described in a previous study (5). General information regarding HCC patients is shown in Table 1. Of 129 HCC patients, 90 (69.8%) were male, and 39 (30.2%) were female. Mean age was 58.2 ± 12.0 years (range, 26–77 years). Ninety-six (74.4%) patients were positive for HBV, 13 (10.1%) patients for HCV, and 7 (5.4%) patients for both HBV and HCV. Eighty-seven (67.4%) patients had previous history with CH, 22(17.1%) patients with LC, and 20 (15.5%) patients had no previous history with either CH or LC. Based on the Chinese guideline for liver cancer, 40 (31.0%) patients were in clinical stage I, 27(20.9%) in stage II, 37(28.7%) in stage III, 13(10.1%) in stage IV, respectively and 12 (9.3%) patients had no available data on clinical stages. One hundred and twenty-three (95.3%) patients were histologically confirmed, and all the HCC sera had available information regarding serum alpha fetoprotein (AFP). All HCC sera were collected at the time of initial cancer diagnosis, when the patients had not yet received treatment with any chemotherapy or radiation therapy. The patients with CH and LC had been followed for at least 18 months since the date of blood collection to exclude individuals with primary biliary cirrhosis and asymptomatic HCC. Normal human sera were collected during annual health examinations from people who had no obvious evidence of malignancy. Due to the regulations concerning studies of human subjects, the patient's name and identification number were blinded to investigators, and some clinical information for sera used in this study was not currently available. This study was approved by the Institutional Review Board of Xia'men University and collaborating institutions. Monoclonal mouse anti-RalA was commercially purchased (BD Biosciences, San Diego, CA).

2.2. Expression and purification of RalA recombinant protein

cDNA encoding RalA was amplified by PCR from a human EST (expressed sequence tag) clone (#BM560822). For the expression and purification of recombinant protein, the full length cDNA of RalA was subcloned into the pET28 expression vector which was designed to produce a fusion protein with N-terminal 6x histidine and T7 epitope tags. Recombinant RalA protein

was further expressed in *E. coli* BL21 (DE3) cells and purified using nickel column chromatography. The protocol used for high-level expression and purification of 6x His-tagged proteins were performed as described (QIAGEN Inc, Valencia, CA). Elution buffer (8M urea, 0.1M NaH₂PO₄, 0.01M Tris, pH4.5) was used to elute the recombinant protein. The purified recombinant proteins were further analyzed by electrophoresis on SDS-PAGE, and identified by Western blot using anti-histidine antibody and also by commercial monoclonal anti-RaLA antibody.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Purified recombinant protein was diluted in phosphate-buffered saline (PBS) to a final concentration of 0.5µg/ml and coated onto a 96 well microtiter plate (Dynatech Laboratories, Alexandria, VA). Human sera diluted 1:200 were incubated in the antigen-coated wells. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Caltag Laboratories, San Francisco, CA) and the substrate 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim GmbH, Mannheim, FRG) were used as detecting reagents. Each serum sample was tested at least 2 times, and the average OD value at 405nm was used for data analysis. The cutoff value designating positive reaction was the mean OD of 82 normal human sera (NHS) +3SD. Each run of ELISA included 8 NHS selected to represent a range of absorbance above and below the mean of 82 NHS, and whose OD average is close to the mean OD of 82 normal human sera. The inclusion of these normal controls is to normalize all absorbance values in other runs of ELISA. All positive sera were further confirmed by Western blotting.

2.4. Western blotting

Western blotting was performed essentially as described (6). Purified recombinant RaLA protein was electrophoresed on SDS-PAGE and transferred onto a nitrocellulose membrane paper. After pre-blocking with PBS containing 5% non-fat dry milk and 0.05% Tween-20 (PBST) for 30 min at room temperature, the nitrocellulose strips were incubated for 90 min at room temperature with a 1:200 dilution of serum. HRP-conjugated goat anti-human IgG was applied as secondary antibody at a 1:3,000 dilution. Immunoreactive bands were detected using the ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

2.5. Indirect immunofluorescence assay

Identification of autoantibody against RaLA in HCC sera was performed using methanol-and acetone-fixed HepG2 cell slides. In brief, HepG2 cells were grown on coverslips, fixed for 5 min at -20°C in 100% methanol, and permeabilized for 3 min at -20°C in 100% acetone. Monoclonal anti-RaLA antibody (25µl) at 1:2,000 dilutions was added as positive control. Human cancer sera (1:200 dilution) which show positive reactivity in ELISA and the same sera which have been preabsorbed with antibody were added to HepG2 cell-coated wells on the slide. Normal human serum and PBS were used as negative and blank controls, respectively. After incubation of one hour in a humidity chamber, the FITC-conjugated goat-anti-human IgG (Caltag Laboratories, San Francisco, CA) was applied as secondary antibody (1:80 dilution). The slides were examined with a confocal laser-scanning microscope (LSMS PASCAL, Carl Zeiss GmbH, Jena, Germany).

2.6. Absorption of antibodies with recombinant protein

Sera were diluted (1:200) and incubated for 2 hours at room temperature with 15µg recombinant proteins. The mixture was centrifuged at 10,000×g for 10 min, and the supernatant was used for ELISA analysis and immunofluorescence assay. The experiment was repeated four times, and average OD values were used for data analysis.

2.7. Immunohistochemistry (IHC) with tissue array slides

Superfrost plus tissue slides which contain 60 paraffin-embedded HCC specimens, 40 LC tissue specimens and 30 normal liver tissue specimens were commercially purchased (Cybrdi, Bethesda, MD) and used for RalA antigen detection. Antigen retrieval is performed by microwave-heating methods in a citrate-based antigen retrieval solution (BioGenex, San Ramon, CA) according to the manufacturer's recommendation. Non-specific protein binding sites were blocked by 1.5% normal horse serum for 30 min in a humidifier. The sections were incubated with monoclonal anti-RalA antibody at optimal concentration (1:2,000 dilution) which was determined by initial immunohistochemistry on cancer tissue slides (William Beaumont Medical Center in El Paso). Biotinylated secondary antibody, ABC (Avidin: Biotinylated enzyme Complex), and DAB (3,3'-diaminobenzidine) substrate were used as detecting reagents according to the manufacturer's recommendation (Vector Laboratories, Burlingame, CA). The slides were counterstained with hematoxylin, fixed by Scott's solution and dehydrolyzed with different concentration of EtOH and Citrisolvent. Finally, the slides were mounted with permount mounting medium and observed under microscope. All staining results were also confirmed by our collaborator from the University of Texas Medical Branch in Galveston, Texas, who is a medical pathologist.

2.8. Statistical analysis

The frequency of antibodies in each cohort of patients' sera was compared using the χ^2 test with Yate's correction, and two significant levels (0.01 and 0.05) will be used. Sensitivity and specificity were calculated as described(7).

3. Results

3.1. Prevalence of autoantibody to RalA in HCC

As described in Materials and Methods, the cDNA encoding RalA was amplified by PCR from a human EST clone, and the PCR products were subcloned into the pET28 expression vector which was designed to produce a fusion protein with N-terminal 6x histidine and T7 epitope tags. In the subsequent study, the full-length recombinant RalA protein was used as coating antigen in ELISA to detect antibodies against this protein in sera from patients with different conditions. Sera from 129 patients with HCC, 30 patients with LC, and 30 patients with CH as well as 82 NHS were available for this study. As demonstrated in Table 2, among 129 HCC sera analyzed, the frequency of autoantibody to RalA was 20.1% (26/129), which were significantly higher than that in sera with LC (3.3%), CH (0%), and NHS (0%) ($P<0.01$). The sensitivity and specificity of anti-RalA antibody as marker in detection of HCC was 20.1% and 99.3%, respectively. Most of positive HCC sera showed OD values over 2 folds above the cut-off (mean+3S.D. of NHS), indicating that antibody responses to the antigen in some HCC patients were quite robust and not just mildly elevated. The ELISA result was confirmed by Western blotting analysis. Figure 1 shows four representative HCC sera which have positive reaction to RalA in ELISA, and also have strong reactivity in Western blotting analysis. The specificity of recombinant RalA in ELISA was also confirmed by antibody absorption study (data was not shown). In further study, the data showed that 67 of 129 (51.9%) HCC sera had abnormal serum AFP level ($>100\text{ng/ml}$). If anti-RalA and AFP were simultaneously used as diagnostic markers, 79 of 129 (61.3%) HCC patients could be correctly identified.

3.2. Immunofluorescence staining pattern of RalA in HepG2 cells

To further confirm the specificity of autoantibody response to RalA in HCC sera, and also explore the intracellular location of RalA protein, HCC sera with anti-RalA positive in ELISA were also examined using HepG2 cells as substrate by indirect immunofluorescence assay. As shown in Figure 2, a representative HCC serum with anti-RalA antibody positive in ELISA

had a cytoplasmic staining pattern with more intense staining in the perinuclear regions, which was a similar pattern as monoclonal RalA antibody showed. The same HCC serum was preabsorbed with recombinant RalA protein, and subsequently applied for immunofluorescence assay, and then the fluorescent signal was significantly reduced.

3.3. Expression of RalA in HCC tissues by immunohistochemistry with tissue array

In the current study, the expression profile of RalA in HCC, LC and normal liver tissues were examined by immunohistochemistry with tissue array slides. Tissue array slides including 60 HCC tissue specimens, 40 LC tissue specimens, as well as 30 normal liver tissue specimens were commercially available for this study. The concentration of monoclonal anti-RalA used for immunostaining the tissue specimens were determined by initial immunohistochemistry study on tumor tissue slides, and subsequently, the tissue-array slides were stained with monoclonal anti-RalA antibody. The H&E (hematoxylin and eosin) staining showed the histopathology of the same specimen. As shown in Table 3, the results indicated that there was a stepwise increased frequency of RalA overexpression in HCC tissues (63.3%, 38/60), LC tissues (45.0%, 18/40) and normal liver tissues (26.7%, 8/30). The frequency of RalA expression in HCC tissues was significantly higher than that in normal liver tissues ($p < 0.01$). The frequency of RalA positive staining in HCC grade I, II and III were 62.5% (5/8), 69.23% (27/39), and 46.1% (6/13), respectively. Due to the small sample size of tissues with different grades in this tissue array slide, it is difficult to establish a statistical association between RalA expression and tumor grade. Figure 3 showed the representative positive and negative immunostaining pattern of RalA in HCC tissues. Because of lack of the data regarding different clinical stages of HCC tissues in this commercial tissue array slide, it was not able for us to establish a statistical correlation between RalA expression and clinical stages in the present study.

4. Discussion

Ral proteins are small GTPase and work down stream of Ras protein signaling pathway(8). The monomeric Ral (Ras-like) GTPases have been indirectly implicated in mitogenic regulation and Ras-mediated oncogenic transformation (9,10). Ral-GEFs (Ral guanine nucleotide exchange factors), one of the primary Ras binding proteins or effectors propagate oncogenic Ras signals by activating the RalA and RalB (11,12). It was showed that RalA and RalB collaborate to maintain tumorigenicity through regulation of both proliferation and survival (13,14). The mechanism by which Ral GTPase may contribute to proliferation and transformation was still poorly understood. Henry and his co-workers have provided one possible explanation that the expression of activated Ral in quiescent rodent fibroblasts was sufficient to induce activation of NF- κ B-dependent gene expression and cyclin D1 transcription, two key convergence points for mitogenic and survival signaling (15,16). Another study also found a constitutively activated form of RalA GTPase activated the transcription factor Stat3 through the activation of c-Src (17). In the current study, RalA was identified as a possible tumor-associated antigen by immunoscreening a cDNA expression library with serum antibodies from a HCC patient. In subsequent study, the purified recombinant RalA protein was used as target antigen, and the autoantibody response to this protein in sera from patients with HCC and precursor conditions such as liver cirrhosis and chronic hepatitis was investigated.

RalA was more commonly activated compared to other major Ras effector pathway in distinct cancer cell lines, and knockdown of RalA expression impeded, if not abolished the ability of human cancer cells to form tumors. Thus activation of RalA plays a critical role in Ras-induced tumorigenesis (18), RalA was also reported to play an important role in epidermal growth factor (EGF)-mediated cell motility and it has potential contribution to tumor metastasis in human

cancer (19–21). Taken together with the evidence provided by our study that the frequency of antibody to RalA was significantly higher in HCC sera (20.1%) compared to other conditions (LC, CH and NHS), it suggested that RalA might contribute to liver malignant transformation, and could be used as a potential tumor marker in HCC detection. Data from our study also demonstrated that if both RalA and AFP were simultaneously used as diagnostic markers, 61.3% HCC patients could be correctly identified, suggesting that RalA might be an independent but also a supplementary serological marker for the diagnosis of HCC.

Due to the small size of samples, whether the antibody response to RalA in HCC sera is correlated with the clinical significance remains to be established. In the current study, the expression profiles of RalA in HCC, liver cirrhosis, and normal liver tissues were extensively evaluated by immunohistochemistry assay with tissue microarray (TMA) slides. Our results showed that the expression of RalA in HCC tissues (63.3%) was significantly higher than that in normal liver tissues (26.7%). Analysis on the frequency of positive RalA staining in HCC tissues with different grades has not revealed any significant correlation between RalA expression and cancer grades due to the limited number of tissue specimens in this study, especially the small sample size of tissues with grade I and III. The finding also suggests that the emerging of the expression of RalA is associated with HCC progression, and may play a role in HCC transformation. The higher immunogenicity of RalA in HCC sera is probably related to the abundant expression of RalA in HCC tissue, which makes them more accessible for presentation within MHC molecules and thus, more available to the immune system for immune recognition. The mechanism for RalA contributing to cell proliferation and transformation is still unknown, but evidence from other studies supported that expression of gain-of-function of RalA can affect several mitogenic regulatory cascades, including nuclear factor- κ B (16), Src (17), and phospholipase D1 (PLD1) (22). Although there are strong indications that RalA may be functionally associated with HCC tumorigenesis, however, the non-restricted HCC expression, especially the higher expression in benign condition such as liver cirrhosis, tempered its usefulness as a target for HCC immunotherapy.

With the rapid development of new techniques in searching TAAs, a bunch of novel antigenic proteins have been identified as potential tumor antigens. A systemic analysis to validate the target antigens and assessment of clinical application is essentially important, especially in the area of biomarkers for diagnosis and the targets for immunotherapy. As mentioned above, despite the improvement of therapeutic method in HCC, such as surgery, chemo-therapy, radio-therapy, ethanol injection therapy, and trans-arterial embolization, a high case fatality rate still remains a problem, and it is partially due to the precursor liver diseases that limit the use of chemotherapeutic drugs, rapid intra-liver spread and out-liver metastasis. Therefore, new approach such as immunotherapy is eagerly needed. However, the critical selection of immunotherapeutic targets relies on the restricted tumor expression of tumor antigens that will be recognized by host immune system leading to tumor rejection. The lower expression in normal tissue and vital organs, thus avoid the autoimmune damage accompanied with immunotherapy. Our study demonstrated that there was a significantly increased autoimmune response to RalA in HCC sera compared to normal human sera. Whether or not RalA expression is specifically in HCC, and can contribute ideal targets for HCC immunotherapy still need to be established. Many studies demonstrated that immunohistochemistry approach can provide such a method to analyze the expression profile of targeted tumor antigen by staining tissue specimens with monoclonal or polyclonal antibody (23). In the present study, the expression profile of RalA in different tissue types, such as the tissues from patients with HCC and LC as well as normal liver tissues, was examined and evaluated in tissue microarray (TMA) slides using immunohistochemistry approach. The tumor antigens which show restricted HCC expression are assumed to be ideal candidates for diagnostic markers as well as potential targets for cancer immunotherapy. Tissue microarray technology takes advantage of tissue cylinders (diameter 0.6 mm) derived from hundreds of different primary tumor blocks and subsequently

brought into one empty “recipient” paraffin block. It allows high throughput molecular profiling of diagnostic or predictive markers in cancer specimens and rapid validation of novel potential candidates identified from genomic and proteomic analyses in a large number of tumor samples by immunohistochemistry. Most importantly, data from tissue microarray can be evaluated in combination with databases containing pathological and clinical information related to individual specimens (24). The functional significance of individual tumor antigen, thus, can be assessed at a global scale of cancer development. Due to the higher immunogenicities of RalA in HCC sera, we proposed that the increased HCC-specific expression of the antigen may strengthen its usefulness not only as diagnostic markers but also feasible targets for HCC immunotherapy.

In summary, data from this study demonstrated that RalA had a significantly higher immunogenicity in HCC patients compared to patients with liver cirrhosis and chronic hepatitis as well as normal individuals. Whether RalA can be used as diagnostic marker in HCC detection, and the underlining mechanism of how this antigen induces humoral immune response in HCC patients remain to be investigated. Further study with a comprehensive analysis on RalA expression profile in HCC may provide more insight into how it is involved in the tumorigenesis of HCC.

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Figure 1.

Western blotting analysis showing representative HCC sera recognizing RalA recombinant protein. Lanes 1–4, four representative HCC sera which were positive in ELISA, and also have strong reactivity with RalA recombinant protein in Western blotting analysis; lane 5, a normal human serum was used as negative control; The monoclonal anti-RalA antibody was used as positive control.

Figure 2.

Representative immunofluorescence staining pattern of anti-RaLA antibody positive HCC serum. **A.** Phosphate-buffered saline (PBS) was used as blank control; **B.** A normal human serum (NHS) was used as negative control; **C.** Monoclonal anti-RaLA antibody which showed a perinuclear immunofluorescence staining pattern was used as positive control; **D.** A representative anti-RaLA antibody positive HCC serum demonstrated similar perinuclear immunofluorescence staining pattern; **E.** The same HCC serum which was used in panel D was pre-absorbed with recombinant RaLA protein, and subsequently utilized for immunofluorescence assay. The fluorescent signal was primarily removed.

Figure 3.

Expression of RalA in HCC tissues examined by immunohistochemistry (IHC). Tissue-array slide was stained with monoclonal anti-RalA antibody at a 1:2,000 dilution. H&E (hematoxylin and eosin) staining was performed to show the histopathology of the tissue specimens. A. a representative RalA negatively stained HCC tissue (40x); B. a representative RalA positively stained HCC tissue (40x). A positively stained area (arrow) was enlarged (100x).

Table 1

Characteristics of 129 patients with HCC

Variables	Results
Mean age (mean $\pm$ SD, year)	58.2 $\pm$ 12.0
Age range (year)	26–77
<i>Gender</i>	
Male (%)	90 (69.8%)
Female (%)	39 (30.2%)
<i>Hepatitis virus infection</i>	
HBV (%)	94 (74.4%)
HCV (%)	13 (10.1%)
HBV + HCV (%)	7 (5.4%)
<i>Background</i>	
Chronic hepatitis (%)	87 (67.4%)
Liver cirrhosis (%)	22 (17.1%)
<i>Clinical stages</i>	
Stage I (%)	40 (31.0%)
Stage II (%)	27 (20.9%)
Stage III (%)	37 (28.7%)
Stage IV (%)	13 (10.1%)
Histological diagnosis (%)	123 (95.3%)

Note: Abbreviation: HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 2

Frequency of autoantibody to RalA in human sera by ELISA

Type of sera	No. tested	Autoantibody to RalA (%)
HCC	129	26 (20.1)**
LC	30	1 (3.3)
CH	30	0
NHS	82	0

Note: Cutoff value: Mean + 3 SD of NHS; P value relative to NHS:

**
 $P < 0.01$

Abbreviations: CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NHS, normal human sera

Table 3

Expression profile of RalA in HCC, LC, and normal liver tissues

Type of tissues	No. tested	Frequency of RalA expression (%)
HCC	60	38 (63.3) **
LC	40	18 (45.0)
Normal	30	8 (26.7)

Note: P value relative to NHS:

**
 $P < 0.01$