Original Article

Diagnostic and Prognostic Impacts of Six Autoantibodies against Multiple Tumor-Associated Antigens with Hepatocellular Carcinoma

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ABSTRACT

Introduction: Despite improvements in surgical techniques and other treatment modalities, the prognosis of hepatocellular carcinoma (HCC) remains poor because of late diagnosis. Recently, multiple molecular biomarkers were reported to be potential diagnostic tools for HCC.

Methods: We analyzed serum samples from 152 healthy controls and 94 HCC patients at the Chiba Cancer Center using an enzyme-linked immunosorbent assay. Recombinant proteins were expressed in Escherichia coli (*E. coli*), purified, and then used for enzyme-linked immunosorbent assay (ELISA) kit construction. For the expression and purification of recombinant proteins, the full-length cDNA of tumor-associated antigens (TAAs), including heat shock protein 70 (Hsp70), Galectin-1 (Gal-1), KM-HN-1, HCC-22-5, RalA, and NY-ESO-1, was amplified using polymerase chain reaction.

Results: Among HCC patients, the highest sensitivity of autoantibodies against TAAs was observed in 22 patients (23%) for Hsp70. The combination assay increased the sensitivity for all six autoantibodies to 56% (53/94). The specificity of all autoantibodies was >95% in both cohorts. Results using cutoff values for individual autoantibodies from the validation cohort yielded similar results in the test cohort. The sensitivity of the six-

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autoantibody panel combined with α-fetoprotein (AFP) and/or protein induced by absence of vitamin K or by antagonist-II (PIVKA-II) was significantly higher than sensitivity achieved with AFP and/or PIVKA-II only (P = 0.005). There were no statistically significant differences in the 3-year overall survival between the autoantibody-positive and autoantibody-negative groups.

Conclusions: This study showed that a panel of six autoantibodies against Hsp70, Gal-1, KM-HN-1, HCC-22-5, RalA, and NY-ESO-1 increased the sensitivity of conventional tumor markers used to detect HCC.

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KEYWORDS: autoantibody, biomarker, diagnosis, hepatocellular carcinoma, prognosis

Introduction

Approximately 850,000 people worldwide die of hepatocellular carcinoma (HCC) annually, and HCC is the second most common cause of death by cancer.[1] Surgical techniques and other treatment modalities have undergone improvement; however, the prognosis of HCC remains poor because of late diagnosis. Therefore, methods for early detection are of utmost importance.[2] Protein induced by vitamin K absence or antagonist-II (PIVKA-II), α-fetoprotein (AFP), and other tumor markers is currently used for the diagnosis of HCC.[3] These markers, especially for early stage HCC, have low sensitivity and specificity. It has been reported recently that HCC could be diagnosed using multiple potential molecular markers.[4]

The immune response of cancer patients causes the production of autoantibodies.[5] Autoantibodies to tumor-associated antigens (TAAs) were recently reported as being promising serum tumor markers. Furthermore, serum autoantibodies against TAAs are induced during the early stages of cancer.[6] Notably, several TAAs, including heat shock protein 70 (Hsp70), p53, RalA, and HCC-22-5, can be used to identify HCC.[4, 7, 8] Indeed, HCC and healthy control sera were satisfactorily differentiated using IgG autoantibodies, such as NY-ESO-1, p53, RalA, and Galectin-1 (Gal-1).[9–11] A single TAA cannot reliably detect HCC, therefore, the use of a TAA panel was suggested to improve detection rates. Previous studies, including our own pilot study using 18 TAAs, showed that Hsp70, Gal-1, KM-HN-1, HCC-22-5, RalA, and NY-ESO-1 were strongly immunogenic, and that the sensitivity of a multiple autoantibody panel was significantly higher than the sensitivity of a single autoantibody.[9, 10, 12–17] However, there are no reports on the relationship among the autoantibodies, PIVKA-II, and AFP. Moreover, there are no reports on the relationship between autoantibodies and the HCC prognosis.

Here, we evaluated the additive effect of a panel of six TAAs, including Hsp70, Gal-1, KM-HN-1, HCC-22-5, RalA, and NY-ESO-1, in order to detect HCC on conventional serum markers in a Japanese population. Moreover, we analyzed the prognostic significance of the autoantibodies.

Patients and Methods

Collection of serum samples

Serum samples were obtained from Biobank Japan or collected at the Department of Gastroenterological Surgery, the Chiba Cancer Center. Samples were stored at −80°C until use. HCC was defined using computed tomography (CT) and was confirmed using histopathology. Tumor stage was clinically determined with CT and was defined according to the 7th edition of the American Joint Committee on Cancer Staging Manual.[18] Healthy controls were without any previous malignant disease and without hepatitis B or C infection. This study was retrospective and the cohorts were characterized as follows: The test cohort group registered serum samples from Biobank Japan of (I) 50 HCC patients; and (II) 79 healthy controls. Validation cohort group registered: serum samples of (I) 44 HCC patients from the Chiba Cancer Center; and (II) serum samples from 73 healthy controls. Using an optimal combination panel of autoantibodies against the six selected TAAs, we assayed a total of 246 samples from 152 healthy controls and 94 HCC patients.

Recombinant TAA purification and enzyme-linked immunosorbent assay (ELISA)

Full-length cDNA of the TAAs Hsp70 (GenBank accession number: NM 004134), Galectin1 (NM 1001867), KM-HN-1 (NM 152775), HCC-22-5 (NM 004683), RalA (BM 560822), and NY-ESO-1 (NM 001327) were amplified via polymerase chain reaction, as previously described.[12] Briefly, the recombinant proteins were expressed in Escherichia coli BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA,

USA). Each 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. DNA sequencing confirmed that the correct gene was inserted into the constructed plasmid.

Serum samples from patients and healthy controls were analyzed using an ELISA, as previously described.[19] Serum AFP and PIVKA-II were also evaluated as previously described.[20]

Assay cutoff values

The optical density value greater than the mean ± 3 standard deviations of normal controls from the test cohort was defined as the cutoff value indicating a positive response.[16] The specificity of the assay was calculated as the percentage of the healthy controls from whom a negative result was obtained.

Ethical approval

Informed patient consent was obtained, and the study was approved by the Ethics Committee of the Chiba Cancer Center (no. 21-26) and the Toho University School of Medicine (#26-095). This clinical study was registered in the UMIN Clinical Trials Registry (UMIN000014530).

Statistical analysis

Statistical analysis was performed using JMP statistical software (version 12; SAS Institute, Cary, NC, USA). The number and proportion of positive samples are presented with 95% exact confidence intervals (CIs) for binomial proportions. The false positive rate, false negative rate, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio, all with 95% CIs, were calculated. Comparisons between unpaired groups for antibody titers were conducted using the Mann-Whitney *U*-test. Comparisons between two groups for clinicopathological variables were conducted using Fischer's exact test. Overall survival was calculated using the Kaplan-Meier product limit estimate. Differences between groups regarding survival were analyzed using the log-rank test. Significant predictors identified by univariate analysis were assessed by multivariate analysis using the Cox proportional hazards model. In all tests, we considered *P*-values of <0.05 (two-sided) to indicate statistical significance.

Results

Sensitivities and titers of serum autoantibodies against the six-TAA panel in HCC patients

Among the 94 HCC patients, autoantibodies to TAAs were detected for Hsp70 in 22 patients (23%), Gal-1 in 20 patients (21%), KM-HN-1 in 13 patients (14%), HCC-22-5 in 11 patients (12%), RalA in 11 patients (12%), and NY-ESO-1 in 10 patients (11%). The titers of autoantibodies to all six TAAs are shown in Fig. 1. All autoantibodies to these TAAs were clearly elevated in serum samples from patients with HCC when compared with sera from healthy controls in both cohorts (all $P < 0.05$). The sensitivities of autoantibodies in HCC patients from both cohorts are shown in Fig. 2, and the sensitivities of autoantibodies to TAAs in test and validation cohorts of HCC patients and normal controls are shown in Table 1. The specificities of all autoantibodies were higher than 95% in both cohorts. In the validation cohort, the sensitivity to Hsp70 (30%) was the highest, followed by that of Gal-1 (27%).

Sensitivity, specificity, and diagnostic accuracy of serum autoantibodies against each TAA and the six-TAA panel

The sensitivity of Hsp70 was the highest among the six autoantibodies: 23% (22/94) overall; 18% (9/50) in the test cohort; and 30% (13/44) in the validation cohort. The combination assay increased sensitivities to: 38% (36/94) for Hsp70 and/or Gal-1; 46% (43/94) for Hsp70, Gal-1, and/or KM-HN-1; 51% (48/94) for Hsp70, Gal-1, KM-HN-1, and/or HCC-22-5; 53% (50/94) for Hsp70, Gal-1, KM-HN-1, HCC-22-5, and/or RalA; and 56% (53/94) for all six TAAs. Using all six autoantibodies provided an enhanced panel sensitivity of 48% (95% CIs, 35%-62%) and 66% (95% CIs, 51%-78%) in the test and validation cohorts, respectively. Results using cutoff values for individual autoantibodies from the validation cohort yielded similar results in the test cohort (Table 2). The sensitivities of autoantibodies to TAAs at each cancer stage in both cohorts are shown in Fig. 3. The sensitivity of autoantibodies for stage I was 62% (8/13), for stage II it was 50% (19/38), for stage III it was 39% (7/18), and for stage IV it was 71% (5/7).

Clinicopathological characteristics and autoantibody status in patients with HCC

Clinicopathological characteristics of the validation cohort are shown in Table 3. The patient characteristics were not significantly related to the presence of autoantibodies (Table 3a). Autoantibodies were raised to a second

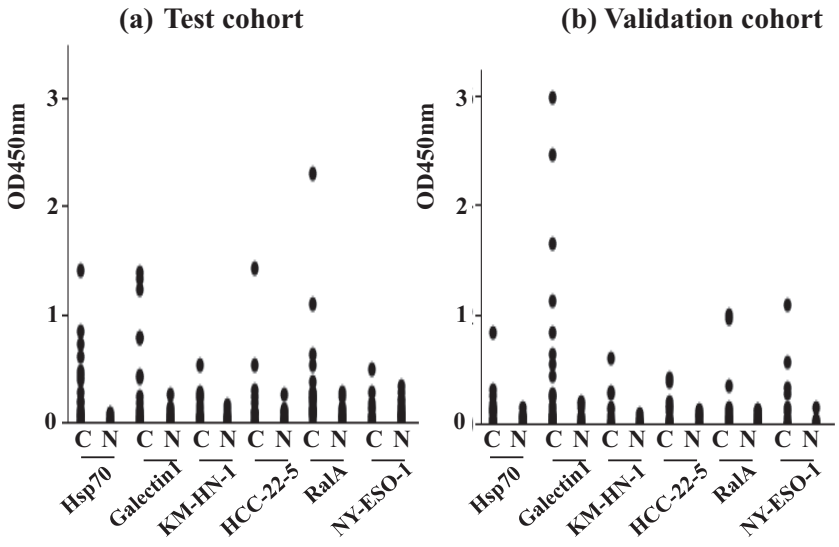

Fig. 1 ELISA antibody titers of individual HCC patients and normal controls for tumor-associated antigens.
Scatter plots of optical density values of autoantibodies in sera from patients with HCC (n = 50) and healthy controls (n = 79) in the test cohort (a) and from patients with HCC (n = 44) and healthy controls (n = 73) in the validation cohort (b). C, cancer patients; N, normal controls

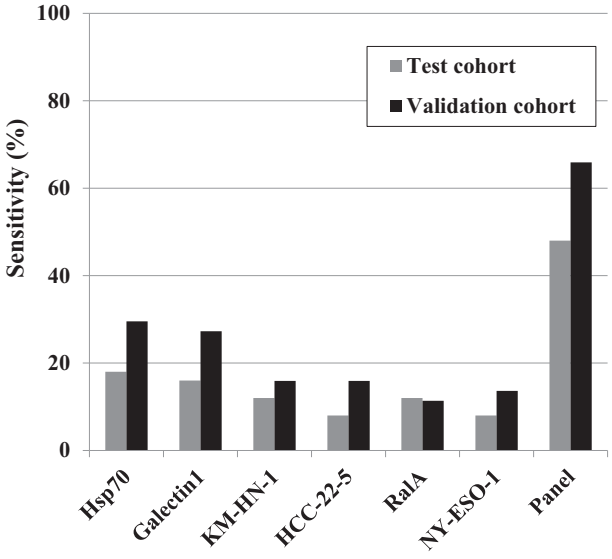

Fig. 2 Sensitivity of individual tumor-associated antigens (TAAs) and the panel of six TAAs

antigen in 32% of the patients. We compared the clinicopathological characteristics between zero or one antibody-positive patients and two or more antibody-positive patients. The characteristics were not significantly related to the number of positive autoantibodies (0, 1, or ≥ 2) (Table 3b).

Sensitivities of the serum autoantibody panel and conventional serum markers to detect HCC

In order to confirm the diagnostic power of the panel of six autoantibodies, we compared its sensitivity with the sensitivities of the conventional tumor markers AFP and PIVKA-II (Fig. 4). The sensitivities of AFP and PIVKA-II were 66% and 76%, respectively, whereas the sensitivity of the six-autoantibody panel was 62%. There was no significant difference between the sensitivities of the panel and conventional tumor markers. The sensitivity of the six-autoantibody panel combined with AFP and/or PIVKA-II was significantly higher than sensitivity of AFP and/or PIVKA-II ($P = 0.005$).

Prognostic impact of serum autoantibodies on patients with HCC

The prognostic significance of autoantibodies in the validation cohort is shown in Fig. 5. There was no statistically significant difference in the three-year overall survival between the autoantibody-positive and autoantibody-negative groups (Fig. 5a). We also divided the patients into subgroups: patients negative or positive to one autoantibody; and patients positive for two or more autoantibodies. Although the difference was not statistically significant, the ≥2 autoantibody-positive group had poorer overall survival than the autoantibody-negative or one autoantibody-positive group (median survival time 39 vs.

Table 1 Frequency of autoantibodies to tumor-associated antigens in test and validation cohorts of hepatocellular carcinoma patients and normal controls

Group	Hsp70	Galectin1	KM-HN-1	HCC-22-5	RalA	NY-ESO-1	Panel
Test cohort							
Sensitivity	18.0 (9.8－30.8)	16.0 (8.3－28.5)	12.0 (5.6－23.8)	8.0 (3.2－18.8)	12.0 (5.6－23.8)	8.0 (3.2－18.8)	48.0 (34.8－61.5)
Specificity	97.4 (91.2－99.3)	98.7 (93.2－99.7)	98.5 (91.8－99.7)	98.7 (93.2－99.7)	98.7 (93.0－99.7)	94.9 (87.7－98.0)	92.4 (84.4－96.5)
Validation cohort							
Sensitivity	29.5 (18.1－44.2)	27.3 (16.3－41.8)	15.9 (7.9－29.4)	15.9 (7.9－29.4)	11.4 (5.0－24.0)	13.6 (6.4－26.7)	65.9 (51.1－78.1)
Specificity	98.6 (92.6－99.7)	95.8 (88.5－98.6)	97.2 (90.3－99.2)	98.6 (92.6－99.7)	98.6 (92.5－99.7)	97.2 (90.3－99.2)	90.4 (81.5－95.3)

All values are given in percentage positivity with 95% confidence interval in each group.
Panel, autoantibody positivity to any one of the six antigens

Table 2 Diagnostic ability of the autoantibody panel in the diagnosis of hepatocellular carcinoma

Group	Sensitivity	Specificity	FPR	FNR	PPV	NPV	PLR	NLR
Test cohort	48.0 (34.8－61.5)	92.4 (84.4－96.5)	7.6 (3.5－15.6)	52.0 (38.5－65.2)	80.0 (62.7－90.5)	73.7 (64.3－81.4)	6.32 (2.96－14.22)	0.56 (0.47－0.70)
Validation cohort	65.9 (51.1－78.1)	90.4 (81.5－95.3)	9.6 (4.7－18.5)	34.1 (21.9－48.9)	80.6 (65.0－90.2)	81.5 (71.7－88.4)	6.87 (3.57－13.82)	0.38 (0.28－0.52)

All values are given with 95% confidence interval in each group.
FPR, false positive rate; FNR, false negative rate; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio.

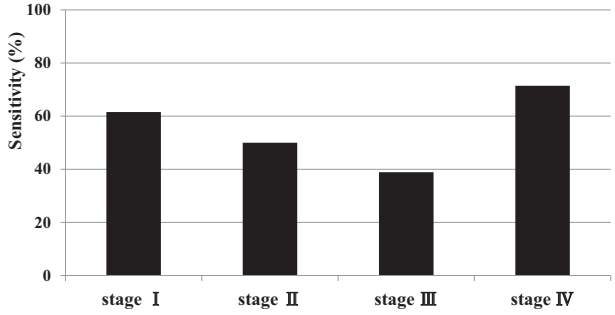

Fig. 3 Sensitivities of autoantibodies to TAAs at each cancer stage

26 months) (Fig. 5b).

Discussion

The sensitivity of Hsp70 was the highest among all six autoantibodies. Overall sensitivity of the six-autoantibody panel was 66%. For stage I, the sensitivity of the six-autoantibody panel was 62%. The presence of autoantibodies was not associated with conventional tumor markers; thus, the autoantibody panel increased overall sensitivity when combined with conventional tumor markers.

The reason for separating the test cohort and the validation cohort was that the test cohort was offered from Biobank, so we needed to verify reproducibility. Indeed, the test cohort and the validation cohort showed similar results. It is important to note that the specificity of the six-autoantibody panel was over 90%. Generally, the sensitivity of the six-autoantibody panel in the patients with malignant tumors was affected by the profiling of tumor antigens rather than the tumor volume.[21, 22] Therefore, positive rate and/or number of positive autoantibodies were not associated with tumor progression. In fact, the sensitivity of stage IV was similar to the sensitivity of stage I and II. This result had similar results even in gastric cancer and colorectal cancer.[23, 24] Indeed, because the sensitivity of the six-autoantibody panel exceeded 50% even in stages I and II, it may be useful for early detection of HCC.

The relationship between autoantibody and prognosis in various cancers has not been reported, precisely. Although we initially speculated that sero-positive group had a good prognosis because of the immunogenicity, we could not find statistical difference. Therefore, autoantibody reaction, in advanced cancer in patients with HCC, did not

Table 3 Patient details of panel positive in validation cohort

(a)

Panel	Total	(−)	(+)	P value [a]
Number	44	15 (34.1)	29 (65.9)	
Gender, n (%)				
Male	36 (81.8)	11 (25.0)	25 (56.8)	0.294
Female	8 (18.2)	4 (9.1)	4 (9.1)	
Age, n (%)				
＜65	18 (40.9)	5 (11.4)	13 (29.5)	0.462
≧65	26 (59.1)	10 (22.7)	16 (36.4)	
Depth of tumor invasion, n (%)				
T1, 2	23 (52.2)	6 (13.6)	17 (38.6)	0.24
T3, 4	21 (47.8)	9 (20.5)	12 (27.3)	
Lymph node metastasis, n (%)				
Positive	2 (4.5)	0 (0.0)	2 (4.5)	0.19
Negative	42 (95.5)	15 (34.1)	27 (61.4)	
Distant metastasis, n (%)				
Positive	5 (11.4)	1 (2.3)	4 (9.1)	0.462
Negative	39 (88.6)	14 (31.8)	25 (56.8)	
TNM stage, n (%)				
I, II	21 (47.8)	5 (11.4)	16 (36.4)	0.166
III, IV	23 (52.2)	10 (22.7)	13 (29.5)	
AFP, n (%) [b]				
Positive	25 (65.8)	11 (28.9)	14 (36.8)	0.077
Negative	13 (44.2)	2 (5.3)	11 (28.9)	
PIVKA-II, n (%) [b]				
Positive	29 (76.3)	11 (28.9)	18 (47.4)	0.373
Negative	9 (23.7)	2 (5.3)	7 (18.4)	

[a] Fisher's exact test.
[b] Total n = 38

(b)

Number of antigen positive	Total	0, 1	≥2	P value [a]
Number	44	30 (68.2)	14 (31.8)	
Gender, n (%)				
Male	36 (81.8)	25 (56.8)	11 (25.0)	0.706
Female	8 (18.2)	5 (11.4)	3 (6.8)	
Age, n (%)				
＜65	18 (40.9)	12 (27.2)	6 (13.6)	0.857
≧65	26 (59.1)	18 (40.9)	8 (18.2)	
Depth of tumor invasion, n (%)				
T1, 2	23 (52.2)	16 (36.4)	7 (15.9)	0.836
T3, 4	21 (47.8)	14 (31.8)	7 (15.9)	
Lymph node metastasis, n (%)				
Positive	2 (4.5)	1 (2.3)	1 (2.3)	0.572
Negative	42 (95.5)	29 (65.9)	13 (29.5)	
Distant metastasis, n (%)				
Positive	5 (11.4)	4 (9.1)	1 (2.3)	0.531
Negative	39 (88.6)	26 (59.1)	13 (29.5)	
TNM stage, n (%)				
I, II	21 (47.8)	14 (31.8)	7 (15.9)	0.836
III, IV	23 (52.2)	16 (36.4)	7 (15.9)	
AFP, n (%) [b]				
Positive	25 (65.8)	17 (44.7)	8 (21.1)	0.938
Negative	13 (44.2)	9 (23.7)	4 (10.5)	
PIVKA-II, n (%) [b]				
Positive	29 (76.3)	20 (52.6)	9 (23.7)	0.896
Negative	9 (23.7)	6 (15.8)	3 (7.9)	

[a] Fisher's exact test.
[b] Total n = 38

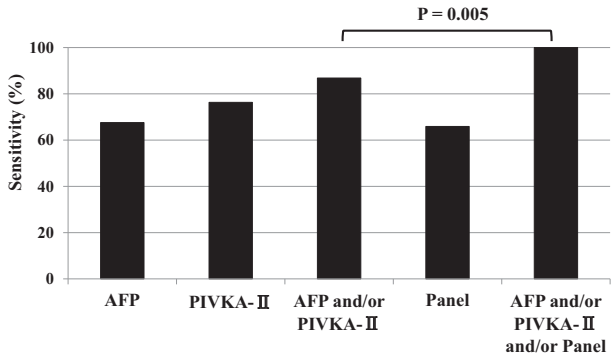

Fig. 4 Sensitivity of autoantibody panel and the traditional tumor markers; AFP and PIVKA-II

affect patients' survival.

A limitation of our present study was that the sample size was too small to evaluate the prognostic significance of autoantibodies. In our previous study of patients with gastric cancer, a panel of autoantibodies to TAAs, including s-p53-Abs and Hsp70, showed a poorer survival rate of the autoantibody-positive group as compared with survival rate in the group that tested negative; however, this difference was not statistically significant.[23] Similar tendency was also observed in our present series of HCC patients. Even though the ≥2 autoantibody-positive group had poorer survival than did the autoantibody-negative or one autoantibody-positive group, the difference was not noted to be statistically significant. Such correlation between the presence of certain autoantibodies with prognosis in patients with HCC should be evaluated in larger examinations and long-term follow-up. An additional prospective multi-institutional study is now underway to evaluate the clinical utility of this autoantibody panel assay.

In conclusion, high positive rates, even in stage I, will be helpful for detecting HCC regardless of tumor stages. This study showed the additive effect of the autoantibody panel, including Hsp70, Gal-1, KM-HN-1, HCC-22-5, RalA, and NY-ESO-1, for detecting HCC when combined with conventional tumor markers.

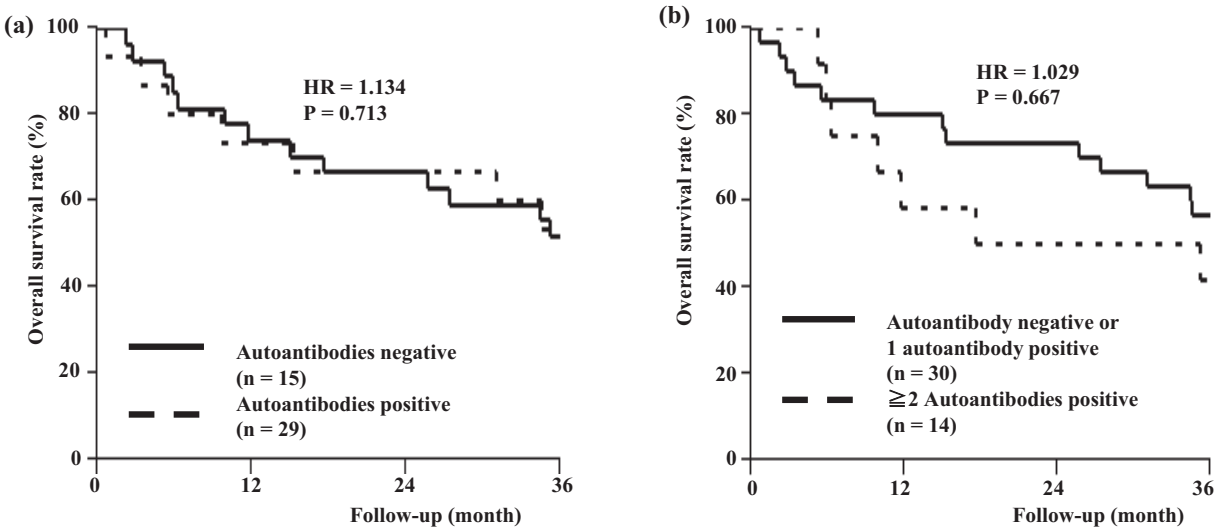

Fig. 5 Overall survival curves of patients with different autoantibody states
(a) Autoantibody-positive and -negative groups; (b) ≥2 autoantibodies positive group and autoantibody-negative or 1 autoantibody-positive group

Disclaimer: Hideaki Shimada is one of the Editors of Toho Journal of Medicine. He was not involved in the editorial evaluation or decision to accept this article for publication at all.

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