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Detection of Novel Autoantibodies to Nucleolin's RNA-binding Domains as a Serum Tumor Biomarker Through ELISA

Fatemeh Ezzatifar[1,2,3], Alireza Rafiei[1,2], Reza Valadan[1,2], Hossein Asgarian-Omran[2], and Mahmood Jeddi-Tehrani[4]

[1] Molecular and Cell biology Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[2] Department of Immunology, School of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[3] Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran
[4]Monoclonal Antibody Research Center, Avicenna Research Institute, Tehran, Iran

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ABSTRACT

Expression and location of nucleolin are often abnormal in malignancies, which may result in the production of autoantibodies. Despite this, the identification of such autoantibodies may be essential for the early diagnosis and prognosis of cancers.

In this investigation, the recombinant nucleolin protein was generated using an *Escherichia coli* expression system and was used an indirect enzyme-linked immunosorbent assay to detect anti-nucleolin autoantibodies in cancer patients' sera.

Lung cancer patients' autoantibodies displayed the highest seroreactivity with the recombinant protein, with area under the curve of 0.948 and sensitivity and specificity of 85% and 96.67%, respectively (accuracy=92%). Anti-nucleolin autoantibodies were linked with lung tumor size (r=0.793), tumor, node, metastasis staging (r=0.643), and proliferation (r=0.744).

These autoantibodies distinguished patients with early-stage lung cancer from healthy controls. Since anti-nucleolin autoantibodies are strongly linked to tumor size, clinical staging, and growth, they can be used to measure how well a treatment is working.

Keywords: Autoantibodies; Biomarkers; Enzyme-linked immunosorbent assay; Neoplasms; Nucleolin

INTRODUCTION

Cancer is one of the leading causes of death and a major, barrier to increasing life expectancy. Lung, breast prostate, colorectal, pancreatic, hepatic, and ovarian cancers will be the leading causes of mortality and morbidity in 2022.[1] Also, over 70% of cancer patients are detected at an advanced stage when treatment options are limited.[2] On the other hand, as the serum biomarkers are noninvasive and reproducible screening techniques, they would boost diagnostic capabilities to complement existing diagnostic assays. Numerous investigations have shown that sera from cancer patients include autoantibodies (AAbs) that react with a distinct collection of autologous cellular antigens referred to as tumor-associated antigens (TAAs).[3,4] The immune response emerges months to years before the clinical diagnosis of a tumor, which is induced by the release of

Corresponding Author: Alireza Rafiei, DVM, PhD;
Department of Immunology, School of Public Health, Mazandaran University of Medical Sciences, P.O.Box : 48471-91971, Sari, Iran. Tel: (+98 11) 3354 3614, Fax: (+98 11) 3354 3087, E-mail: rafiei1710@gmail.com

TAAs from tumors in cancer patients, indicating that detection of serum AAbs is well suited for early cancer diagnosis. Early diagnosis is critical for successful cancer care.[5] Similarly, the AAbs have grown in popularity as tumor markers over the past several decades due to their ease of extraction from serum by negligibly invasive blood sampling. They have also been detected with the progression to malignancy and may be useful indicators since they are persistent serological proteins[6] with strong serum levels despite low antigen levels.[7] This makes sample management easier. So, the identification of tumor biomarkers from profiling of body fluids is one of the most effective management approaches in promising cancer therapies. Nucleolin (NCL), with a molecular weight of 110 kDa, is one of the critical proteins found in trace levels in the nucleus of normal eukaryotic cells.[8,9] This multifunctional phosphoprotein is made of four RNA-binding domains (RBDs) and is required for ribosomal organization, rRNA processing, and mRNA stability. It also regulates the production of microRNAs involved in tumor development and metastasis.[10] Additionally, it may act as a ligand for extracellular molecules involved in cell differentiation and adhesion, leukocyte trafficking, inflammation, angiogenesis, and tumorigenesis.[11] This phosphoprotein is overproduced in tumor cells as a result of malignant transformation, with the greatest levels in lung, colon, neck, breast, stomach, thyroid, pancreas, and testis cancer cells.[12] By aggregating on the plasma membrane and migrating to the cytoplasm of the cell[13], it most probably draws the immune system's attention as a TAA, resulting in the generation of AAbs. As a result, the anti-NCL AAbs have the potential to become an intriguing candidate biomarker for the detection of a wide variety of malignancies. Additionally, a high-throughput technology capable of detecting reactive AAbs to tumor-related proteins in cancer patients' sera contributes to the discovery of novel tumor biomarkers. Enzyme-Linked Immunosorbent Assay (ELISA) may now be used to detect the titer of tumor biomarker-specific antibodies by utilizing a well-coated pure recombinant protein.[14] Herein, the main objective of the present study was to evaluate the immunoreactivity of our purified recombinant NCL against the sera of patients with lung, head and neck, colon, breast, stomach, thyroid, pancreas, and testis cancers. In brief, 160 serum samples from patients with various stages of the aforementioned cancers and the healthy sera from 30 devotees were collected and analyzed for NCL reactive autoantibodies using an indirect ELISA approach.

MATERIALS AND METHODS

Using UALCAN and GEPIA2 Online Databases for NCL Gene Analysis

UALCAN (The University of ALabama at Birmingham CANcer data analysis Portal) is a web portal for studying The Cancer Genome Atlas (TCGA) pan-cancer transcriptome data and verifying key genes.[15] UALCAN can find biomarkers, verify candidate genes, and examine the relative expression of query genes in tumor and normal tissues as well as distinct tumor molecular subtypes. RNA sequencing data from TCGA and Genotype-Tissue Expression (GTEx) programs are analyzed by Gene Expression Profiling Interactive Analysis 2 (GEPIA2).[16] We employed UALCAN and GEPIA2 to explore the pattern of the NCL gene expression in LC (lung cancer), H & N-C (head and neck cancer), TC (testis cancer), BC (breast cancer), CC (colon cancer), TyC (thyroid cancer), PC (pancreatic cancer), and GC (gastric cancer) patients. Results were shown in box, stage, and dot plots.

Subjects and Sample Preparation

This study included 190 participants who were assigned to one of the two groups. Patient groups were randomly chosen by counting 160 individuals with diverse types of cancers, including LC, H & N-C, TC,TyC, BC, CC, PC, GC (n=20 for each form of cancer). The healthy control group included 30 healthy people (15 men and 15 women, aged 58.8±31.9 years old). Tissue and blood samples were collected at Imam Khomeini Hospital, Sari, Iran. Table 1 presents the detailed characteristics of the nine groups. Each individual signed an informed consent form. The research protocol was developed in accordance with the 1964 Declaration of Helsinki Criteria and was approved by the Ethics Committee of Mazandaran University of Medical Sciences (IR. MAZUMS: REC.1397.2700). A histopathological examination was performed to confirm the presence of cancerous or premalignant lesions. Moreover, none of the cancer patients should have prior immunosuppressive intervention history, such as surgery, chemotherapy, or radiation treatment. The inclusion criteria included: no history of cancer in the preceding 6 months, no hospitalization for lung, head and neck, testis, thyroid, gastric, colon, pancreatic, or breast surgery or any

disorder including serious hypertension, severe heart disease, heart arrhythmias, kidney and liver diseases, patients with gastrointestinal blockage or other conditions that might cause vomiting, in addition to not having had any chemical or biological treatment in the past year and not having taken juvantia and the exclusion criteria included: Any acute or chronic, infectious or non-infectious illnesses or malignancy, regardless of severity, history of cancer in the preceding 6 months, hospitalizations for lung, head and neck, testis, thyroid, gastric, colon, pancreatic, or breast surgery, autoimmune diseases and blood transfusion during the previous two months, were considered for healthy controls. After collecting all malignant and healthy blood samples, the supernatant sera were separated and kept at -70°C until further usage and analysis. Additionally, 5 healthy subjects with the above-mentioned inclusion and exclusion criteria provided peripheral blood mononuclear cell (PBMC) for recombinant protein production. Briefly, 10 mL of plasma-depleted, heparinized whole blood was diluted in Hank's buffered salt solution (Thermo Fisher Scientific, USA). Then, 30 mL of diluted blood was spread over 15 mL of ficoll–hypaque (Innotriane, Germany). After 30 minutes of continuous centrifugation at 2000 rpm and room temperature, it was easy to get the PBMCs out.

Histopathological Examination

Surgeons and pathologists conducted all morphological and histological analyses of tumor tissues. Tumor size, differentiation, nearby lymph node status, distant metastases, estrogen receptor (ER)/ progesterone receptor (PR) and cytokeratin 7 (CK7) levels, P53 screening for mutations of codon 248 and the Ki-67 index value were the parameters studied. ER, PR, CK7, and Ki-67 were examined using immunohistochemistry (IHC), and P53 was screened using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The antibodies utilized were ER (Monoclonal Mouse Anti-Human Estrogen Receptor, Code: N1575, Clone: 1D5, Dako, USA), PR (Monoclonal Mouse Anti-Human Progestogen Receptor, Code: N1630, Clone: PgR636, Dako, USA), CK7 (Monoclonal Mouse Anti-Human Cytokeratin 7, Clone OV-TL 12/30, Denmark), and Ki-67 (Monoclonal Rabbit Anti-Human Ki-67 Antibody, Clone: IHC167, Eagle Biosciences, USA). Using IHC, the percentages of ER and PR-positive cells were determined according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines (at least 1% stained tumor nuclei). Also, at least 500 tumor cells were counted from the active region to determine the proportion of cells with positive Ki-67 nuclei. Finally, the tumor, node, metastasis (TNM) classification system was employed to grade the tumors according to the size and extent of the tumor (T), the involvement of neighboring lymph nodes (N), and distant metastases (M). Briefly, tumor stages are numbered from T1 through T4, with T4 being the most advanced stage of growth and invasion of the original tumor. A score of N0 indicates the absence of any nodal dissemination in the immediate area, whereas scores of N1 through N3 suggest gradual, distant spread. Also, the tumor is called M0 if it hasn't spread to other parts of the body, and M1 if it has.

Accordingly, stage 1 cancer is localized (T1-T2, N0, M0); stage 2 cancer is locally invasive (early stages, T2-T4, N0, M0); stage 3 cancer is locally invasive (late stages, T1-T4, N1-N3, M0); stage 4 cancer is metastatic (T1-T4, N1-N3, M1).

Purification and Validation of the rRBDs-NCL Protein

The recombinant RNA-binding domains of nucleolin (rRBDs-NCL) protein containing amino acids 307-649 (nucleotides 1050-2078 (1029 bps)) were engineered by cloning in the *Escherichia coli* (*E. coli*) expression system according to our previous work.[17] Briefly, total RNA from harvested PBMCs was produced according to the manufacturer's instructions using a very pure RNA extraction kit (QIAGEN, Germany). A Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis were used to evaluate the quantity and quality of the extracted RNA, respectively. The cDNA synthesis was then performed using a high-capacity cDNA reverse transcription kit (Fermentas cDNA Synthesis Kit, Waltham, Massachusetts, USA) with 10 µL RNA, dNTP, and random primers. Then the target fragment sequence was amplified via PCR using the following primers: Forward:5'GGATCCGGCACAGAACCGACTACG3', Reverse:5'GAATTCTCAGAAGCCACCTTCACCCTTA3'. The amplified sequence and pET-28a expression vector were double digested by *EcoRI* and *BamHI* restriction enzymes to integrate the target gene into the prokaryote system as per the protocol of Sambrook et al.[18] *E. coli BL21 (DE3)* bacteria were transformed with the pET28 expression vector with the target construct.

Table 1. Characterization of eight distinct forms of cancer and healthy control

Groups	HC	LC	H & N-C	TC	BC	CC	TyC	PC	GC
Sex									
Male (%)	15 (50)	8 (33)	13 (65)	20 (100)	-	11 (55)	8 (33)	9 (45)	6 (30)
Female (%)	15 (50)	12 (67)	7 (35)	-	20 (100)	9 (45)	12 (67)	11 (55)	14 (70)
Age (Mean ±SD)	58.8±31.9	71±6.2	54.3±12.5	34±9.1	54.17±11.3	63.9±8.8	60±12.5	66.8±23.9	67.3±16.5
Min	25	66	41	26		56	44		48
max	91	77	66	45	44	73	73	41	89
					62			90	
Tumor size	-								
< 3 cm (%)		15 (75)	17 (85)	12 (60)	14 (70)	10 (50)	4 (20)	6 (30)	3 (15)
≥ 3 cm (%)		5 (25)	3 (15)	8 (40)	6 (30)	10 (50)	16 (80)	14 (70)	17 (85)
TNM staging	-								
1 (%)		4 (20)	4 (20)	5 (25)	4 (20)	5 (25)	3 (15)	5 (25)	4 (20)
2 (%)		8 (40)	7 (35)	5 (25)	8 (40)	6 (30)	7 (35)	4 (20)	6 (30)
3 (%)		5 (25)	6 (30)	7 (35)	4 (20)	6 (30)	6 (30)	7 (35)	6 (30)
4 (%)		3 (15)	3 (15)	3 (15)	4 (20)	3 (15)	4 (20)	4 (20)	4 (20)
Differentiation	–								
Poor (%)		5 (25)	6 (30)	8 (40)	6 (30)	6 (30)	5 (25)	4 (20)	5 (25)
Moderate (%)		12 (60)	7 (35)	5 (25)	8 (40)	6 (30)	7 (35)	12 (60)	3 (15)
High (%)		3 (15)	7 (35)	7 (35)	6 (30)	8 (40)	8 (40)	4 (20)	12 (60)
Ki 67	-								
< 20% (%)		6 (30)	9 (45)	12 (60)	10 (50)	7 (35)	11 (55)	7 (35)	6 (30)
≥ 20% (%)		14(70)	11 (55)	8 (40)	10 (50)	13 (65)	9 (45)	13 (65)	14 (70)
P53 mutation	-								
Positive (%)		8 (40)	7 (35)	2 (10)	8 (40)	7 (35)	8 (40)	14 (70)	10 (50)
Negative (%)		12 (60)	13 (65)	18 (90)	12 (60)	13 (65)	12 (60)	6 (30)	10 (50)
CK7	-								
Positive (%)		9 (45)	10 (50)	-	20 (100)	11 (55)	-	10 (50)	8 (40)
Negative (%)		11 (55)	10 (50)	20 (100)	-	9 (45)	20 (100)	10 (50)	12 (60)
ER rate	-								
< 1% (%)		6 (30)	18 (90)	16 (80)	3 (15)	15 (75)	11 (55)	20 (100)-	15 (75)
≥ 1% (%)		14 (70)	2 (10)	4 (20)	17 (85)	5 (25)	9 (45)		5 (25)
PR rate	-								
< 20% (%)		12 (60)	16 (80)	20 (100)	6 (30)	20 (100)	19 (95)	7 (35)	20 (100)
≥ 20% (%)		8 (40)	4 (20)	-	14 (70)	-	1 (5)	13 (65)	-

HC: healthy control; LC: lung cancer; H & N-C: head and neck cancer; TC: testis cancer; TyC: thyroid cancer; BC: breast cancer; CC: colon cancer; PC: pancreatic cancer; GC: gastric cancer; TNM: tumor (T), nodes (N), and metastases (M); CK: cytokeratin 7; ER: estrogen receptor; PR: progesterone receptor

The expression of the recombinant pET-28a/NCL plasmid vector process was performed by induction of 0.125 mg/ml IPTG in 400 ml of Luria Broth (LB) medium containing kanamycin and chloramphenicol antibiotics at 170 rpm and 37°C overnight. Following the dissolution of the harvested plate in the lysis buffer and treatment with ultrasound sonication, the supernatant was used for purification with Ni-NTA column resin (QIAGEN, Germany) according to the protocol of Sambrook et al. After evaluation of the purified protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 12%,

the purity of the final product was confirmed via immunoblotting with the ECL kit (Amersham Phamacia Biotech Inc, USA).

ELISA Testing of the Purified rRBDs-NCL Immunoreactivity with Cancer Patient Serum

Using an ELISA reader (BioTek, USA), the immunoreactivity of the 190 serum samples from cancer patients and healthy controls was tested against the purified protein. ELISA polystyrene plates (Maxisorp SPL) were coated with 100 µL of diluted rRBDs-NCL (5 µg/mL in PBS, *p*=7.2). After blocking non-specific binding sites with 3% BSA, patient and healthy group sera were added. Then 100 µL of anti-human IgG-HRP (1:4000 diluted Sigma, Germany) was added. Tetramethylbenzidine (TMB) substrate (Sigma, Germany) was added at room temperature in the dark after incubation to create a dye reaction. After 20 minutes, H_2SO_4 stopped the reaction. Optical densities (ODs) at 450 and 630 nm were measured. Mean delta OD of 450 nm was used for data analysis. On the receiver operating characteristic (ROC) curve where Youden's index (sensitivity + specificity-1) was greatest, the cut-off for positive responses was identified.

Statistical Analysis

All experiments were done twice in duplicate. The anti-rRBDs-NCL AAb seroreactivity data were given as mean±SD of OD. GraphPad Prism version 7.0 (San Diego, CA) was used for statistical analyses and graphics. After ascertaining the normal distribution of the data (via Shapiro-Wilk's test), parametric one-way analysis of variance (ANOVA) and student's t-test were used to compare groups. In GEPIA2, one-way ANOVA was used to compare cancer and healthy sample gene expression. In UALCAN's stage plot, Z-values represent standard deviation (SD) from the sample median. Pearson's test correlated AAb levels with tumor size and TNM staging. Using a single-variable ROC analysis, malignant samples were distinguished from healthy ones, yielding area under the curve (AUC) values with a 95% confidence interval (CI). Also, a *p value* less than 0.05 was deemed statistically significant.

RESULTS

Pan-cancer Expression Levels of NCL Protein

We assessed NCL gene transcription in each TCGA tumor. The overexpression of this gene in malignancies varies, and a comparison with normal control samples demonstrates significant NCL expression in most tumor types (Figures S1a and S1b). An study of the GEPIA2 dataset in respect to the eight malignancies with substantial NCL expression including BRCA (breast invasive carcinoma), COAD (colon adenocarcinoma), HNSC (head and neck squamous cell carcinoma), THCA (thyroid carcinoma), PAAD (pancreatic adenocarcinoma), STAD (stomach adenocarcinoma), TGCT (testicular germ cell tumor), and LUAD (lung adenocarcinoma) found that NCL overexpression was statistically significant in COAD, PAAD, STAD, and TGCT tumors, despite being considerably higher in all cancer groups compared to matched normal controls (Figure 1). We also evaluated the NCL levels by cancer stage. UALCAN stage maps demonstrate that NCL expression is high in cancer samples (particularly COAD, BRCA, HNSC, LUAD, PAAD, and TGCT) and rises as the disease advances (Figure S2).

Expression and Purification of the rRBDs-NCL Protein

After transforming T7 pET-28a-RBDs-NCL into *E coli* BL21 (DE3), production of rRBDs-NCL was stimulated with 0.125 mg/ml isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C overnight. Fast protein liquid chromatography (FPLC) (Kenauer, Germany) purified the protein. The BCA test kit (DNAbiotech, IRAN) assessed the rRBDs-NCL protein to be 1 mg/ml. Also, the immunoblotting and SDS-PAGE indicated the purification of a 37.9-kD recombinant protein (Figure 2).

The AAb Against the 37.9 kD rRBDs-NCL Protein Was Found in the Serum Samples of Patients with Cancers

Figure 3 displays rRBDs-NCL AAbs in age-and sex-matched healthy people and the eight cancer groups. First, there was no statistically significant difference in age between the sex-matched healthy participants and the cancer patients (LC: *p*=0.8703, H & N-C: *p*=0.9581, TC: *p*=0.5761, BC: *p*=0.5201, CC: *p*=0.8994, TyC: *p*=0.9002, PC: *p*=0.8693, GC: *p*=0.7398). Second, the immunoreactivity study revealed that, with the exception of GC, AAbs against the recombinant protein were higher in LC, H & N-C, TC ($p<0.0001$), BC, CC ($p<0.001$), TyC ($p<0.01$), and PC ($p<0.05$) sera. ELISA results showed a high level of rRBDs-NCL-specific AAbs in LC sera (1.034±0.567), which was substantially

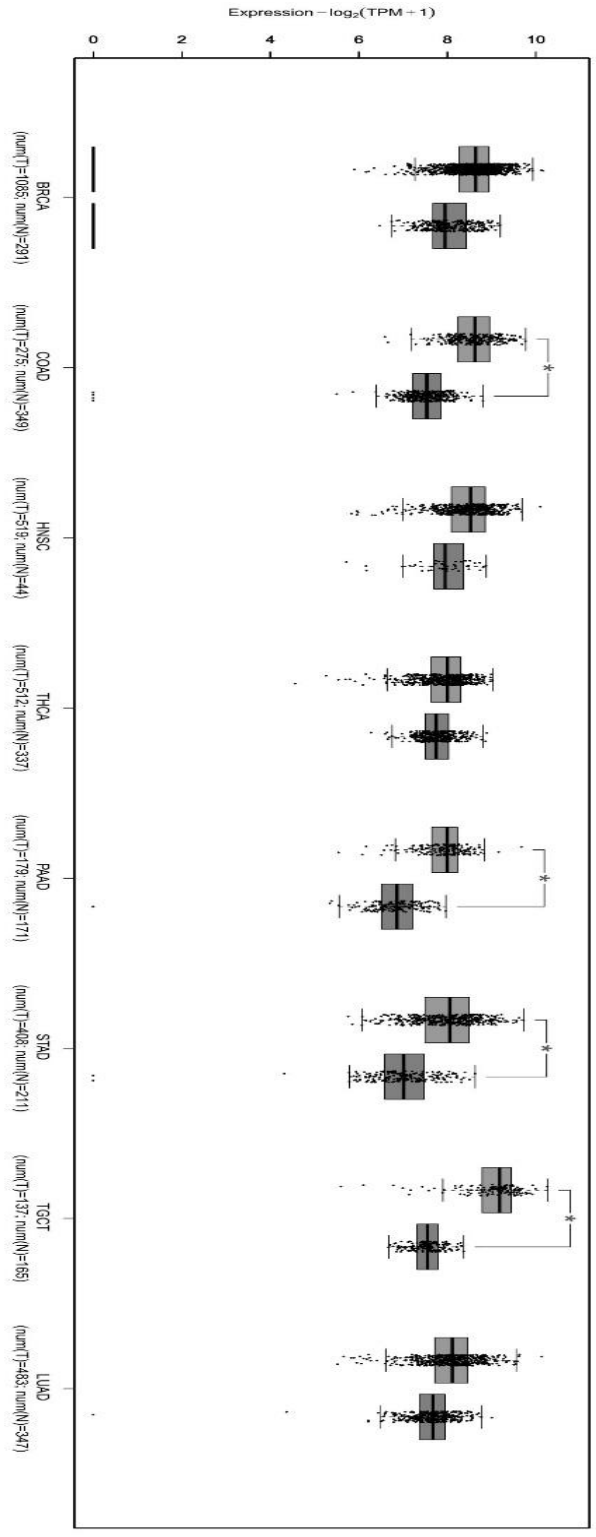

Figure 1. The box plot compares the nucleolin (NCL) expression levels across eight different cancers types and their paired normal tissues (light gray left boxes) by GEPIA2. BRCA: breast invasive carcinoma; COAD: colon adenocarcinoma; HNSC: head and neck squamous cell carcinoma; THCA: Thyroid carcinoma; PAAD: pancreatic adenocarcinoma; STAD; stomach adenocarcinoma; TGCT: testicular germ cell tumor; LUAD: lung adenocarcinoma, * $p<0.05$

higher than other malignancies and healthy controls. After that, H & N-C (0.826 ± 0.494), TC (0.756±0.437), BC (0.720±0.426) and CC (0.708±0.418) sera had modest levels of certain rRBDs-NCL AAbs.

Within the next tier, TyC (0.679±0.400), and PC (0.573±0.307) serum samples demonstrated the lowest level of reactivity with the generated recombinant protein and, thus, the least level of specific AAbs. However, healthy donor sera (0.205±0.154) exhibited no substantial seroreactivity.

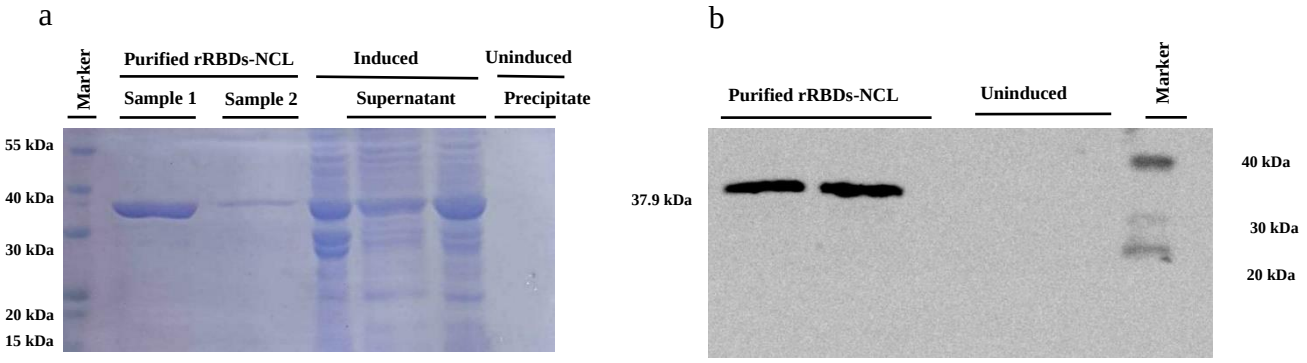

Figure 2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses of isolated recombinant RNA binding domains of nucleolin (rRBDs-NCL) protein. (a) The SDS-PAGE analysis displayed the 37.9 kDa rRBDs-NCL protein following substrate induction both in the supernatant and precipitate. Also, the same sharp band was revealed in the undiluted (1) and diluted (2, 1:10) purified protein samples. (b) Duplicate western blot analysis also showed a high-efficiency synthesis of 37 kDa rRBDs-NCL protein, while in the uninduced condition, no product and then a particular 37.9 kDa protein band were seen.

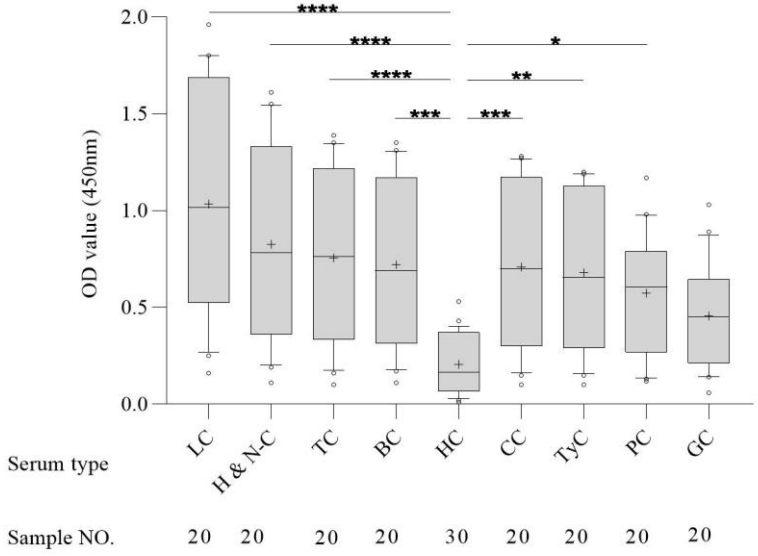

Figure 3. Autoantibody (AAb) levels to the recombinant RNA binding domains of nucleolin (rRBDs-NCL) protein. Box and Whisker plots for serum levels of AAb against rRBDs-NCL in the sera of HC: healthy control, LC: lung cancer, H & N-C: head and neck cancer, TC: testis cancer, TyC: thyroid cancer, BC: breast cancer, CC: colon cancer, PC: pancreatic cancer and GC: gastric cancer groups. The "+" sign and the line crossing the box show the mean and median, respectively. The end of each box marks the 10th and 90th percentiles. The bars show the upper and lower extremes. One-way analysis of variance (ANOVA) test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Potential of the AAb Against the rRBDs-NCL Protein in Early Cancer Diagnosis

In LC, H & N-C, and TC patients, the difference between late and early-stage sera was statistically significant (p<0.05) (Table S1). In contrast to other cancer sera, early-stage LC sera had identical immunoreactivity to rRBDs-NCL protein (p=0.0009) as advanced-stage sera (p<0.0001). However, the serum from other early-stage tumor patients did not interact significantly (p>0.05) (Table 2). We performed ROC analysis to evaluate anti-rRBDs-NCL AAb's LC diagnostic value. The overall accuracy of anti-rRBDs-NCL AAb in differentiating LC patients from matched healthy persons was 92% (AUC=0.948). By adopting the cut-off of 0.460, this AAb may identify LC patients with the highest sensitivity (85%) and specificity (96.66%) compared to HC (95% CI: 88.40 to 100, p<0.0001). However, this AAb's capacity to detect other cancers decreased, and its sensitivity and specificity for diagnosing GC reached 60% and 93.3%, respectively (95% CI: 67.43 to 92.73, p=0.0004) (Table 3).

Correlation of the AAb Against the rRBDs-NCL Protein with the Clinicopathological Features

Anti-rRBDs-NCL antibody levels were higher in patients with larger tumors (≥3cm) of LC (p=0.0007), H & N-C ($p=0$.0190), and TC ($p=0$.0018) than in patients with smaller tumors (<3 cm) (Table S1). Additionally, only serum samples from the same cancer patients in late (3 and 4) stages showed at least a 1.7 fold increase in the immunoreactivity with the resultant recombinant protein compared to individuals with early (1 and 2) stages ($p=0$.0231 for the LC, $p=0$.0067 for the H & N-C, and $p=0$.0249 for the TC). (p=0.0231 for the LC, p=0.0067 for the H & N-C, and p=0.0249 for the TC).

Table 2. Comparing the immunoreactivity of the early and late stages cancer sera against the recombinant RNA binding domains of nucleolin (rRBDs-NCL) protein

Group (NO.)	Mean	Standard deviation	Comparison (NO.)					
HC (30)	0.205	0.154	Early stage	[a] *p value*	[b] *p value*	Late stage	[a] *p value*	[b] *p value*
LC (20)	1.034	0.567	LC (12) vs HC (12)	*0.8434*	*0.0009****	LC (8) vs HC (8)	*0.9874*	*<0.0001*****
H & N-C (20)	0.826	0.494	H & N-C (11) vs HC (11)	*0.9075*	*0.1509*	H & N-C (9) vs HC (9)	*0.9104*	*<0.0001*****
TC (20)	0.756	0.437	TC (10) vs HC (10)	*0.8985*	*0.2559*	TC (10) vs HC (10)	*0.7308*	*0.0013***
BC (20)	0.720	0.426	BC (12) vs HC (12)	*0.7012*	*0.0612*	BC (8) vs HC (8)	*0.8009*	*0.0010***
CC (20)	0.708	0.418	CC (11) vs HC (11)	*0.9342*	*0.0628*	CC (9) vs HC (9)	*0.9541*	*0.0056***
TyC (20)	0.679	0.400	TyC (10) vs HC (10)	*0.8231*	*0.2215*	TyC (10) vs HC (10)	*0.8004*	*0.0020***
PC (20)	0.573	0.307	PC (9) vs HC (9)	*0.9501*	*0.4380*	PC (11) vs HC (11)	*0.9118*	*0.0589**
GC (20)	0.455	0.262	GC (10) vs HC (10)	*0.9219*	*0.8556*	GC (10) vs HC (10)	*0.8502*	*0.4155*

Early stage: stages 1 and 2; late stage: stages 3 and 4; HC: healthy control; LC: lung cancer; H & N-C: head and neck cancer; TC: testis cancer; TyC: thyroid cancer; BC: breast cancer; CC: colon cancer; PC: pancreatic cancer; GC: gastric cancer

[a] Statistical significance of Age between sex-matched healthy control group and cancer patients

[b] Statistical significance of seroreactivity between age and sex-matched healthy control group and cancer patients

[a and b] one-way analysis of variance (ANOVA) test.

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Table 3. Evaluation of (autoantibody) AAb against recombinant RNA binding domains of nucleolin (rRBDs-NCL) in cancer diagnosis

Cancer	AUC	Sensitivity (%)	Specificity (%)	[a] *p value*	Cut-off	Youden's Index	95%CI	Accuracy (%)	NPV (%)	PPV (%)
LC	0.948	85.0	96.67	*<0.0001*****	0.460	0.816	88.40 to 100	92	90	94.4
H & N-C	0.895	75.0	93.33	*<0.0001*****	0.410	0.683	80.50 to 98.5	86	84.8	88.23
TC	0.883	70.0	93.33	*<0.0001*****	0.405	0.633	78.70 to 97.97	84	82.3	87.5
BC	0.870	70.0	93.33	*<0.0001*****	0.405	0.633	76.98 to 97.02	84	82.3	87.5
CC	0.865	65.0	93.33	*<0.0001*****	0.405	0.583	76.19 to 96.81	82	80	86.6
TyC	0.858	65.0	93.33	*<0.0001*****	0.415	0.583	75.24 to 96.43	82	80	86.6
PC	0.850	65.0	93.33	*<0.0001*****	0.405	0.583	74.28 to 95.89	82	80	86.6
GC	0.800	60.0	93.33	*0.0004****	0.405	0.533	67.43 to 92.73	80	77.7	85.7

HC: healthy control; LC: lung cancer; H & N-C: head and neck cancer; TC: testis cancer; TyC: thyroid cancer; BC: breast cancer; CC: colon cancer; PC: pancreatic cancer; GC: gastric cancer; PPV: positive predictive value; NPV: negative predictive value; YI: Youden's Index

[a] Pearson's correlation coefficient

Furthermore, our findings show that, with the exception of GC, the immunoreactivity of other cancer sera rises significantly with increased proliferative capacity (expression of > 20% Ki-67). Cancer sera from people of different ages did not show a significant difference in immunoreactivity or other clinicopathological indicators, such as ER and PR expression, CK7, and common P53 mutation, as well as tumor differentiation level (Table S1). On the other hand, there was a strong positive correlation between serum levels of anti-rRBDs-NCL AAb and tumor size in the LC (r=0.793, $p<0.0001$), H & N-C (r=0.696, p=0.0006) and TC (r=0.605, p=0.004) (Figures 4a, c and e). Furthermore, serum levels of anti-rRBDs-NCL AAb demonstrated a somewhat favorable correlation with advanced stages of LC (r=0.643, p=0.002) and H & N-C (r=0.629, p=0.002) malignancies, as well as a moderately positive correlation (r=0.544, p=0.013) with the sera of TC patients (Figures 4b, d and f). Further analysis of the serum level of anti-rRBDs-NCL AAb and highly proliferative Ki-67 positive cells revealed a strong positive correlation with LC patients' sera (r=0.722, p=0.0002) (Figure S3a), except for GC, its levels revealed a moderate positive correlation (0.4<r<0.6) (Figure S3b-h).

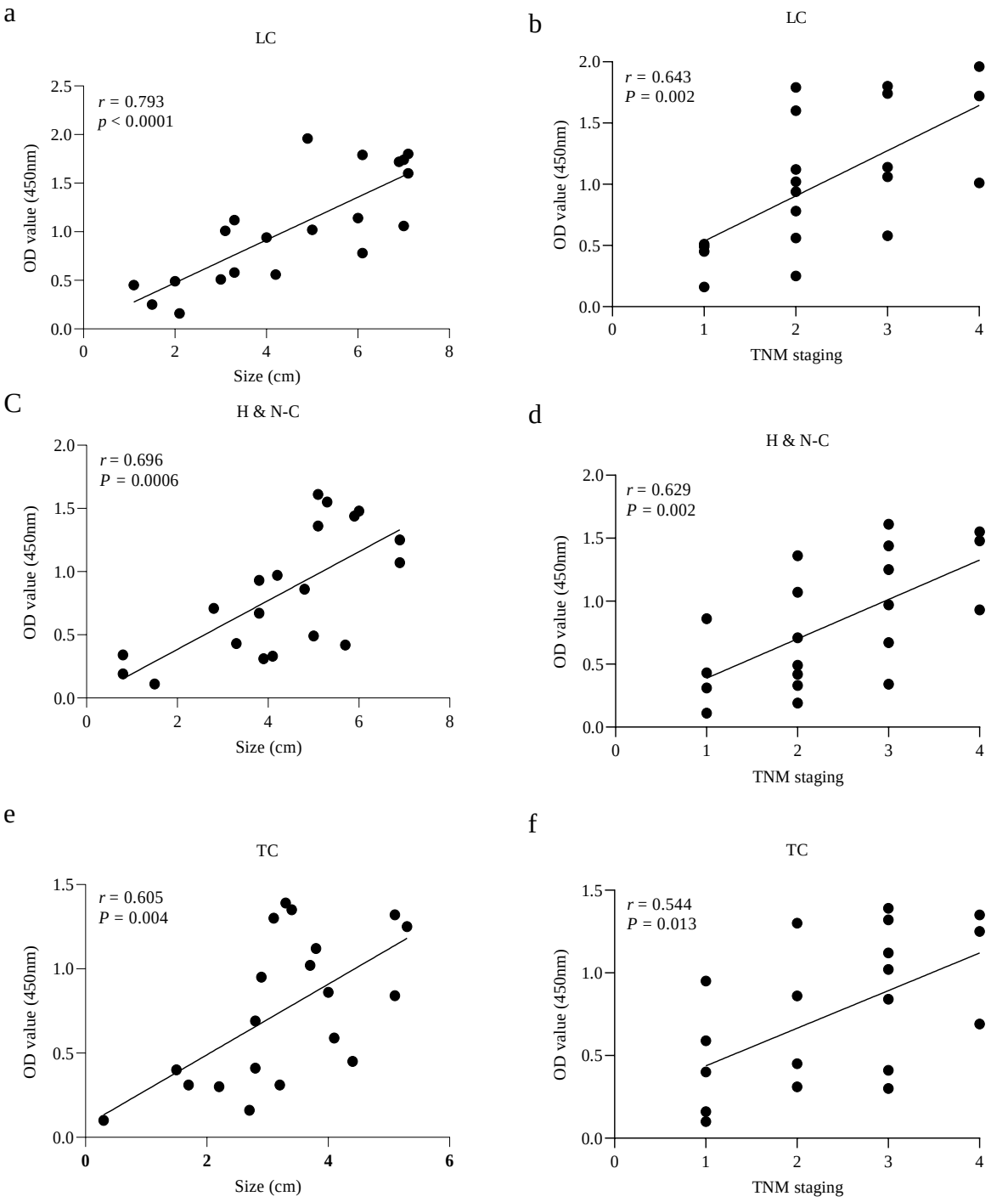

Figure 4. Correlation of autoantibody (AAb) to recombinant RNA binding domains of nucleolin (rRBDs-NCL) protein with tumor size and tumor, node, metastasis (TNM) stage. Scatter graph for serum levels of anti-rRBDs-NCL in tumor size (a, c and e) and tumor, node and metastasis (TNM) stage (b, d, and f). Pearson's test was used to evaluate the correlation of AAb level with tumor size and TNM stage. LC: lung cancer; H & N-C: head and neck cancer; TC: testis cancer.

DISCUSSION

Firstly, using the GEPIA2 and UALCAN datasets, we found higher NCL levels in most cancers relative to normal tissues. This was especially true for BRCA, COAD, HNSC, THCA, PAAD, STAD, TGCT, and LUAD. Moreover, NCL overexpression in certain cancer samples was contradictory with the stage plot in that there was no substantial increase as the disease stage advanced. This may be due to phenotypic alterations related to tumor cell differentiation. Therefore, in this investigation, after the in vitro purification of the 37.9 kDa His-tagged rRBDs-NCL protein, we examined the immunoreactivity of different stages of cancer patients' sera against it using an indirect ELISA technique. The in vitro analysis indicated that, compared to HC, all cancer sera except for GC included highly reactive antibodies against NCL, suggesting that their OD levels were substantially greater than the Youden's index cut-off and could discriminate age and sex matched healthy and malignant sera. The highest interaction was observed between LC, H & N-C and TC sera, which corresponded to GEPIA2 and UALCAN datasets on NCL overexpression and significant levels in these cancers. This may indicate the overproduction and significance of this protein in cancer development and immune system activation to create AAbs. Few studies have linked blood antibodies to tumor or disease prognosis. Dai et al, examined the titers of AAbs against the ECH1 and HNRNPA2B1 autoantigens in individuals with LC. They found that ECH1 AAbs, with a sensitivity of 62.2% and a specificity of 95.5%, were capable of discriminating LC patients from control samples. Additionally, this AAb correlated negatively with tumor size. Also, AAbs against HNRNPA2B1 with sensitivity and specificity of 72.2% and 95.5%, respectively, were able to differentiate patients with LC from healthy control samples and showed a negative relationship with lymph node metastasis. In addition, AAbs against ECH1 with 60.0% sensitivity and 89.3% specificity could detect patients in early stages of LC 2 years before the symptom onset.[19]

Katchman et al, found AAbs against 11 tumor-related antigens, including ICAM3, CTAG2, p53, STYXL1, PVR, POMC, NUDT11, TRIM39, UHMK1, KSR1, and NXF3 in the serum of patients with ovarian cancer that exhibited a 45% sensitivity and 98% specificity in distinguishing high-grade serous ovarian cancer from healthy controls.[20] Also, a study by Zayakin et al, found a 45-AAb signature that could discriminate GC patients from healthy controls with 59% sensitivity and 90% specificity.[21]

In 2015, Mattioni et al, examined the prognostic role of p53 AAb in the sera of non-small cell LC patients. They found a significant relationship between serum p53 AAbs and high levels of p53 expression in cancer cells.[22] In 2010, Yuta Koike et al, evaluated anti-survivin AAb levels in patients with systemic lupus erythematosus (SLE) and observed that IgG AAb levels in serum patients were significantly higher than in healthy controls.[23] Nevertheless, diagnosis via detecting AAb against the NCL protein has been performed in only a few autoimmune diseases such as SLE[24] and multiple sclerosis.[25]

Considering the AUC value of 0.948, it is obvious that the screening of AAbs against RBDs-NCL has a high ability to discriminate LC patients from healthy persons, with a 92% accuracy.

However, there was minimal variation in seroreactivity of other patients. One of the primary causes of the rise in anti-rRBDs-NCL AAbs in the sera of patients with LC is the lymphatic tissue's arrangement.

As a potential immunological organ, lung tissue has a diverse array of diffuse and structured immune tissues and cells that defend the lungs from foreign invaders and malignant cells. On the other hand, the constant drainage of lung lymphatic fluid and the flow of huge volumes of blood via the pulmonary arteries may bring even the tiniest alterations in lung cells to the immune system's attention. As a result of their continual contact with immune system components, lung tumors seem to have a high potential for immunological activation. They are thus prone to developing large amounts of AAbs. Accordingly, to determine whether there is a difference in the interaction of sera from patients with early and late stages of cancer, after dividing the cancer sera into early (TNM stages 1 and 2) and late stages (stages 3 and 4), their interaction with the resulting recombinant protein was investigated.

The intergroup analysis revealed that despite the total decreased immunoreactivity of the sera from early-stage malignancies compared to late-stage ones, only the sera of patients with early-stage LC interacted strongly with the resultant rRBDs-NCL protein compared to healthy sera (p=0.0009). Therefore, it can be concluded that lung tumors can be detected by stimulating the considerable production of anti-NCL AAbs due to their

specific anatomical position and continuous interaction with immune cells from the early stages of the disease. On the other hand, the intragroup analysis revealed a substantial rise in the level of this AAb only in the serum of patients in the late stages of LC, H & N-C, and TC. Further studies on the relationship between the clinicopathological features of tumor specimens and the interaction of corresponding cancerous sera showed that among all the studied cancers, only in LC, H & N-C and TC did the amount of anti-rRBDs-NCL AAbs increase with the tumor size and, consequently, the interaction with our recombinant protein. In fact, it resembles those bigger tumors possibly stimulate the immune system continuously and further with a higher volume of NCL antigen as the tumor size increases.

In an intriguing new study that is align with our results, researchers employed a G-quadruplex structure termed rG4 to discover elevated levels of NCL in LC patients' tissue samples and liquid biopsies. Curiously, it assisted in the diagnosis, prognosis, and treatment response of these individuals.[26] Also, in most of our studied cancers, the faster proliferation of tumor cells, which was associated with higher expression of Ki-67, led to the production of more anti-rRBDs-NCL AAbs. However, it remains unclear if these antibody responses against the NCL protein would benefit the patient. Generally, the prognosis and treatment of tumors depend on CK7, ER, and PR expression by cancer cells. Thus, their excessive expression worsens the patient's health and increases the likelihood of developing malignant and metastatic tumors. Also, mutations at codon 248, located in domain IV of the p53 gene's highly conserved region, are strongly linked to both sporadic malignancies and hereditary cancer syndrome. As such, understanding the functional impact of a mutation at codon 248 is important. Nevertheless, there was no correlation between the expression level of this AAbs and other clinicopathological factors, including age, ER, PR, CK7 and P53 mutation.

Based on existing information, this is the first research to evaluate serum AAb levels against NCL in patients with diverse malignancies. In comparison with the healthy group, cancer patients' sera had the greatest rise in autoantibody levels. This evidence revealed that AAbs to rRBDs-NCL might serve as possible biomarkers for identifying individuals at risk for developing LC. Besides, there is a common temporal shift in positive reactivity throughout the prediagnostic period, so additional work is required to analyze further how to clinically utilize these anti-rRBDs-NCL AAb responses.

Furthermore, in order to enhance the accuracy and precision of the findings, it is advised that the aforementioned research be conducted on a considerably larger population, including age-and gender-matched control samples for each tumor subgroup. On the other hand, proving that the expression of NCL goes up in tumor tissue or liquid biopsies from patients greatly increases the sensitivity and specificity of the quality factor, which were the project's limits.

Consequently, it is probable that the discovery of anti-rRBDs-NCL autoantibodies might benefit the diagnosis and monitoring of the treatment response of patients with these tumors; nevertheless, it seems that the greatest potential for using these AAbs rests in LC sera. To evaluate the status of cancer patients and eliminate false positives and negatives, more research is necessary to determine the specificity and sensitivity of the serum anti-rRBDs-NCL test in a larger study population comprised of healthy sera that have been thoroughly matched. Accordingly, the serological biomarker is a viable method for distinguishing between various diseases, notably cancers, using the least costly methods.

STATEMENT OF ETHICS

The research protocol was developed in accordance with the 1964 Declaration of Helsinki Criteria and was approved by the Ethics Committee (Approved letter number: 1468) of Mazandaran University of Medical Sciences, Sari, Iran (IR. MAZUMS: REC.1397.2700). Also, an informed consent form was signed by each subject.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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