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Original Article

Novel combined tumor autoantibody detection in serological diagnosis of gastric cancer

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Abstract: Objective: Gastric cancer (GC) is a highly prevalent malignancy, yet its early diagnosis rate is generally low. Therefore, we have established a serum-based combined detection method based on tumor autoantibodies aimed at improving the diagnostic rate of gastric cancer. Methods: Through clinical studies, we selected a series of proteins aberrantly expressed in gastric cancer patients, including RalA, Survivin, NY-ESO-1, p53, Cyclin B1, and Koc, and expressed and purified them using prokaryotic expression and nickel column chromatography. Results: The levels of autoantibodies in the serum of gastric cancer patients and healthy individuals were measured using enzyme-linked immunosorbent assay (ELISA), and the diagnostic value of the combined detection of tumor autoantibodies for gastric cancer was evaluated through receiver operating characteristic (ROC) curve analysis. The levels of autoantibodies against RalA, Survivin, NY-ESO-1, p53, and Cyclin B1 in the serum of gastric cancer patients were significantly higher than those in healthy individuals ($P < 0.05$), while the level of Koc showed no significant difference between the two groups ($P > 0.05$), suggesting that Koc may not be suitable for serological diagnosis of gastric cancer. ROC analysis of the combined levels of autoantibodies against RalA, Survivin, NY-ESO-1, p53, and Cyclin B1 for gastric cancer diagnosis achieved a sensitivity of 73.68% and specificity of 78.13%, with an AUC value of 0.8767. Conclusion: The combined tumor autoantibody detection established in this study may have promising potential applications in early screening and diagnosis of gastric cancer.

Keywords: Gastric cancer, antoantibody, serological diagnosis

Introduction

Cancer ranks as the second leading cause of death globally, claiming over 10 million lives annually [1]. GC stands as one of the most prevalent malignant tumor types and ranks third in cancer-related mortalities. Currently, the primary methods for clinically diagnosing GC involve gastroscopy and gastric mucosal tissue biopsy. However, these examinations are challenging to perform, and cause significant harm in patients as well as being costly. Hence, there is a critical need to develop simple and rapid methods for early-stage screening and prognostic diagnosis of GC [2].

Research has revealed that the serum of tumor patients contains certain cell proteins capable of eliciting an autoimmune response, known as tumor-associated antigens (TAAs), with the antibodies induced by these antigens termed tumor-associated autoantibodies [3, 4]. Numerous studies have demonstrated the presence of antibodies against TAAs in samples from patients with various malignancies. For instance, biomarkers such as CA15-3, CA27-29, and carcinoembryonic antigen (CEA) have been employed in the diagnosis of breast cancer [5, 6]. Autoantibodies targeting p53 have been extensively documented across various cancer types, including colorectal cancer (CRC), breast cancer (BC), ovarian cancer, and GC, among others. Their production has primarily been linked to missense mutations and the accumulation of p53 in patients with malignancies [6]. Therefore, autoantibodies targeting TAAs hold promise as potential biomarkers for cancer diagnosis, prognosis, and therapeutic

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drug monitoring [7]. Nevertheless, the systematic investigation and characterization of autoantibodies targeting TAAs in GC remain inadequately addressed. The sensitivity of individual autoantibodies for the diagnosis of GC was found to be low, with a median value of 12.35%. Additionally, the proportion of patients positive for various autoantibodies varies across different stages of GC [8, 9]. Oshima et al. employed ELISA to compare the positivity rates of anti-NY-ESO-1 antibodies between cancer patients and healthy controls. Their findings revealed that the positivity rate of anti-NY-ESO-1 antibodies in serum from esophageal cancer patients reached 31%, significantly higher than that observed in other groups, while it was found to only be 10% in GC cases [10, 11]. Although individual autoantibody detection lacks the specificity and sensitivity required for cancer screening and diagnosis, tumor-associated autoantibodies possess several unique advantages. These include high stability of autoantibodies in the bloodstream, early production and detection of autoantibodies generated by tumors, and the ability to conduct autoantibody-related testing solely by collecting patient serum, thus enabling cancer screening [12]. Therefore, tumor-associated autoantibodies can be utilized for the screening and diagnosis of early-stage cancer patients.

Material and methods

Basic information

For this study, conducted between July 2022 and November 2023, 35 GC patients diagnosed via pathology were selected as the study participants. Among them, there were 21 males and 14 females, with an average age of (61.0±11.68) years. Inclusion criteria were: (1) Age ≥ 18 years; (2) Male or female; (3) Final clinical diagnosis was determined according to the most advanced findings of endoscopy and pathological examination. Staging was performed according to the 8th edition of the gastric cancer TNM staging system, revealing 8 cases in stage I, 2 cases in stage II, 10 cases in stage III, and 15 cases in stage IV [13]. The healthy control individuals were eligible blood donors in the test cohort without any previous malignant disease. All research subjects provided informed consent before participation.

Exclusion criteria were: Diagnosed with tumors other than cancer; A disease state that could not be controlled clinically; Active autoimmune diseases that require systemic treatment within the past 2 years (i.e., the use of disease regulating drugs, corticosteroids, or immunosuppressive drugs); Allogeneic tissue/solid organ transplantation had been performed; Pregnancy status (blood HCG positive) or during a lactation period; Lack of complete clinical data or inability to be followed up; Patients diagnosed with immune dysfunction or infection; According to the researcher's judgment, were not suitable to participate in this study.

Methods

Plasmid construction: Reference full-length gene sequences of p53 (Accession Number: NM_000546.6), NY-ESO-1 (Accession Number: NM_139250.2), RalA (Accession Number: NM_005402.4), Cyclin B1 (Accession Number: NM_031966.4), Koc (Accession Number: U76705.1), and Survivin (Accession Number: HM625836.1) were synthetic gene fragments synthesized from the GenBank database. These synthesized gene fragments were then cloned into expression vectors using BamHI/XhoI double digestion to construct pGEX-6P-1-p53, pET28a-NY-ESO-1, pET28a-RalA, pET28a-cyclin B1, pET28a-KOC, and pET28a-SUMO-Survivin. The plasmids used in this study were all synthesized by Beijing Qingke Biotechnology Co., Ltd.

Construction of recombinant expression strains: After transforming the recombinant products into Escherichia coli Rosetta competent cells, they were cultured in ampicillin-resistant bacterial medium and incubated at 37°C. After 14-16 hours, individual colonies were picked and transferred to test tubes containing 5 mL of ampicillin-resistant bacterial medium. These tubes were then cultured on a shaker at 37°C for another 14 hours before being sent to Genewiz (Shanghai) Co., Ltd. for sequencing identification.

Protein expression and purification: We inoculated 2 mL of sequenced-correct positive clone strains into 200 mL of LB medium supplemented with ampicillin and cultured them at 37°C while shaking at 220 rpm for 2.0-2.5 hours until reaching an OD600 of 0.6-0.8. Next, we added 0.1 μM IPTG to induce expression, and continue incubating for 18-20 hours. After induction, we centrifuged the culture at 8000

rpm, 4°C, for 5 minutes to collect bacterial cells. Following sonication for cell lysis, we centrifuged the lysate at 8000 rpm, 4°C, for 10 minutes, and collected the supernatant. Next we filtered the supernatant using a 0.22 μm filter and proceed with protein purification according to the instructions provided with the His-tagged protein purification resin. Then the purified protein samples were collected and separation of collected protein samples was performed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA detection of autoantibodies: We diluted the recombinant antigen to 2 μg/ml and added it to a 96-well ELISA plate at 100 μl per well, and then incubated it overnight at 4°C. Next, we discarded the liquid from the wells and washed twice with 300 μl PBST. We diluted the BSA to 1% in PBST and added 300 μl per well, and then incubated it at 37°C for 3.5 hours. The liquid was discarded from the wells and washed twice with 300 μl PBST. The serum samples were diluted 10-fold in PBST and we added it to the wells, and then incubated it at 37°C for 1 hour. The liquid was discarded from the wells and washed three times with 300 μl PBST. The HRP-conjugated mouse anti-human IgG secondary antibody was diluted to 1:3000 in PBST containing 1% BSA, adding 100 μl of it per well, and then it was incubated at 37°C for 1 hour. The liquid was discarded from the wells and cells were washed four times with 300 μl PBST. In a dark environment, we added 100 μl TMB to each well and incubated at 37°C for 5 minutes. Fifty μl of stop solution was added per well, then we used an enzyme-linked immunosorbent assay (ELISA) reader to measure the absorbance at 450 nm for each well.

To establish diagnostic criteria for GC utilizing autoantibody markers in this study, we performed statistical analyses to determine critical thresholds. Specifically, clinically confirmed GC patients served as positive references, while healthy individuals acted as negative controls. ROC analysis was conducted on the obtained ELISA data. For individual autoantibody markers, we aimed to select combinations that exhibit specificity exceeding 80% while maintaining high sensitivity in our analytical results. However, in the analysis of multiple marker combinations, we accept a slight reduction in specificity to enhance detection sensitivity.

Statistics

Data analysis and graphing were performed using SPSS 22.00 and GraphPad Prism 9. Normally distributed continuous variables were presented as mean ± standard deviation (SD), and the t-test was used for comparisons between the two groups. Receiver operating characteristic (ROC) curves were constructed, and logistic analysis was conducted to evaluate the diagnostic value of serum tumor autoantibodies for gastric cancer. A p-value < 0.05 was considered statistically significant.

Results

Expression and validation of antigen proteins

Following protein purification, SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining revealed the relative molecular weights of the expressed recombinant proteins: p53 protein at 70.5 kDa, NY-ESO-1 protein at 21.5 kDa, RalA protein at 27.1 kDa, Cyclin B1 protein at 51.9 kDa, Koc protein at 67.3 kDa, and Survivin protein at 30.2 kDa (**Figure 1**).

Levels of the six tumor autoantibodies in the serum of gastric cancer patients and healthy individuals

ELISA was performed to detect serum samples from 35 gastric cancer patients and 35 healthy individuals. The results revealed significantly elevated levels of NY-ESO-1, RalA, Survivin, Koc, and Cyclin B1 autoantibodies in the gastric cancer patient group compared to the healthy control group, with statistical significance ($P < 0.05$). However, there was no significant difference in the levels of Koc autoantibodies between the two groups ($P > 0.05$) (**Figure 2**).

The effectiveness of combined detection of tumor autoantibodies in the diagnosis of gastric cancer

ROC curves were generated based on the levels of RalA, Survivin, NY-ESO-1, p53, and Cyclin B1 autoantibodies as well as the combination of these five tumor antigens (**Figure 3**). Due to the lack of a significant difference in Koc autoantibody levels between the gastric cancer group and the healthy control, Koc autoantibody levels were not included in the combined analysis. The results indicate that the AUC value of the combined analysis (0.7355) was

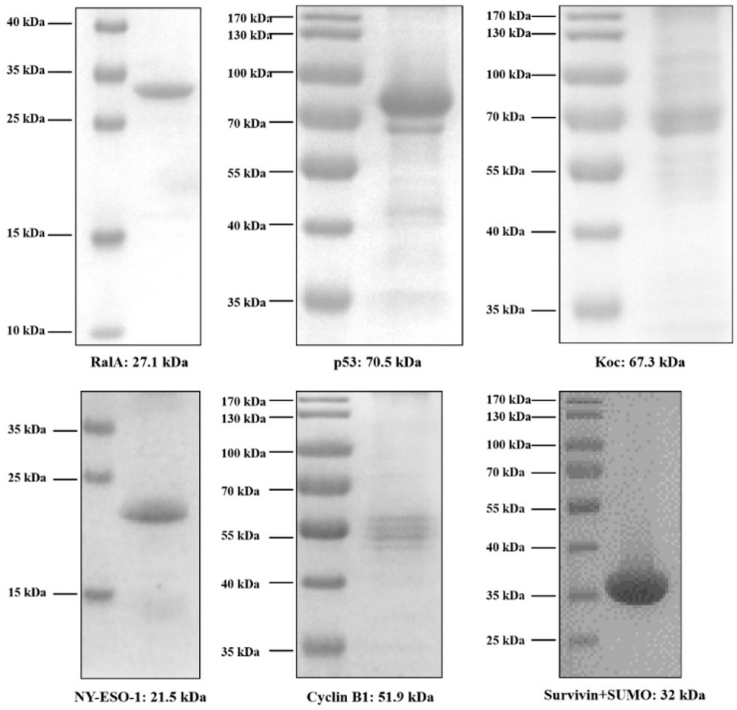

Figure 1. SDS-PAGE gel electrophoresis verification of tumor associated antigens.

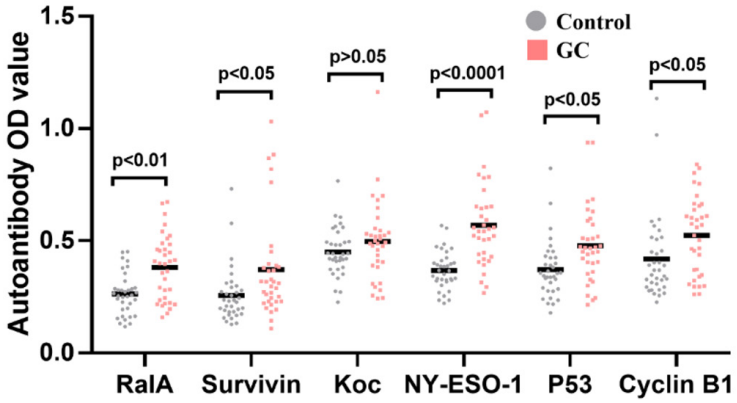

Figure 2. Serum levels of TAAs autoantibodies in the healthy control group and gastric cancer group. GC, gastric cancer.

higher than the AUC values obtained from individual tests of each indicator (**Table 1**), suggesting that the diagnostic efficacy of combined detection surpasses that of individual tests for each indicator. The critical diagnostic values were determined as follows: OD= 0.3916 for RalA, OD=0.3178 for Survivin, OD=0.4948 for NY-ESO-1, OD=0.4363 for p53, and OD=0.4555 for Cyclin B1. The sensitivity and specificity of each indicator are presented in **Table 1**. Although combined detection reduced specificity, it increased sensitivity.

Discussion

GC is one of the most common malignant tumor types that occurs in China, ranking third nationwide in both incidence and mortality, with early-stage GC often presenting no obvious symptoms [14]. Currently, gastroscopy is the conventional method for early screening of GC; however, it is somewhat invasive. Several common serum biomarkers tests (such as CA72-4 and CA19-9) have been utilized for GC detection, but their sensitivity is relatively low, making them unsuitable for clinical monitoring [15]. During cancer development, aberrantly expressed tumor-associated antigens (TAAs) can trigger the immune system to produce corresponding autoantibodies. Increasing evidence suggests that TAA autoantibodies hold potential diagnostic value in GC, colorectal cancer, lung cancer, and various other cancer types [16-18].

In this study, six tumor autoantibodies (p53, NY-ESO-1, Cyclin B1, RalA, Survivin, Koc) were selected for GC diagnosis [19]. Some tumor antigens are expressed at high proportions in tumors, to the extent that they are referred to as “universal antigens”. Antigens expressed in over 50% of tumor types and capable of inducing immune responses include p53, NY-ESO-1, and Survivin. p53, for instance, is a tumor suppressor gene, and mutations in p53 occur in over 50% of malignant tumors [20]. NY-ESO-1 (New York esophageal squamous cell carcinoma 1), a cancer-testis antigen (CTA), is a commonly observed cancer-associated protein found in various aggressive tumors, with widespread expression across multiple cancers [21]. Cyclin B1, also known as

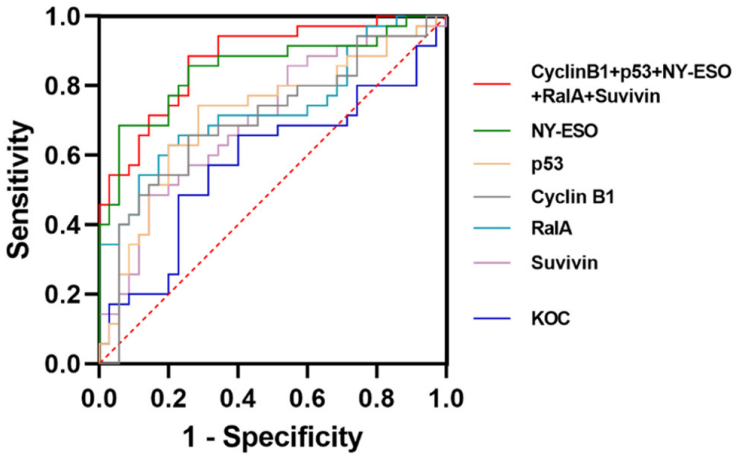

Figure 3. Single and combined prediction of cancer ROC curves by RalA, Survivin, NY-ESO-1, p53, and Cyclin B1.

cell cycle protein B1, is a member of the Cyclin protein family. Several studies have found overexpression of Cyclin B1 in tissues such as esophageal cancer, non-small cell lung cancer, and colorectal cancer. It is believed that the overexpression of Cyclin B1 may play a significant role in the occurrence and progression of cancer [22-24]. RalA is a member of the small GTPase Ras superfamily, and this enzyme is aberrantly induced by the key molecule Ras during the process of epithelial-mesenchymal transition in tumor development, particularly in GC cells [25]. It has been reported that RalA and RalB are crucial cancer phenotypic markers as well as biomarkers for cell migration, invasion, and metastasis [26, 27]. Koc is an insulin-like growth factor II (IGF-II) messenger RNA-binding protein that plays a role in regulating cell growth, differentiation, and post-differentiation functions [18, 28]. Previous studies have found that the positive rate of Koc antibodies in the serum of 777 tumor patients was 11.2%, which is higher than that in normal populations and patients with autoimmune diseases [29]. The positivity rate of the anti-apoptotic protein Survivin in 1137 tumor patients was 8.4%, which was higher than that in the normal population and patients with autoimmune diseases [30]. Although these biomarkers play a significant regulatory role in tumorigenesis and progression, they have been incorporated into numerous studies as candidate molecules for predictive diagnosis, yet it is evident that these tumor markers are not exclusively expressed in GC. This indicates that the combination of these tumor markers or autoantibodies does not necessarily reflect tissue specificity. This represents a critical issue that remains unresolved by existing research, potentially leading to misdiagnoses in clinical practice. To achieve tissue-specific diagnosis, it is imperative to identify biomarkers that are aberrantly expressed solely in GC, such as the recently reported CA72-4 protein [31], which will be a key focus of our future investigations.

The determination of the cutoff value is essential for ensuring detection validity, necessitating a balance between sensitivity and specificity. In this study, for individual autoantibody markers, we prioritized high specificity during screening to ensure detection accuracy, as evidenced by the data statistics showing that nearly all autoantibody markers exhibited specificity greater than 80%. However, in the analysis of multiple marker combinations aimed at enhancing the detection rate of positive samples, we shifted our focus from strictly prioritizing specificity to slightly sacrificing some degree of specificity in order to maximize sensitivity. It is important to note that due to sample size limitations, the cutoff value established in this study may still be subject to further optimization.

In a study by Liu, a combination test was conducted to measure the levels of autoantibodies against Imp1, p62, Koc, p53, and c-myc in the serum of colorectal cancer patients and healthy individuals. The levels of autoantibodies against these five antigens were all higher in colorectal cancer patients compared to normal individuals. ROC analysis indicated a sensitivity of 60.9% and a specificity of 89.7% for the diagnosis of colorectal cancer [32]. In our study, the levels of autoantibodies against RalA, Survivin, NY-ESO-1, p53, and Cyclin B1 in the serum of GC patients were significantly higher than those in healthy individuals ($P < 0.05$), while the level of Koc showed no significant difference between the two groups, suggesting that Koc may not be suitable for diagnosing GC. In Zhu's study, by measuring the levels of autoantibodies against RAE1, the AUC values for GC diagno-

Table 1. Diagnostic value of RalA, Survivin, NY-ESO-1, p53, Cyclin B1 single and combined prediction of cancer

Biomarkers	AUC (95% CI)	*P* value	Sensitivity (95% CI)%	Specificity (95% CI)%	Cutoff
RalA	0.7355 (0.6170-0.8540)	0.0007	54.29 (38.19-69.53)	88.57 (74.05-95.46)	0.3916
Survivin	0.7012 (0.5786-0.8239)	0.0038	48.57 (32.99-64.43)	85.71 (70.62-93.74)	0.3178
NY-ESO-1	0.8498 (0.7563-0.9433)	< 0.0001	68.57 (52.02-81.45)	94.29 (81.39-98.98)	0.4948
p53	0.7159 (0.5925-0.8394)	0.0019	62.86 (46.34-76.83)	80 (64.11-89.96)	0.4363
Cyclin B1	0.7037 (0.5787-0.8287)	0.0034	65.71 (49.15-79.17)	74.29 (57.93-85.84)	0.4555
CyclinB1+p53+NY-ESO+RalA+Survivin	0.8767 (0.7975-0.9560)	< 0.0001	73.68 (73.18-74.18)	78.13 (77.63-78.13)	

sis at different stages were 0.710, 0.745, and 0.804, respectively.

In Qin's study, a recursive partitioning method was used to screen nine tumor-associated antigens (c-Myc, p16, HSPD1, PTEN, p53, NPM1, ENO1, p62, HCC1.4). The sensitivity of detecting was 64.9%, with a specificity of 70.5% and an AUC of 0.737, significantly lower than the combined diagnostic results in this study (sensitivity: 73.68%, specificity: 78.13%). This suggests that merely increasing the number of markers does not necessarily correlate with enhanced sensitivity and specificity.

In conclusion, tumor autoantibody detection holds potential diagnostic value for early screening of GC. However, our study still has some limitations, such as a relatively small sample size, necessitating further validation with a larger sample size in the next step. Additionally, optimization is needed for the selection of tumor-associated proteins for combined analysis. In the future, we hope to achieve more accurate diagnosis of GC through the establishment of diagnostic models.

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Disclosure of conflict of interest

None.

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