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NY-ESO-1 autoantibody as a tumor-specific biomarker for esophageal cancer: Screening in 1969 patients with various cancers

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Abstract

Background: NY-ESO-1 has been cloned as an esophageal cancer specific antigen, and serum autoantibodies against this antigen have been detected in patients with esophageal cancer. However, the presence of serum NY-ESO-1 antibodies has not been well documented in a large series of patients with different types of cancers. **Patients and methods:** A total of 1969 cancer patients [esophageal cancer (n = 172), lung cancer (n = 269), hepatocellular carcinoma (n = 91), prostate cancer (n = 358), gastric cancer (n = 313), colorectal cancer (n = 262), breast cancer (n = 365)] and 74 healthy individuals were included in the study. Serum samples were obtained from the patients, before treatment, and analyzed using originally developed enzyme-linked immunosorbent assay systems. A cut-off optical density value, determined as the mean + 3 standard deviations of sera from the healthy controls, was fixed at 0.165.

Results: The positive rates of serum NY-ESO-1 antibodies in the patients and healthy controls were as follows; esophageal cancer (31.3%), lung cancer (13.0%), hepatocellular carcinoma (10.5%), prostate cancer (10.2%), gastric cancer (10.1%), colorectal cancer (8.0%), breast cancer (7.3%), and healthy controls (0%). The positive rate of serum NY-ESO-1 antibodies in patients with esophageal cancer was significantly higher than that in the other patients.

Conclusions: The positive rate of serum NY-ESO-1 antibodies was significantly higher in patients with esophageal cancer. Based on its high specificity and sensitivity, the NY-ESO-1 antibody may be the first choice as a serum marker in esophageal cancer.

Key words: NY-ESO-1, serum autoantibody, tumor marker, esophageal cancer, enzyme-linked immunosorbent assay

List of abbreviations used

CEA, carcinoembryonic antigen

SCC-Ag, squamous cell carcinoma antigen

SCC, squamous cell carcinoma

OD, optical density

Competing interests

Hideaki Shimada has received research grant from Medical & Biological Laboratories Co., Ltd.

Introduction

The serum tumor markers, carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC-Ag), and serum p53 antibody, have been approved for use in patients with esophageal squamous cell carcinoma (SCC) [1, 2]; however, due to the lack of expression of these markers in several tumors, they are not suitable for use in early cancer diagnosis.. Moreover, these tumor markers are not specific for esophageal SCC. The immune system is capable of recognizing tumor cells, very early on [3, 4], including a mutated version of p53 tumor suppressor protein over-expressed in esophageal cancer [5]. NY-ESO-1 was cloned as an esophageal SCC specific antigen [6], in order to induce serum antibodies during the early stages of esophageal cancer [7]. Recently, patients with several other types of cancers [8], lung cancer [9, 10], breast cancer [11], hepatocellular carcinoma [12, 13], gastric cancer [14], prostate cancer [15], and colorectal cancer [16], also produce serum anti-NY-ESO-1 antibodies. However, most of these studies involved small number of patients, with only one type of cancer. Therefore, in the present study, 1969 patients with various types of cancers were evaluated for the presence of serum anti-NY-ESO-1 antibodies in patients with esophageal SCC.

Patients and methods

Collection of sera

Before starting the treatment, sera was obtained from a total of 1969 patients with various types of cancer involving the esophagus (n = 176), stomach (n = 348), large intestine (n = 289), liver (n = 114), prostate (n = 372), breast (n = 386), and lung (n = 284), and 74 healthy donors. Each serum sample was centrifuged at 3,000 x g for 5 min, and the resulting supernatant was stored at −80°C

until further use. Repeated thawing and freezing of the samples were avoided. This study was approved by the institutional review boards of Chiba Cancer Center (#21-26) and Toho University School of Medicine (#22-112, #22-047).

Purification of recombinant NY-ESO-1 protein

For the expression and purification of recombinant protein, full-length NY-ESO-1 cDNA (GenBank accession Number: NM 001327) obtained from human testis was amplified by polymerase chain reaction. The amplified gene was inserted between the *Bam*HI and *Xho*I sites of pGEX-4T-1 plasmid (GE Healthcare UL Ltd, Buckinghamshire, UK), expressed as a N-terminal GST fusion protein. Because of the insolubility of the recombinant NY-ESO-1 protein expressed in *E.coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA), the homogenized inclusion body was boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and fractionated on SDS-PAGE. NY-ESO-1 antigen was purified by eluting from the gels. The expression and purity of recombinant protein were examined in 12.5% SDS-PAGE. The integrity of the genes inserted into the plasmids was confirmed by DNA sequencing analysis.

Enzyme-linked immunosorbent assay to detect serum NY-ESO-1 antibodies

Sera from a total of 1969 patients and 74 healthy controls were analyzed by enzyme-linked immunosorbent assay as described previously [6]. Briefly, purified recombinant proteins were coated on to 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY). NY-ESO-1 was diluted in phosphate buffered saline (PBS) to a final concentration of 1.25 μg/ml, and added to the plates (100 μl/well), which were then incubated overnight at 4°C. PBS was used as control. After two washes with PBS, the proteins were blocked with 200 μl of PBS containing 1% bovine serum albumin (BSA) and 5% sucrose at room temperature for 3 hours. All human sera were diluted (1:100) in PBS

containing 0.15% Tween 20, 1% casein, and 0.2 mg/ml *E.coli* extract. Then, 100 µl of the diluted sera was added to each NY-ESO-1 or PBS coated well and incubated at room temperature, 250 rpm for 60 min. After washing with PBS containing 0.05% Tween-20 (PBST) 4 times, 100 µl of horseradish peroxidase-conjugated antihuman IgG (1:5000; MBL, Nagoya, Japan) diluted in 20 mM HEPES, 135 mM NaCl, 1% BSA, and 0.1% hydroxiphenylacetic acid was added to each well as a secondary antibody and incubated at room temperature, 250 rpm for 60 min. The wells were washed four times, with PBST buffer, and autoantibodies were detected by addition of 100 µl of 3, 3′, 5, 5′-tetramethylbenzidine substrate. After incubation at room temperature for 30 min, the reaction was stopped by the addition of 0.25 N sulfuric acid (100 µl/well). Absorbance value was measured at 450 nm using a SUNRISE Microplate Reader (Tecan Japan Co., Ltd, Kawasaki, Japan). NY-ESO-1 signals were evaluated by calculating the difference in absorbance values between the wells containing NY-ESO-1 and PBS.

Statistical analyses

Fisher's exact (two-sided) probability test and the Mann–Whitney U test were used to determine the significant differences between the two groups. All statistical analyses were carried out using the Stat View 5.0J program for Windows (SAS Institute Inc., Cary, NC). P values lower than 0.05 were considered statistically significant.

Results

Serum titers of anti-NY-ESO-1 antibodies

Optical density (OD) values (mean and standard deviation) of serum anti-NY-ESO-1 antibodies were significantly higher ($P < 0.001$) in patients with esophageal SCC (0.625 ± 1.260) than in the healthy donors (Figure 1). Mean OD values in other types of

cancers were as follows: 0.232 in lung cancer, 0.053 in liver cancer, 0.229 in prostate cancer, 0.156 in gastric cancer, 0.122 in colorectal cancer, and 0.119 in breast cancer.

Positive rates of serum anti-NY-ESO-1 antibodies

Serum anti-NY-ESO-1 antibody levels were divided into two groups; normal OD values that were below the border level of 0.165 (calculated as the mean + 3 standard deviations of the values in healthy donors) and abnormal, or positive, values that were higher than 0.165. The positive rate of serum anti-NY-ESO-1 antibody level in patients with esophageal SCC was 32.0% and was the highest among all the cancer types analyzed in this study (Figure 2). The positive rate of the antibody was higher than 10% in patients with lung cancer, hepatocellular carcinoma, gastric cancer, and prostate cancer, whereas the rates were found to be less than 10% in patients with colorectal and breast cancer

Discussion

In the present study, 1969 patients with various cancers were evaluated for the presence of serum anti-NY-ESO-1 antibodies. Patients with esophageal cancer demonstrated significantly higher positive rates for the antibodies. Interestingly, the distribution of serum OD values from liver and breast cancer patients, in the present study, did not show normal distribution compared with that from other types of cancers. As only a few reports have analyzed the distribution of autoantibody titer data so far [13], further studies evaluating its clinical significance, is warranted. In the present study, the positive rate of serum anti-NY-ESO-1 antibodies in patients with esophageal SCC was 32.0%, which is comparable to that reported in another recent study [7]. The positive

rate of this antibody has been well analyzed in patients with lung cancer and reported to be from 6% to 13% [9, 10]. Other types of cancers have also demonstrated similar positive rates of around 5% to 10% [8-16].

Although the positive rate of serum anti-NY-ESO-1 antibodies in patients with esophageal carcinoma was not significantly higher than that in patients with SCC-Ag [17], it was found to be 0% in the healthy donors, indicating very low false-positive rates. Moreover, SCC-Ag usually presents with high positive rates in patients with other types of cancers, such as lung squamous cell carcinomas [18]. Serum p53 antibodies also showed high positive rates in patients with colorectal and breast cancer [19]. Therefore, based on specificity to esophageal carcinoma, serum anti-NY-ESO-1 antibodies seem to be the first choice as serum tumor marker in esophageal SCC.

Recent clinical trials have shown promising treatment and antibody responses to NY-ESO-1 vaccine, in patients with advanced esophageal SCC [20]. Immunoreactivity of NY-ESO-1 in tumor specimens was one of the essential criteria for selection, in those studies. Due to the association between serum NY-ESO-1 antibodies and immunoreactivity, they can be used as an alternative biomarker for such vaccine treatments in future trials.

In conclusion, of the 1969 patients with various types of cancers, in the present study, those with esophageal cancer showed significantly higher positive rates of serum NY-ESO-1 antibodies. Based on its high specificity and sensitivity, the NY-ESO-1 antibody may be the first choice as a serum marker in esophageal cancer. However, prospective multi-institutional studies comparing the sensitivity and specificity of this antibody with other conventional serum tumor markers, including CEA, SCC-Ag, and serum p53 antibodies are mandatory.

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Figure legends

Figure 1. Graph showing distribution of antibody titers in patients with various solid tumors. A total of 1969 patients with cancer and 74 healthy controls were evaluated for serum anti-NY-ESO-1 autoantibody titers before treatment. The position of a cut-off value (mean of healthy controls + 3 standard deviations = 0.165) is shown.

Figure 2. Bar graph showing positive rates of serum NY-ESO-1 antibodies in patients with different types of cancer.

Supplemental Figure S1. Purification of recombinant NY-ESO-1 proteins

For the expression and purification of recombinant protein, full-length of NY-ESO-1 cDNA from human testis was amplified by PCR. Amplified gene was inserted between *Bam*HI and *Xho*I sites of pGEX-4T-1 plasmid and expressed as N-terminal GST fusion protein. The homogenized inclusion body was boiled in SDS-PAGE sample buffer and fractionated on SDS-PAGE. NY-ESO-1 antigen was purified by eluting from the gels. The expression and purity of recombinant protein were examined in 12.5% SDS-PAGE. All constructed plasmids confirmed that these inserted genes were correct by DNA sequencing analysis.

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