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ORIGINAL ARTICLE

Multi-panel assay of serum autoantibodies in colorectal cancer

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Abstract

Background Although serum p53 autoantibodies (s-p53-Abs) are induced even in the early stages of colorectal cancer, their positive rate is only approximately 20%. Therefore, we assessed the possibility of using other serum autoantibodies to increase the positive rates for detecting colorectal cancer.

Methods Autoantibodies against 17 tumor antigens (p53, RalA, HSP70, Galectin1, KM-HN-1, NY-ESO-1, p90, Sui1, HSP40, CyclinB1, HCC-22-5, c-myc, PrxVI, VEGF, HCA25a, p62, and Annexin II) were evaluated in 279 patients with colorectal cancer and 74 healthy controls. Cutoff values were fixed at mean + 3 standard deviations of serum titers in healthy controls.

Results Autoantibodies with the highest positive rates were p53 (20%), RalA (14%), HSP70 (12%), and Galectin1 (11%). Combination assays using multiple autoantibodies increased the positive rates based on the number of autoantibodies used. Positive rates of 56, 62, 66, 71, and 73% were obtained with 6, 9, 11, 14, and 17 antibodies, respectively, for the overall disease. Moreover, these autoantibodies showed relatively high positive rates even during stage 0/I disease (55 and 70% with 6 and 17 antibodies, respectively).

Conclusion The measurement of set of 17 autoantibodies allowed autoantibody profiling in patients with colorectal cancer. The combination assay of six tumor antigens (p53, RalA, HSP70, Galectin1, KM-HN-1, and NY-ESO-1) achieved a positive rate of 56%. Such high positive rates will be helpful for detecting colorectal cancer regardless of tumor stages.

Keywords Colorectal cancer · Autoantibody · p53 · RalA · HSP70

Introduction

Serum tumor markers have been reported to be useful for monitoring treatment responses and/or recurrence after colorectal surgery [1, 2]. The immune system recognizes tumor cells even in the early stages of cancer [3], including a mutated version of the p53 tumor suppressor protein that is overexpressed in colorectal cancer [4]. Although the combination of carcinoembryonic antigen, carbohydrate antigen 19-9, and serum p53 antibodies (s-p53-Abs) had a positive rate of approximately 60%, the remaining patients tested triple negative for this combination [4, 5].

Such autoantibodies have been considered useful for detecting early stage cancers [6–8]. Although single autoantibodies are unable to achieve sufficient sensitivity levels, the combined use of multiple autoantibodies may improve sensitivity [9, 10]. Screening of multiple autoantibodies against a panel of tumor-associated antigens (TAAs) leads to the development of new markers. Recently, Chen et al.

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[11] analyzed 64 autoantibodies in colorectal cancer using multiplex bead-based serological assays. Negm et al. [12] analyzed 32 autoantibodies in colorectal cancer from three different cohorts. Both of the aforementioned studies showed the possibility of the combined use of multiple autoantibodies. In total, 17 autoantibodies were screened in patients with colorectal cancer. We had recently developed a panel of 17 TAAs for gastric cancer or hepatocellular carcinoma [3, 13]. This TAA panel included p53, RalA [14], HSP70, Galectin1 [15], KM-HN-1, NY-ESO-1 [16], p90, Sui1, HSP40, CyclinB1, HCC-22-5, c-myc, PrxVI, VEGF, HCA25a, p62, and Annexin II.

In the present study, autoantibody titers were screened against the panel of 17 TAAs in patients with colorectal cancer to verify the appropriate combination of autoantibodies for detecting colorectal cancer.

Patients and methods

Patients

Samples from 279 patients with colorectal cancer (100 patients from BioBank and 179 patients from Chiba Cancer Center) were analyzed for serum antibodies against 17 TAAs. In total, 74 healthy control samples were also obtained. Healthy volunteers were not screened by colonoscopy. BioBank samples were anonymous, with no other patient information except disease stage (number of cases at stage I/II: 50/50). Among the 179 surgically treated patients from Chiba Cancer Center, 112 were men (63%) and 67 were women (37%), with a median age of 63 (range 27–84) years. The TNM stage for colorectal cancer was identified based on the general rules for the clinical and pathological study of primary colorectal cancer (7th edition) [17]. Among the 179 patients, 26, 34, 24, 42, and 38 had stage 0, I, II, III, and IV disease, respectively, whereas 15 had recurrence. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Chiba Cancer Center (No. 21–26) and Toho University School of Medicine (Nos. 22–112 and 22–047). This clinical study has been registered in the UMIN Clinical Trials Registry (UMIN000014530).

Purification of recombinant TAAs and ELISA for detecting serum antibodies

For purifying recombinant protein, full-length cDNA of the TAAs p53 (GenBank accession number: AB082923), RalA (AF493910), HSP70 (NM 004134), Galectin1 (NM_002305), KM-HN-1 (NM152775), NY-ESO-1 (AJ003149), p90 (AF334474), Sui1 (NM_005801), HSP40 (NM 006145), CyclinB1 (NM 031966), HCC-22-5 (NM 004683), c-myc (K02276), PrxVI (NM 004905), VEGF (AF486837), HCA25a (AF469043), p62 (AF057352), and Annexin II (NM 001002858) were amplified via polymerase chain reaction, as previously reported [3, 13]. The recombinant p53 protein was expressed in Sf21 cells, using the Bac-to-Bac® Baculovirus Expression System (invitrogen, Carlsbad, CA, USA). The cells were dissolved in radio-immunoprecipitation assay (RIPA) buffer, and the target protein was fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For the other genes, each gene was inserted into a plasmid expressed as a tag, and these recombinant proteins were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA, USA) and were dissolved in phosphate-buffered saline (PBS). TAA extract was added to Ni Sepharose™ 6 Fast Flow (GE Healthcare UL, Buckinghamshire, UK), and the column was washed with 50 mM imidazole in PBS. Purified TAA recombinant proteins were eluted with 200 mM imidazole in PBS. NY-ESO-1 was expressed in BL21-CodonPlus (DE3)-RIL and dissolved in PBS. The antigen was purified by eluting from the gels similar to p53. The expression and purity of the recombinant proteins were examined with 12.5% SDS-PAGE. DNA sequencing analysis confirmed whether the correct gene had been inserted into the constructed plasmid [3].

Serum samples from patients and healthy controls were analyzed via ELISA, as previously described [6]. In brief, purified recombinant proteins were coated on 96-well microtiter plates (Maxisorp; Nunc, Rochester, NY, USA). TAAs were diluted in PBS to final concentrations of 0.5–5.0 μg/mL and added to the plates for fixation. Sample sera diluted 100-fold were added to TAA- or PBS-coated wells and incubated at room temperature for 60 min at 250 rpm. Subsequently, horseradish peroxidase-conjugated antihuman IgG (1:5000; MBL, Nagoya, Japan), a secondary antibody, was added to detect autoantibodies. Absorbance was measured at 450 nm using a SUNRISE Microplate Reader (Tecan Japan Co., Ltd, Kawasaki, Japan). TAA signals were evaluated by calculating the difference in absorbance between wells containing TAAs and those containing PBS [3].

Cutoff values for serum antibody titers

Optimized antibody titer cutoff values and a standard cutoff value corresponding to a value greater than mean + 3 standard deviations (SDs) of the 74 healthy control cohorts were used for each of the 17 antibodies while maintaining a specificity of > 95% [18, 19]. Details regarding the 3 SD values of each autoantibody titer have been previously described [13]. The specificity of the assay was determined as the percentage of healthy controls showing negative results.

Statistical analysis

Data were expressed as mean ± SD. Comparisons of paired groups were performed using Fisher's exact test. Statistical analyses were performed using EZR statistical software [20]. Two-tailed p values of < 0.05 were considered statistically significant.

Results

Positive rates of serum autoantibodies in patients with colorectal cancer

Positive rates of each autoantibody against TAAs in patients with colorectal cancer are shown in Fig. 1. p53 (20%) had the highest positive rate, followed by RalA (14%), HSP70 (12%), and Galectin1 (11%). Although VEGF is one of the key target molecules in colorectal cancer, its positive rate was only 4.7% (Fig. 1). Combination assays using multiple autoantibodies increased the positive rates based on the number of autoantibodies used (Fig. 2a). False positive rates also increased based on the number of autoantibodies used. The correlation between the overall positive and false positive rates is shown in Fig. 2a. Positive rates of 39, 56, 62, 66, 71, and 73% were obtained with 3, 6, 9, 11, 14, and 17 antibodies, respectively. On the other hand, false positive rates of 7, 15, 23, 24, 31, and 37% were obtained with 3, 6, 9, 11, 14, and 17 antibodies, respectively. Based on the receiver operating characteristic (ROC) curve, the optimal point for sensitivity and 1 − specificity was 11 antibodies (Fig. 2b). The combination assay with 11 antibodies yielded

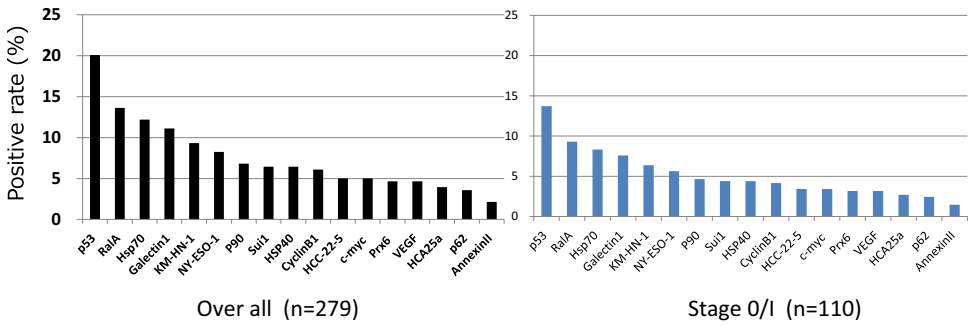

Fig. 1 Positive rates of each autoantibody against tumor-associated antigens in patients with colorectal cancer. Cutoff values corresponding to mean + 3 standard deviations of the healthy control cohort were used in each of the 17 antibodies while maintaining a specificity of > 95%

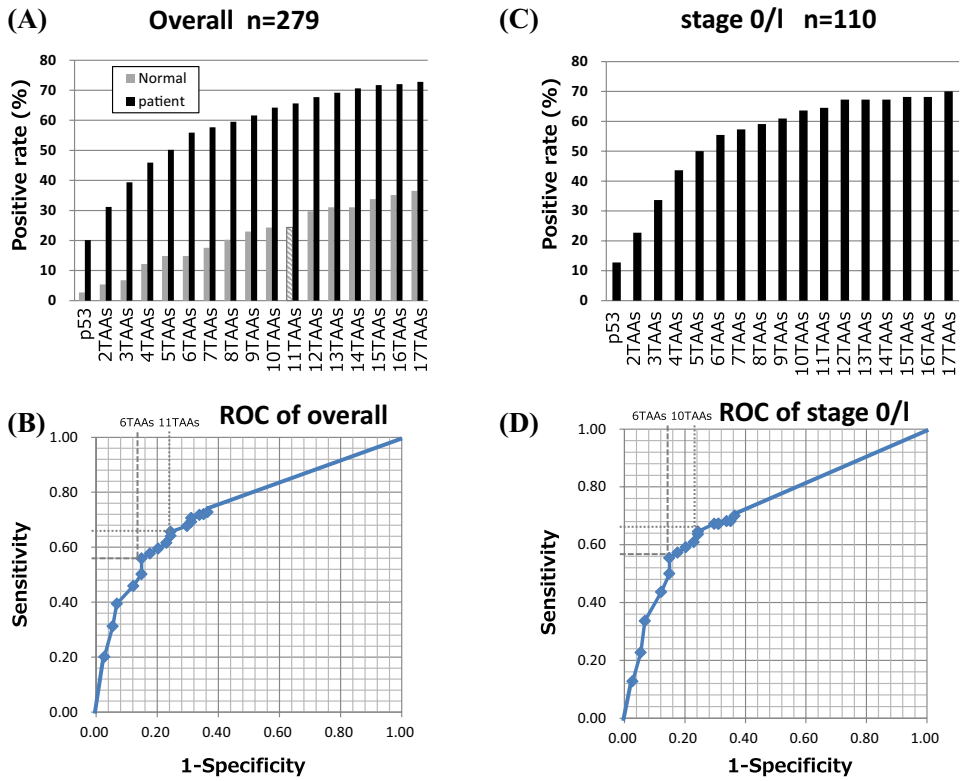

Fig. 2 **a** Overall positive and false positive rates using combination assays for multiple autoantibodies. **b** Overall sensitivity and 1 − specificity for multiple autoantibodies. Based on the ROC curve, the optimal point for sensitivity and 1 − specificity is using a combination of 11 antibodies. **c** Stage 0/I positive and false positive rates using combination assays for multiple autoantibodies. **d** Stage 0/I ROC curve with sensitivity and 1 − specificity for multiple autoantibodies

a sensitivity and specificity of 0.66 and 0.76, respectively. At this point, however, the false positive rate was 24%. Focusing on stage 0/I tumors ($n = 110$) (Fig. 2c), similar trends for sensitivity/specificity were observed in the ROC curve for the combination assay (Fig. 2d). Based on the ROC curve, the optimal point for sensitivity and specificity was 10 antibodies. The combination assay with 10 antibodies yielded a sensitivity and specificity of 0.64 and 0.76, respectively (Fig. 2d).

Distribution of the number of positive autoantibodies in each patient

Among the 279 patients, 76 (27%) were negative for all 17 autoantibodies (Fig. 3). In total, 121 (39%) patients were positive for a single antibody, 55 (20%) were positive for two antibodies, and 36 (14%) were positive for more than three antibodies. Two patients were positive for 6 and 10 antibodies. Positive rates of the combination assay with all 17 autoantibodies according to tumor stages are shown in Fig. 4. Positive rates for stages 0, I, II, III, and IV were 69, 70, 74, 69, and 76%, respectively, whereas that for recurrence was 87%. The positive rates of a single antibody for stages 0, I, II, III, and IV were 58, 35, 39, 33, and 47%, respectively, whereas that for recurrence in each stage was 47% (Fig. 4).

Positive rates and serum titers of the top four autoantibodies according to tumor stages

Positive rates of the top four autoantibodies (p53, RalA, HSP70, and Galectin1) according to tumor stages are shown in Fig. 5. Similar to the overall autoantibodies, positive rates of these top four autoantibodies did not increase depending on stage progression. Even at stage 0/I/II, these autoantibodies showed relatively high positive rates (> 10%). The

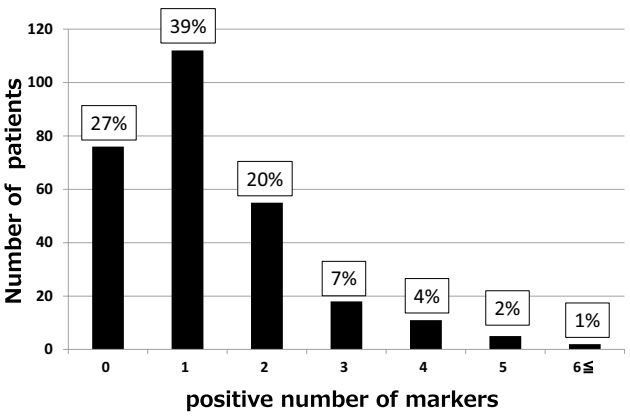

Fig. 3 Distribution of the number of positive autoantibodies in each patient

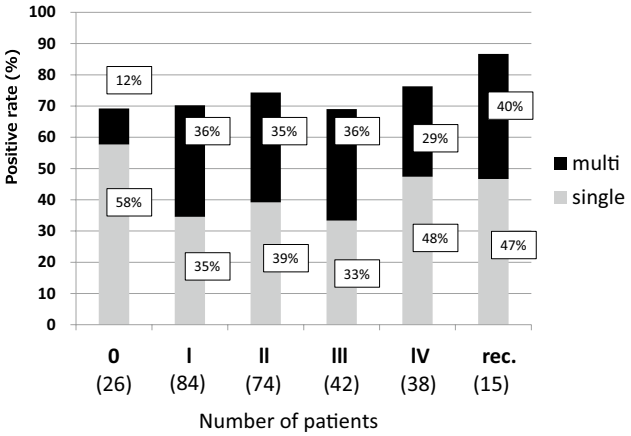

Fig. 4 Distribution of patients who tested positive for single and multiple antibodies according to the disease stage

distribution of the titers for the top four autoantibodies with high positive rates is shown in Fig. 6. Similar to positive rates, the distribution of titers did not significantly increase depending on the tumor stage.

Significance of the highest titers in each antibody

The distribution of serum titers is presented according to TAAs while focusing on patients who showed the highest values for each antibody (Fig. 7). Three patients showed the highest titers in 2 of the 17 antibodies, and 14 patients showed the highest titers in at least 1 antibody. Among these 14 patients, 7 had stage IV or recurrent disease, none had stage III disease, 3 had stage II disease, three had stage I disease, and 1 had a stage 0 disease. Unsurprisingly, the 53 patients with stage IV or recurrent disease more frequently revealed higher titers than the

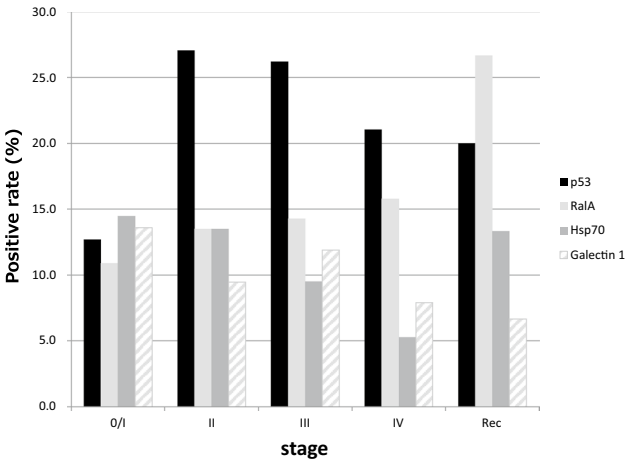

Fig. 5 Antibody titers for p53, RalA, Hsp70, and Galectin1 according to the disease stage

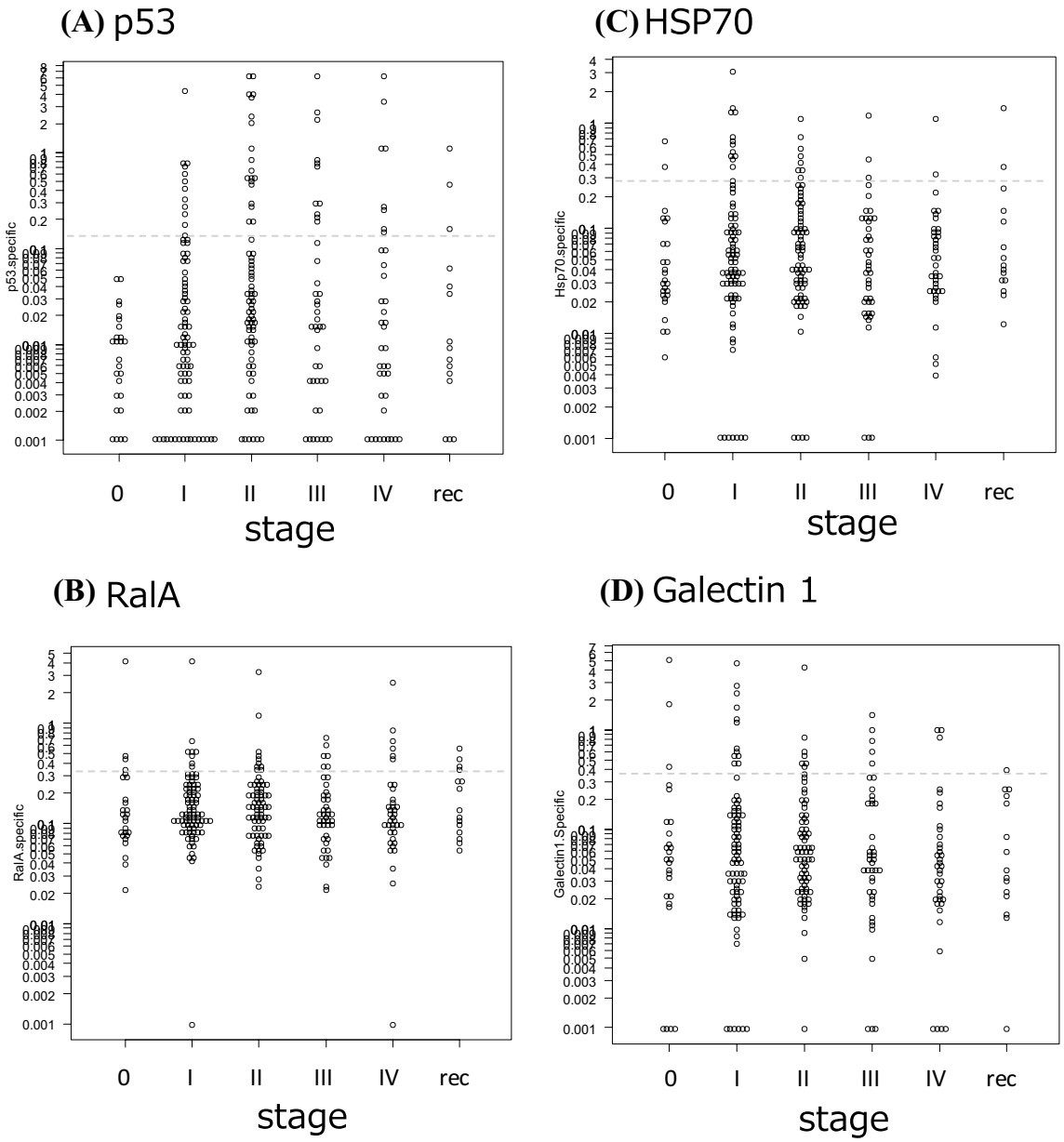

Fig. 6 Distribution of titers according to disease stage focusing on the top four autoantibodies

110 patients with stage 0/I disease (13 vs. 4%, $p=0.04$). Some patients showed high autoantibody responses even during the early stages of tumor. Interestingly, four patients with stage 0/I disease showed the highest titers in six antibodies (RalA, Hsp70, Galectin1, Sui1, Cyclin B1, and HCC-22-5) (Fig. 7).

Discussion

In total, 17 autoantibodies responding to tumor antigens allowed autoantibody profiling in patients with colorectal cancer. The positive rate of stage 0/I tumors was 56% using the combination assay of six of our 17 autoantibodies. The positive rate of overall was equal to the positive rate of stage 0/I tumors. This combination of six autoantibodies may be useful for detecting colorectal cancer regardless of the disease stage.

Recently, Chen et al. [11] reported a combination assay with another set of six autoantibodies against TP53, IMPDH2, MDM2, and MAGEA4 for colorectal cancer. They reported a sensitivity and specificity of 0.39–0.42 and 0.86–0.89, respectively. Using our set of six antibodies, we obtained a sensitivity and specificity of 0.56 and 0.85, respectively, in the present study. Although the specificity

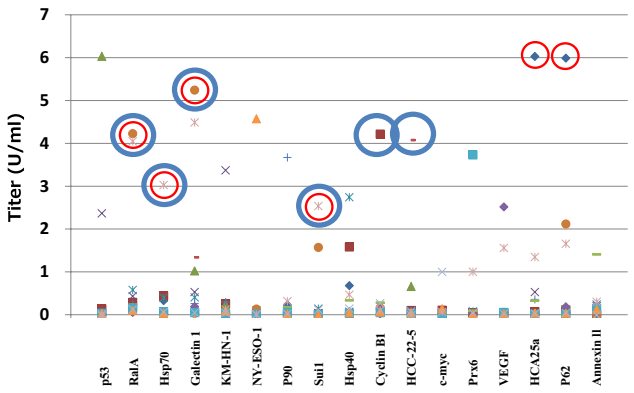

Fig. 7 Distribution of autoantibody titers for patients showing the highest value for each antibody. Red circles indicate three patients who showed the highest titers for two antibodies. Blue circles indicate four patients with stage 0/I disease who showed the highest titers for one antibody

was slightly lower than that in the report by Chen et al. [11], the sensitivity of our set of autoantibodies was higher than Chen's set of six autoantibodies. The sensitivity of our set of six autoantibodies was comparable across all tumor stages.

Overall, 33% of the patients have two or more autoantibodies. Positive rates were similar across tumor stages; moreover, the proportion of patients who tested positive for single or multiple antibodies was similar in all disease stages except stage 0. Same as s-p53-Abs [23], autoantibodies response to each tumor antigen showed similar levels among tumor stages. Positive rates of the top four autoantibodies (p53, RalA, HSP70, and Galectin1) were more than 10% even at early stages. In fact, it has been reported that s-p53-Abs and s-HSP70-Abs are useful tumor markers for the early detection of esophageal cancer [9, 21]. Interestingly, positive rates of p53, HSP70, and Galectin1 were lower in stage IV patients than in stage III patients. Such paradoxical tendencies were also observed in previous studies for p53 in colorectal cancer [22] and Galectin1 in hepatocellular carcinoma [15]. On the other hand, the positive rate of s-RalA-Abs increased depending on tumor stage. This tendency was also observed in patients with esophageal cancer [14]. Similar to positive rates, the distribution of serum titers of the top four autoantibodies did not significantly increase depending on the tumor stage. Such tendency was also observed in s-p53-Abs of patients with esophageal carcinoma [23].

Focusing on patients who showed the highest titers for each antibody, four patients with stage 0/I disease showed the highest titers in six antibodies (RalA, Hsp70, Galectin1, Sui1, Cyclin B1, and HCC-22-5), interestingly. These autoantibodies may be potential marker candidates for the early detection of colorectal cancer.

One limitation of the current study was the relatively high positive rates in healthy controls. Because the healthy volunteers were not screened by colonoscopy, we could not exclude early colorectal cancer among the healthy controls. Because the cutoff values of each autoantibody were mean + 3 SD of healthy volunteers, false positive rates were 0–6% for each antibody in healthy group. Combinatory usage of pleural autoantibodies might increase false positive rates. Although ROC curve indicated that six autoantibodies were the best combination, three or four autoantibodies might be better to reduce false positive rates. The other limitation was that no data were available on the impact of autoantibodies on prognosis. We could not analyze the relationship between positive antibodies and prognosis because the samples were anonymous. Therefore, it is imperative that future prospective studies analyze this relationship.

In conclusion, the measurement of set of 17 autoantibodies allowed autoantibody profiling in patients with colorectal cancer. The combination assay of six tumor antigens (p53, RalA, HSP70, Galectin1, KM-HN-1, NY-ESO-1) achieved a positive rate of 56%. Such high positive rates will be helpful for detecting colorectal cancer regardless of tumor stages.

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Compliance with ethical standards

Conflict of interest Hideaki Shimada received research Grants from Medical & Biological Laboratories Co., Ltd., Nagoya, Japan. Akiko Kuwajima is an employee of the Medical & Biological Laboratories Co., Ltd., Nagoya, Japan. The other authors declare that there are no conflicts of interest associated with the present study.

Informed consent Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Chiba Cancer Center (no. 21-26) and Toho University School of Medicine (nos. 22-112 and 22-047). This clinical study has been registered in the UMIN Clinical Trials Registry (UMIN000014530).

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