ORIGINAL ARTICLE

Detection of *Candida albicans* Sap2 in cancer patient serum samples by an indirect competitive enzyme-linked immunosorbent assay for the diagnosis of candidiasis

Yicun Wang, Xiang Gao, Zhi gang JU, Jingyuan Liu, Shuai Dong, Li Wang

Institute of Genetics and Cytology, School of Life Sciences, Northeast Normal University, 5268 Renmin Street, Changchun, Jilin Province, P.R. China

Address for correspondence:
Dr. Li Wang, Institute of Genetics and Cytology, School of Life Sciences, Northeast Normal University, 5268 Renmin Street, Changchun, Jilin Province - 130 024, P.R. China. E-mail: wangli@nenu.edu.cn

ABSTRACT

Background: The secreted aspartyl proteinases 2 (Sap2) of *Candida albicans* (*C. albicans*) is a potential marker of candididasis. It is a virulence factor associated with adherence and tissue invasion. **Aim:** In order to detect Sap2 in clinical sera, we developed an indirect competitive enzyme-linked immunosorbent assay (ELISA). **Materials and Methods:** Polyclonal antibodies were produced for Sap2 by injecting Sap2 into a New Zealand White inbred rabbit. They could be used at a dilution exceeding 1:1200 in an indirect ELISA, and detected Sap2 concentration up to 1 ng/mL. **Results:** Of the 286 cancer serum samples tested, 16.8% were found as candidiasis. The test was simple and economical to perform and had a level of sensitivity for detection of low-titer positive sera; thus, it may be proven to be of value in epidemiological studies on candidiasis.

KEY WORDS: Antibody, cancer, *Candida albicans*, ELISA, Sap2

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INTRODUCTION

Candida albicans is an opportunistic pathogen of humans with an increasing medical relevance, causing superficial as well as systemic infections in susceptible individuals.[1-3] The secreted aspartyl proteinases (Saps) have been recognized as a virulence-associated trait of the *C. albicans* pathogen. *C. albicans* possesses a gene family encoding Saps that have different functions during infection.[4] It was also found that the Saps were essential for the fungal nutrition process and important for localized and disseminated candidal infections.[5]

Sap2 is one of the common antigens found in the vast majority of *C. albicans* strains. The crystal structure of Sap2 indicates that the *C. albicans* proteinase family is unique among the aspartyl proteinases. It has a very broad spectrum of activity, degrading many human proteins, such as extracellular matrix, keratin and collagen. Not only could this provide essential nitrogen for growth but it could also enhance attachment, colonization and penetration of host tissue by the removal of host barriers.[6,7] It was found that Sap2 could induce immune modulation and had been successfully used as an immunogen to reduce mucosal candidasis in mouse or rat models.[8] People with candidiasis have high titers of antibodies to aspartyl proteinases, especially Sap2, with soluble antigens present in their serum.[5]

Candidiasis did not have significant clinical symptoms; therefore, its diagnosis was difficult. Currently available serological diagnosis lacked the desired sensitivity, specificity or speed.[4] In the absence of an appropriate diagnosis, possible therapy was often delayed and morbidity and mortality was increasing. Patients with hematologic cancer and recipients of marrow transplant with Candida colonization had poor survival.[9] In China, growth of cancer morbidity was noticeable.[4] Candidiasis was a common side-effect of anticancer chemotherapy for malignant cancer patients.[10,11] It could be uncredibility to detect the antibodies to Candida antigens for natural levels of anti-Candida antibodies in normal people.[12] There were many *C. albicans* antigens detected, such as arabintol and enolase, but none has yet achieved broad validation.[13] So far, no test had been described to detect Sap2 for disseminated candidiasis in different kinds of cancer sera.[14]

In this work, we used anti-Sap2 antibodies raised against highly purified recombination Sap2 (rSap2) and showing no cross-reaction with sera ingredients to develop and evaluate an indirect competitive enzyme-linked immunosorbent assay (ELISA) for Sap2 detection in serum from cancer. These results may be useful for cancer adjuvant therapy.

MATERIALS AND METHODS

Serum, Strains and Animals

A total of 286 patients (129 men and157 women) with cancer were enrolled in this study. Those patients were treated at the China-Japan Union Hospital of Jilin University, Changchun, Jilin, from September 2004 to November 2010. Patients ranged in age from 29 to 91 years, with a mean age of 54.8 years. All patients gave informed consent prior to the collection of their serum samples and the samples were stored at –80°C until assayed. The health sera were kindly provided by the Northeast Normal University Affiliated Hospital. Serum samples from a panel of 450 healthy volunteers (248 men and 202 women), with a mean age of 54.4 years (range, 24-82 years), were used to determine the suppression rate and reliability of the ELISA method for the detection of Sap2. Three serum samples with candidiasis, at a mean age of 42.7 years (range, 26-58 years), were used as positive serum. Eleven serum samples with candidiasis were used for sensitivity. All cases have been analyzed by clinicians. PET28a-Sap2 in this work were stored in our laboratory; two male rabbits (2.5 kg) were obtained from the pecific-Pathagen-Free animal Facility of Jilin University, China. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000.

Preparation of the immunogen

The pET28a-Sap2 has been cloned in our lab and then transformed into *Escherichia coli* BL21. The rSap2 protein was prepared and purified in our previous research.[15]

Production of polyclonal antibodies

The rSap2 in sterile 0.05 M phosphate-buffered saline (PBS) was emulsified with an equal volume of complete Freund's adjuvant and injected into a New Zealand White inbred rabbit subcutaneously on the dorsal side at multiple sites. Subsequent immunizations were given with incomplete Freund's adjuvant after two immunizations at weekly intervals followed by a booster. After 2 weeks, the rabbit was bled at weekly intervals and the titer checked by indirect competitive ELISA. Booster injections were given when a drop in the titer was noticed. Serum was stored at –20°C until utilized. The polyclonal antibody was purified by Protein G. To evaluate the cross-reactivity of the antibody with sera, several sera were used for testing the specificity of antibody of Sap2 by Western Blot.

Monitoring antibody titers

An indirect ELISA procedure similar to that reported for aflatoxins was used.[16] Microtiter plate wells (Nunc, Roskilde, Denmark) were coated with rSap2 (3 μg/mL) in 0.2 mol/L sodium carbonate buffer, pH 9.6 (100 μL/well) and incubated overnight in a refrigerator. Subsequent steps were performed at 37°C for 1 h. Antibody was diluted in PBS containing 0.05% Tween 20 (PBS-T) and 0.2% BSA (PBS-T BSA) and held for 1 h at 37°C. Goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (Sigma) were used at a 1:5000 dilution to detect rabbit antibodies attached to Sap2. One hundred microliters of tetra-methyl-benzidine (TMB) solution was added to each well and the plate was incubated for 20 min in the dark at room temperature. The reaction was stopped by the addition of 50 μL of 2 mol/L H_2SO_4. The absorbance (A) in each well was measured at 450 nm in a microtiter plate reader (Wellscan 2 and 3 Dragon Labsystem, Labsystem OY, Finland). All samples were measured in duplicate and the average of the duplicate values was taken as the final read out.

Indirect competitive ELISA

An immunoplate was coated overnight with 100 μL/well of Sap2 (1-10 μg/mL) dissolved in 50 mmol/L carbonate buffer (pH 9.6). The plate was washed three times by PBS-T and blocked with 200 μL of PBS-T containing 1% bovine serum albumin (BSA) at 43°C for 1 h to minimize non-specific adsorption; anti-Sap2 antibodies was the mixture with no Sap2 serum for 2 h in the pipette The plate was washed and incubated with the mixture and incubated with goat anti-rabbit IgG-HRP (1:5000 in PBST, 100 μL/well) for 1 h. Subsequently, the plate was washed three times with PBS-T, 100 μL of TMB solution was added to each well and the plate was incubated for 20 min in the dark at room temperature. The reaction was stopped by the addition of 50 μL of 2 mol/L H_2SO_4. The absorbance at 450 nm and 620 nm was then read on a microplate reader. The appropriate coating rSap2 concentration and anti-Sap2 antibody concentration were selected by a microplate reader.

Analysis of the serum samples

The rSap2 standards in 100 μL volume, ranging from 1 ng/mL to 4096 ng/mL, were prepared in no Sap2 sera. Test serum samples were diluted to 1:2 in PBS-T BSA. A 100 μL aliquot of each sample was added to a well containing 50 μL of antibody. Standard curves were obtained by plotting log2 values of Sap2 standards against inhibition rate at A450 and A620. Inhibition levels for the test and control sera were calculated with inhibition rate (IR); inhibition rate of Sap2 in the sample was determined using a formula. The positive sera were defined as above cut-off value. Samples giving IR greater than 2 standard deviations above the mean for samples obtained from the healthy control were considered positive.[14]

$$IR\,(sample) = 1 - [A_{450\text{-}620}(sample + PAbs\ anti\text{-}Sap2)/A_{450\text{-}620}PAbs]$$

Statistical method

For determination of the inter-assay precision, the mean coefficients of variation (CV) were based on three samples performed on 3 days. Each day, a sample was assayed on four separate plates. All calculations were performed using Microsoft Excel. Each assay contained two replicates of each sample. The lower limit of detection (LLD) for the indirect assay was given as three-times the standard deviation plus the black ground of blank wells. The student t-test was used. A value of $P < 0.05$ was considered significant.

RESULTS

Production of antibody

The protocol used for immunization gave an antibody titer of 1:132000, 8 weeks after initiation of the immunization. To determine the cross-reaction of Sap2 with serum, it was decided to test it against the rabbit serum against Sap2, the negative serum, the candidiasis-positive serum and the healthy control serum. These were used in Western Blot. The antibody was specific to rSap2 [Figure 1].

Optimization of the indirect competitive ELISA

The rSap2 was tested at concentrations ranging from 1 μg/mL to 10 μg/mL. In independent tests, an rSap2 concentration of 3 μg/mL was found to be optimum for coating the plates. Antibody at a dilution of 1:1200 gave optimum results.

Stability and sensitivity of the indirect competitive ELISA

The stability of the assay was tested by running the ELISA procedures for three individual times and four replicates on a single day [Table 1]. The relative standard deviation (RSD) of the measured absorbance for replicates at each standard concentration was from 0.7% to 5.2%, indicating the stability of the ELISA. Figure 2 shows the calibration curve generated. In this study, the calibration curve was constructed from 1 to 4096 ng/mL, with a linear range from 1 to 4096 ng/mL ($r^2 = 0.99$), suggesting that the established ELISA was highly sensitive.

Analysis of the serum samples

The positive inhibition rate was determined as 0.33 and the Sap2 concentration threshold was 36.75 ng/mL. The indirect competitive ELISA assay has been applied to determine Sap2 in cancer patients. The information of cancer patients is shown in Table 2. Of the 450 healthy controls, 22 were positive for serum Sap2 in indirect competitive ELISA. Furthermore, we assayed the presence of Sap2 in sera from 105 patients with lung cancer, 67 patients with breast cancer, 40 patients with intestinal cancer, etc. Among 105 patients with lung cancer, 15 (14.2%) patients were positive for serum Sap2. Fifteen (22.3%) patients with breast cancer were positive for serum Sap2, six (15%) patients with intestinal cancer were positive for Sap2, six (27.3%) patients with cervical carcinoma were positive for Sap2, one (5.9%) patient with hepatocarcinoma was positive for Sap2, two (16.7%) patients with gastric cancer were positive for Sap2 and only one (10%) patient with esophageal cancer was positive for Sap2. Two positive sera that were infected with *C. albicans* have been demonstrated by the clinic. Because the other patients left the hospital, we did not determine their real status.

Sera from 450 healthy controls had an inhibition rate between 0 and 0.4 with the indirect competitive ELISA, while the sera of cancer patients ($n = 286$) had an inhibition rate of 0-0.7 [Figure 3]. The percentage of positive patients from a variety of cancer patients is shown in Figure 4. In a separate study, patients with cervical carcinoma and breast cancer had a high proportion of candidiasis positivity, while patients with hepatocarcinoma had a low proportion ($P < 0.05$) of candidiasis positivity.

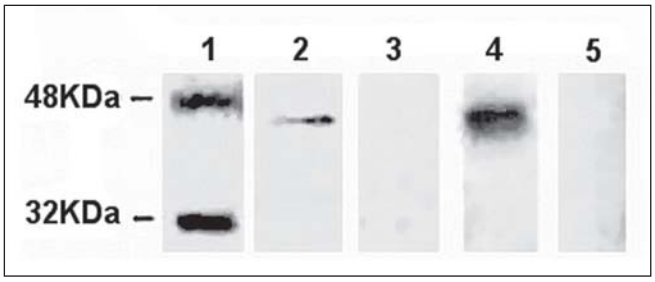

Figure 1: The immunity identification of recombination Sap2 (rSap2). Protein was separated by 12% sodium dodecyl sulfate — polyacrylamide gel electrophoresis and was transferred onto a nitrocellulose membrane. Immunized serum, negative serum, candidiasis serum and normal serum were used to react with protein. The results showed that the recombinant protein can be recognized by antibody in immunized serum and candidiasis serum. However, no antibody in negative serum and normal serum can react with rSap2. Lane 1, marker; lane 2, rSap2 protein against candidiasis serum; lane 3, rSap2 against normal serum; lane 4, rSap2 protein against immunized serum; lane 5, rSap2 protein against negative serum

Table 1: Intra- and inter-assay variation

	Intra-assay		*Inter-assay*	
Added rSap2 into sera (ng/mL)	*Number of replications*	*CV (%)*	*Number of replications*	*CV (%)*
1	4	4.4	3	5.2
2	4	3	3	3.2
4	4	1.4	3	1.6
8	4	1.6	3	1.8
16	4	1.7	3	2
32	4	1.7	3	1.9
64	4	2.1	3	2.1
128	4	1.2	3	1.4
256	4	1.5	3	1.2
512	4	1.2	3	1.1
1024	4	0.7	3	0.9
2048	4	1.3	3	1.9
4096	4	1.4	3	1.6

Intra-assay variation was calculated from four replicates on a single day. Inter-assay variation was calculated from triplicates on three different days. CV: Coefficient of variation

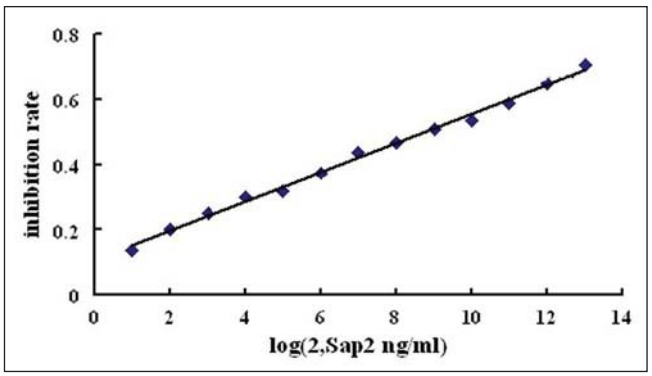

Figure 2: Standard curves for Sap2 by indirect competitive enzyme-linked immunosorbent assay at 1:1200 dilutions of antibodies

DISCUSSION

C. albicans is a harmless commensal pathogen in most healthy people, but it can cause life-threatening systemic infections in patients with compromised immune functions.[17] Sap2 of *C. albicans* has been implicated as an early-onset infection factor.[18-20] It was useful for the diagnosis of candidiasis.

Sap2 has been detected in the serum of animal models.[21] The overall test sensitivity was 83%, but no Sap2 detection was described in the sera of the candidiasis-susceptible population. We have developed a new ELISA to detect the anti-Sap antibody levels before, and the sensitivity and specificity were 77% and 88%.[22] In this study, we prepared the purified rSap2 protein as a coating antigen of ELISA. The detection specificity, defined as the detected negative percentage of the healthy samples, reached 95% (428/450), and the sensitivity, defined as the detected percentage of the candidiasis samples, was 82% (9/11).

Sap2 was detected in 450 healthy patients and 286 cancer patients susceptible for candidiasis. Among the 286 patients, 48 (16.8%) patients were positive for serum Sap2. The level of serum Sap2 in cancer patients was higher than that in health controls, which suggested that cancer patients were the candidiasis-susceptible population and that serum Sap2 could be used as a candidiasis detection marker. The results may be dependent on the differences in the population investigated or the number of samples assayed.

C. albicans colonizes human skin and tracts of the gastrointestinal and urogential systems.[23-25] Nearly three-quarters of all healthy women experience at least one vaginal yeast infection, and about 5% endure recurrent bouts of disease.[26] Candidiasis may have an association with a variety of malignancies.[16,27,28] The study investigated the connection between serum Sap2 and different kinds of cancer. In this study, 22 (27.3%) patients with cervical carcinoma were positive for serum Sap2, six (25%) patients with intestinal cancer and 15 (22.3%) patients with breast cancer were positive for Sap2, while only one (5.9%) patient with hepatocarcinoma was positive for Sap2. By contrast, different

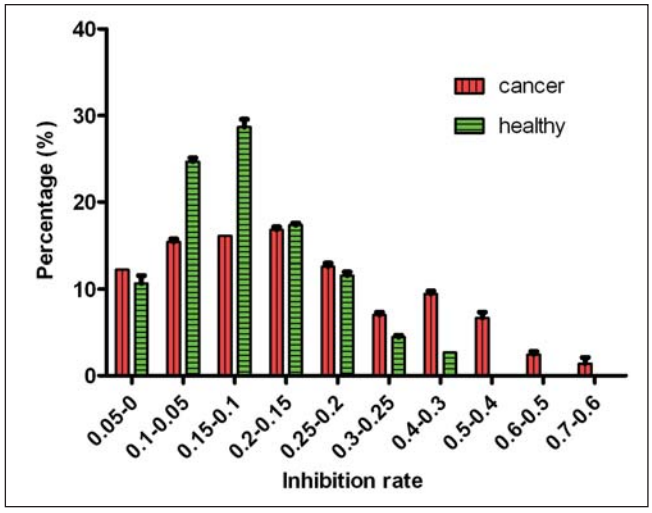

Figure 3: Frequency distribution of inhibition rate obtained with indirect competitive enzyme-linked immunosorbent assay for sera collected from 286 cancer patients and 450 healthy controls. Each data point represents the mean for two independent experiments ± standard deviation

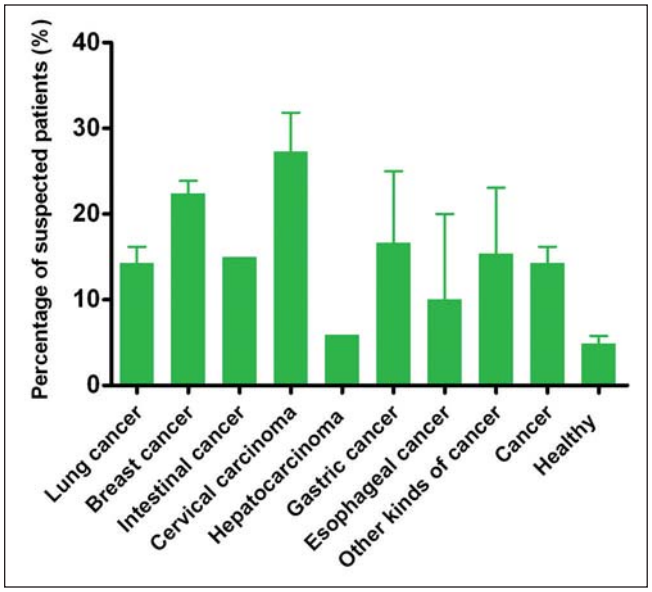

Figure 4: Percentage of Sap2-positive patients obtained with indirect competitive enzyme-linked immunosorbent assay for sera collected from cancer patients and healthy controls. Each data point represents the mean for two independent experiments ± standard deviation

Table 2: Diagnosis of Sap2 in susceptible patients

	Number of people	*Percentage of positive patients (%)*	*Mean inhibition rate*	*Mean Sap2 levels (ng/mL)*	*Mean age*	*Number of male and female*
Lung cancer	105	14.2	0.17±0.14	2.46	59.23±11.2	57/48
Breast cancer	67	22.3	0.22±0.17	4.99	48.4±9.6	3/64
Intestinal cancer	40	15	0.2±0.13	4.24	56.1±10.5	30/10
Cervical cancer	22	27.3	0.23±0.16	6.74	46.3±6.71	0/22
Hepatocarcinoma	17	5.9	0.16±0.08	2.28	56.3±10.53	2/15
Gastric cancer	12	16.7	0.15±0.11	1.96	63.6±9.78	10/2
Esophageal cancer	10	10	0.18±0.07	3.58	54.8±4.68	9/1
Other kinds of cancer	13	2	0.28±0.07	14.58	43.9±13.49	5/8
Cancer	286	16.8	0.19±0.15	3.93	54.8±11.6	129/157

The results of ELISA for sera from cancer patients were shown. The standard was defined as the cut-off value

kinds of cancer had significantly different chances of *C. albicans* infection ($P < 0.05$). The serum Sap2 was associated with a variety of malignancy, suggesting that patients of gynecologic neoplasm and tumors of the intestine are probably predisposed to be infected by *C. albicans*. In conclusion, serum Sap2 for 286 Chinese patients with cancer was detected using the rSap2 and the polyclonal antibody. These results demonstrated that the ELISA method could confirm the Sap2 concentration in patients. This research may provide a useful method to complement conventional clinical diagnosis for patients with cancer.

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