Recognizing Rheumatoid Arthritis: Oncoprotein Survivin Opens New Possibilities

A Population-based Case–Control Study

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Abstract: Survivin is a biomarker of cancer known for its anti-apoptotic and cell-cycle regulating properties. In the context of non-cancer pathology, high levels of survivin may be measured in blood and synovial fluid of patients with rheumatoid arthritis (RA) and associate with early joint damage and poor therapy response.

The aim of the study was to investigate the value of survivin measurements in blood for diagnosis of RA in the frame of the Malaysian epidemiological investigation of rheumatoid arthritis (MyEIRA) study. The study enrolled RA patients from eight rheumatology centres in Peninsular Malaysia. The healthy controls matched by age, gender and ethnicity were recruited on the community basis from the residential area of the patients. Levels of survivin were measured in blood of RA patients (n = 1233) and controls (n = 1566) by an enzyme-linked immuno-sorbent assay (ELISA). The risk for RA was calculated as odds ratio (OR) and 95% confidence intervals in the individuals with high levels of survivin. The risk was calculated in relation to antibodies against cyclic citrullinated peptides (ACPA), detected by ELISA and HLA-DRB1 shared epitope (SE) alleles, identified by the polymerase chain reaction using sequence specific oligonucleotide method.

High levels of survivin were detected in 625 of 1233 (50.7%) RA cases and in 85 of 1566 (5.4%) controls, indicating its high specificity for RA. Survivin was association with an increase in RA risk in the patients having neither SE-alleles nor ACPA (OR = 5.40, 95% CI 3.81–7.66). For the patients combining survivin, SE, and ACPA, the estimated risk for RA was 16-folds higher compared to the survivin negative patients with SE and ACPA(OR = 16.21, 95% CI 5.70–46.18).

To conclude, detection of survivin in blood provides a simple test to improve diagnostic and to increase predictability for RA.

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Abbreviations: ACPA = antibodies against cyclic citrullinated peptides, ELISA = enzyme linked immunosorbent assay, HLA = human leucocyte antigen, IL = Interleukin, MyEIRA = Malaysian epidemiological investigation of rheumatoid arthritis, OR = odds ratio, RA = rheumatoid arthritis, SE = shared epitope.

INTRODUCTION

Rheumatoid arthritis (RA) is a progressive debilitating autoimmune disease, which affects 0.5% to 1% people among all ethnic groups.[1–3] Heritability of RA according to twin studies is about 60%,[4] whereas familial aggregation of RA with siblings has a recurrent risk ratio of between 2 and 17.[5]

Risk of RA is strongly associated with major histocompatibility complex and carriage of the HLA Class II locus, in particular the HLA DR beta chain 1 (*HLA-DRB1*) gene.[6] Several whole-genome studies have confirmed the linkage between the *HLA-DRB1* gene and RA with an estimated contribution of 30% to 35% of the total genetic effect in RA.[7–9] A group of RA-related HLA-DRB1 alleles encoding a conserved amino acid sequence ([70]QRRAA[74] or [70]KRRAA[74] or [70]RRRAA[74]) at position 70 to 74 in the third hypervariable region of the first domain of the DR beta chain was defined as shared epitope (SE).[10] These SE alleles appear to confer high risk of severe joint damaging disease. In contrast, the HLA-DRB1 alleles with negatively charged aspartic acid at residue 70 ([70]DERAA[74]) were considered nonpredisposing DRB1 alleles, potentially protecting from RA or favoring less bone destruction.[11–15]

The exact biological mechanism connecting the SE alleles and RA remains unknown. The activation of CD4+ T cells by autologous antigens is considered an early event in RA pathogenesis. The current view on mechanisms underlying the effect of SE include the activation of CD4+ T cells with arthritogenic self-peptide sequences wherein the amino acid arginine is deiminated to citrulline (citrullinated peptides)[16,17] and expansion of these self-specific T cells in the joints.[10,18] Antibodies specific for citrullinated peptides (ACPAs) have been recognized as important prognostic and diagnostic tool for RA.[19] Though the association of SE alleles with ACPA is documented,[19] the induction of T-cell proliferation and cytokine response was not always associated with ACPA production.[20]

Furthermore, the presence of HLA-DRB1 alleles may also be found in the ACPA-negative RA.[21] This suggests the existence of other factors enhancing the molecular link between SE and aberrant immunological responses in RA.

In the present study, we evaluated survivin, an oncoprotein known as a tissue marker of cancer. Following the initial description of survivin,[22] the studies on survivin are concentrated on its anti-apoptotic and cell cycle-regulatory properties in malignancies.[23] During recent years, the role of survivin in nonmalignant cells attracts increasing attention. Survivin has been shown essential for differentiation, growth, and regeneration of healthy tissues including hematopoetic stem cells.[24] In the immune-competent cells, survivin is required for functional antigen presentation, the hallmark of aberrant immunity in RA disease. Survivin controls the maturation of antigen-presenting dendritic cells and expression of MHC Class II molecules.[25,26] Survivin is important for the formation of a functional T-cell receptor[27,28] in the developing thymocytes and for the differentiation into effector and memory T cells.[29,30] At preclinical phase of RA, high levels of survivin correlate with cytokines assuring formation of aggressive T_H1 and T_H17 cells.[31] In the patients early after the RA diagnosis, survivin predicts joint destructive course of disease,[32] and resistance to anti-rheumatic treatment.[33] Survivin may be measured in blood and synovial fluid of a substantial part of RA patients.[31,32] Thus, we hypothesized that survivin may be specific diagnostic test for RA and for estimation of risk of RA development.

MATERIAL AND METHODS

Study Population

The Malaysian Epidemiological Investigation of Rheumatoid Arthritis (MyEIRA) study was approved by the Medical Research and Ethics Committee, Ministry of Health, Malaysia (KKM/JEPP/02 Jld.1 (86);(14)dlm.KKM/NIHSEC/08/0804/MRG-2005-12). Informed written consent was obtained from all the participants. MyEIRA is a population-based case–control study, which addresses the influence of genes and environment on the development of RA, and comprises 1233 RA patients and 1566 controls enrolled in the study during the period between August 2005 and December 2009. RA cases who fulfilled the American College of Rheumatology classification criteria of RA[34] and had the disease duration <2 years were identified within 8 rheumatology centers in Peninsular Malaysia. The recruitment of controls was done on the community basis with the support of the State and District Health Department and covered the regions corresponding to residential area of the patients. Nurses and healthcare professionals identified the controls matched to the RA cases by the residential area, age, sex, and ethnicity in house-by-house visits. The RA cases and the controls had a structured interview based on the study questionnaire, which collected information on a broad range of issues including lifestyle factors, occupational exposures, health aspects, socioeconomic factors, and demographic data.[35] Blood samples were collected from all the participants.

Assessment of Survivin Protein in Blood

Survivin levels were measured in serum samples diluted 1:10 using a sandwich enzyme-linked immunoassay (DY647, R&D Systems, Minneapolis, MN, USA). The absolute level of survivin in each sample was calculated after the serial dilution of human recombinant survivin (R&D Systems), the detection limit was 100 pg/mL. Samples containing >450 pg/mL of survivin were considered positive based on the cut-off level determined in 104 healthy blood donors.[26]

HLA-DRB1 Genotyping

High-resolution genotyping of HLA-DRB1*01–DRB1*016 alleles was performed in the DNA extracted from the leukocytes of EDTA preserved peripheral blood using Ficoll Hypaque (Lymphoprep™, Axis-Shield PoC AS, Oslo, Norway), by the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). The PCR sequence-specific oligonucleotide probe hybridization method was applied using the LABType*HD Class II DRB1 (One Lambda Inc, CA, USA) as described previously.[36] HLA-DRB1 allele frequencies were obtained by direct counting. The carriers of SE were recognized by the presence of HLA alleles, DRB1*01 (*01:01, *01:02, *01:07), DRB1*04 (*04:01, *04:04, *04:05, *04:08, *04:10), and DRB1*10 (*10:01, *10:03). Individuals carrying 1 or 2 SE alleles were classified as SE-positive.

Autoantibody Measurements

ACPA was measured in serum samples using Immunoscan-RA Mark2 ELISA test (anti-CCP test, Malmö, Sweden). Samples with ACPA ≥25 AU/mL were defined as positive.[37]

Statistical Analysis

The post-hoc calculation of the study power (alpha = 0.05) found that the collected material was sufficient to attain study power of 97%. Student t test was calculated for the comparison of the mean age in the patient and control groups. Prevalence of survivin >450 pg/mL among the RA patients and the controls allowed calculation of specificity and positive predictive value of survivin for RA. The probability of RA among the subjects with high survivin was calculated as odds ratio (OR) and corresponded to the relative risk of RA. The ethnic heterogeneity of the material was taken as a potential confounding factor; therefore, association between survivin and SE alleles and SE sequences was calculated separately for the 3 major ethnical groups comprising MyEIRA Malay, Chinese, and Indian. Samples obtained from individuals with different ethnical background (controls, n = 94, 6.0% and RA, n = 82, 6.7%) were therefore excluded from this analysis. To investigate possible diagnostic basis associated with survivin testing, we repeated the primary analysis among the subjects stratified after ACPA tests.

Ninety-five percent confidence intervals were calculated and P values were 2-sided, with statistical significance set at $P < 0.05$. All analyses were performed using the IBM SPSS Statistics 20.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS

The Prevalence of Survivin in MyEIRA Study Population

Six-hundred twenty-five of 1233 (50.7%) RA patients were survivin-positive and so were 85 of 1566 (5.4%) controls (P = 2.66 × 10E-164). Survivin positivity appeared to have a specificity of 0.95 and positive predictive value of 0.88 for RA. The prevalence of survivin-positive RA patients was comparable between the sexes (50% women and 55% men). Demographic and biomarker characteristics of the RA patients and the controls of 3 major ethnic groups within MyEIRA are presented

TABLE 1. Demographic and Biomarker Characteristics of Patients With RA and Controls in the MyEIRA Study

	Malay			Chinese			Indian		
	RA Patients, n = 515 (%)	Controls, n = 978 (%)	P Value	RA Patients, n = 254 (%)	Controls, n = 206 (%)	P value	RA Patients, n = 382 (%)	Controls, n = 288 (%)	P value
Female:Male, n	440:75	840:138	0.81	210:44	174:32	0.61	332:50	239:49	0.16
Age, mean (SD), years	46.2 (11.8)	46.3 (11.39)	0.87	52.6 (11.2)	50.9 (11.5)	0.11	47.7 (10.9)	48.1 (10.6)	0.63
ACPA-positive, %	60.6	2.5	<0.00001	66.5	3.4	<0.00001	67.3	2.1	<0.00001
Survivin-positive, %	45.8*	5.2	<0.00001	46.1*	5.3	<0.00001	60.5*	6.6	<0.00001
SE-positive, %	35.7	13.0	<0.00001	36.2	12.1	<.00001	48.2	30.2	<0.00001

RA patients (n = 82, 6.7%) and controls (n = 92, 6.0%) of other ethnic groups are not shown. SE-positive was defined by the presence of the HLA-DRB1*01 (*01:01, *01:02, *01:07), DRB1*04 (*04:01, *04:04, *04:05, *04:08, *04:10), and DRB1*10 (*10:01, *10:03) alleles. Individuals carrying 1 or 2 SE alleles were classified as SE-positive. RA = rheumatoid arthritis, SD = standard deviation, SE = shared epitope.

* Statistically significant differences were noted for the survivin-positive frequency between Indian and Malay RA patients ($P = 0.000014$); and between Indian and Chinese RA patients ($P = 0.000354$).

in Table 1. The prevalence of survivin-positive RA patients and controls was significantly higher in the Indian ethnic subgroup (60.5% and 6.6%, respectively) compared with Malays and Chinese (all *P* values <0.0001).

The Association Between Survivin and SE Alleles in RA Patients

The SE carriers comprised 40% of the RA patients and 16% of the controls. Among the SE-positive RA patients, 59% (291/494) were also survivin-positive. The complete genotype of HLA-DRB1*01-HLA-DRB1*16 alleles for all RA patients stratified by the presence of survivin and the controls is shown in Supplementary Table S1 http://links.lww.com/MD/A179. The presence of survivin significantly increased the risk of

RA within the Malays, Chinese, and Indian ethnic groups (Figure 1). The combination of survivin positivity and SE positivity recognized the group wherein the risk of RA was highest. The presence of either survivin or SE separately was associated with lower, but still increased, risk of RA.

The estimated risk of RA related to the presence of specific SE alleles and survivin was also calculated within the Malays, Chinese, and Indian ethnic groups. Overall, the risk of RA was significantly increased in the survivin-positive carriers of DRB1*01 (OR 2.09, 95%CI 1.13–3.88, $P = 0.024$) and DRB1*04 (OR 4.85, 95% CI 2.79–6.20, $P < 0.00001$). In addition, the carriers of DRB1*10 SE alleles had increased RA risk in the survivin-positive (OR 4.77, 95% CI 3.49–6.53, $P < 0.00001$) and in the survivin-negative RA (OR 3.64, 95% CI 2.63–5.04, $P < 0.00001$).
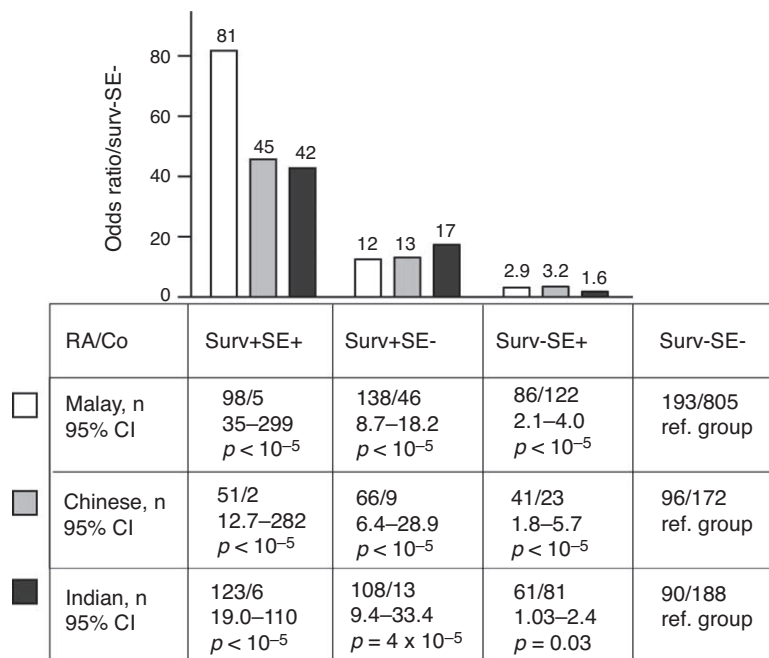
FIGURE 1. The relative risk of RA in subjects with high survivin in 3 major ethnic populations of the MyEIRA study. The study material was stratified after survivin levels (surv+, >450 pg/mL) and the carriage of HLA-DRB1 shared epitope (SE+) alleles within Malay (RA patients, 515, Co 1493), Chinese (RA patients, 254, Co 460), and Indian subjects (RA patients, 382, Co 670). The probability of RA for the subjects with high survivin was calculated as odds ratio in relation to surv-SE- reference group. This corresponded to the relative risk of RA. 95% CI = 95% confidence interval, Co = controls, RA = rheumatoid arthritis.

TABLE 2. The Frequency of Shared Epitope Sequences (QRRAA, QKRAA, and RRRAA) in the Survivin-positive RA Patients

Shared Epitope Sequence*	Malay			Chinese			Indian		
	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
70QRRAA74	77/184	87/851	4.09 (2.90–5.78)	43/75	22/181	4.72 (2.64–8.42)	43/121	26/201	2.75 (1.61–4.70)
70QKRAA74	4/184	4/851	4.63 (1.15–18.66)	1/75	2/181	1.21 (0.11–13.51)	21/121	15/201	2.33 (1.16–4.68)
70RRRAA74	23/184	36/851	2.95 (1.71–5.11)	8/75	2/181	9.65 (2.00–46.53)	71/121	50/201	2.36 (1.54–3.61)

Cases and controls without any of the DRB1-shared epitope-encoding amino acid sequencing (QRRAA, QKRAA, and RRRAA) were used as reference group for calculation of OR and 95% CI. CI = confidence interval, OR = odds ratio, RA = rheumatoid arthritis.

* DRB1-shared epitope-encoding amino acid sequence: QRRAA (*01:01, *01:02, *01:07, *04:04, *04:05, *04:08 and *04:10); QKRAA (*04:01); and RRRAA (*10:01, *10:02, and *10:03).

The analysis of RA risk was performed for the survivin-positive SE carriers in relation to the amino acid sequence of different ethnic subgroups (Table 2). In the Malays survivin-positive patients, the RA risk was associated with the 70QRRAA74 and 70QKRAA74 SE-sequences; in the Chinese survivin-positive patients, the presence of 70RRRAA74 SE-sequence indicated the highest RA risk; and in the Indian survivin-positive patients, the RA risk was comparable between the sequences. The evaluation of other HLA-DRB1 alleles frequent among Asians showed a slight accumulation of survivin-positive patients within the DRB1*09 carriers (OR 2.20, 95% CI 1.50–3.22, $P = 5.9 \times 10E\text{-}5$). The DRB1*14 alleles (eg, *14:02 and *14:06) common among American Indians who originated in northeast Asia were prevalent among the RA patients and were equally distributed in the survivin-positive and survivin-negative groups. The DRB1 alleles containing sequences nonpredisposing to RA DRB1*03, *08, *12, and

*13 were equally represented among the survivin-positive and survivin-negative RA patients (Supplementary Table S1 http://links.lww.com/MD/A179).

The Combination of Survivin and ACPA Increases the Risk of RA

The high levels of ACPA were detected in 83% of the survivin-positive and in 44.7% of the survivin-negative RA patients (Figure 2). The survivin positivity was associated with higher risk of RA when present in combination with ACPA (OR = 16.21, 95% CI 5.70–46.18, $P < 0.0001$); however, the presence of survivin in the ACPA-negative RA patients was associated with a significantly increased risk of RA when compared with the controls (OR = 5.64, 95% CI 4.12–7.70), $P < 0.0001$. The specificity of survivin positivity was comparable between the ACPA-positive RA and ACPA-negative RA (0.89 and 0.95, respectively).
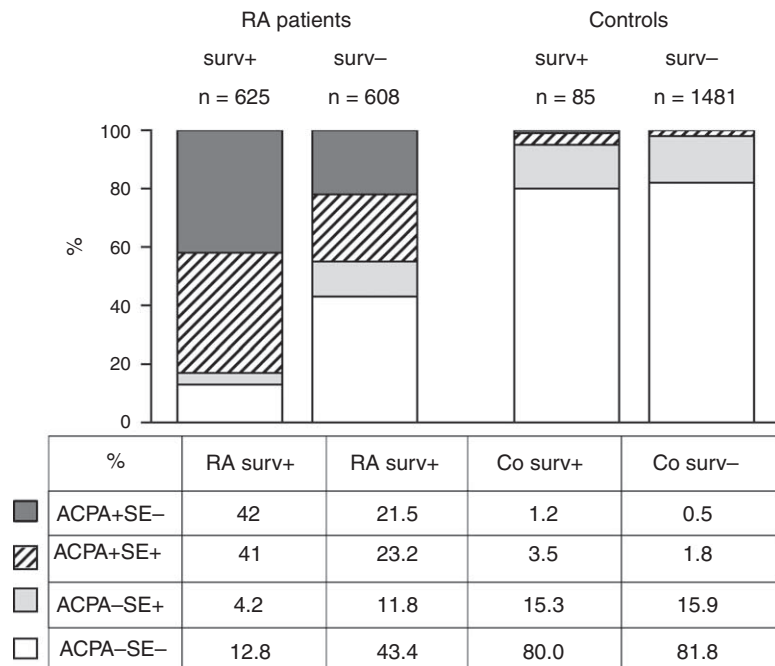

%	RA surv+	RA surv+	Co surv+	Co surv–
ACPA+SE–	42	21.5	1.2	0.5
ACPA+SE+	41	23.2	3.5	1.8
ACPA–SE+	4.2	11.8	15.3	15.9
ACPA–SE–	12.8	43.4	80.0	81.8

FIGURE 2. The composition of survivin-positive and survivin-negative groups among the RA patients and Co of the MyEIRA study. The prevalence of HLA-DRB1 shared epitope alleles (SE) and ACPA is presented in per cent within survivin positive and negative RA patients and Co. ACPA = antibodies to citrullinated peptides, Co = controls, RA = rheumatoid arthritis.

The Added Value of Survivin For Risk of RA in the Presence of HLA-DRB1 SE Alleles and ACPA

Survivin positivity was associated with the overall risk for RA (OR 17.91, 95% CI 14.01–22.89, $P < 10E-10$). The survivin positivity was prevalent in the SE-positive (SE+) and in ACPA-positive (ACPA+) RA patients (Figure 2). The association of survivin with any of these factors indicated the increased risk of RA. To analyze the added value of survivin positivity to the risk of RA, the OR was calculated for the survivin-positive compared with survivin-negative subjects within the groups of SE+ACPA-, SE-ACPA+, SE+ACPA+, and those having neither SE nor ACPA (Figure 3). We observed that survivin positivity was consistently associated with the increased RA risk in each of the studied subgroups (Figure 3). The presence of survivin in the SE- and ACPA-negative subjects was associated with the 5.4-fold higher estimated risk of RA compared with the survivin-negative subjects (OR 5.40, 95% CI 3.81–7.66), whereas the highest estimated risk of RA was in the group of combining all 3 biomarkers (Survivin+SE+ACPA+) (OR 16.21, 95% CI 5.70–46.18).

DISCUSSION

The MyEIRA study is the first large Asian case–control population-based epidemiologic study estimating the predictive value of survivin for RA. The study demonstrates the prevalence of high serum levels of survivin in 50.7% of RA patients and in 5.4% of the controls, indicating its high specificity for RA, which allowed to consistently distinguish RA patients from controls. The MyEIRA study comprises patients with short duration of RA, which explains the high prevalence of survivin similar to the one previously shown in the Swedish cohort of early RA patients.[32]

The study demonstrates that survivin is highly associated with 2 established biomarkers predicting development of RA namely the HLA-DRB1 SE alleles and ACPA. The substantial part of survivin-positive RA cases is carriers of the SE alleles (45%) and additional 42% has production of ACPA. We have previously shown that the presence of DRB1*04 and *10 alleles is associated with the increased risk of ACPA-positive RA in the population of Malaysia.[36] The survivin-positive RA patients are over-represented within the carriers of DRB1*01 and DRB1*04 alleles, whereas the DRB1*10 is associated with survivin-positive and with survivin-negative RA. Additionally, survivin-positive RA patients are shown to be frequent carriers of the SE alleles containing [70]QRRAA[74], [70]QKRAA[74], and [70]RRRAA[74] sequences, whereas DRB1*14 (*14:02 and *14:06) alleles containing the identical SE sequence are not enriched in the survivin-positive patients. In contrast to ACPA-positive RA,[14,15] we observe no negative association between the presence of survivin and the frequency of nonpredisposing DRB1 alleles containing [70]DERAA[74] sequence.

The prevalence of SE and ACPA alone or in combination was significantly higher in the survivin-positive RA patients and may be considered a limitation in this study. To estimate the value of survivin for the development of RA, we validate its role in ACPA-negative patients. The specificity of survivin for RA appears to be high and shows no decline in the absence of ACPA, which indicates that survivin-positivity accounts for a significantly increased risk for development of RA irrespective of the SE and ACPA status. The measurement of survivin may be of unique value for ACPA-negative RA patients where specific biomarkers of the disease are missing.

Selection of RA patients among the hospital-referred subjects with similar baseline characteristics, in which the presence of autoantibodies has high diagnostic value, could be a major concern of this observational study. The internal validity is assured by the broad enrolment programme, which included 8 rheumatology centers of Malaysia. Carefully matched controls are recruited to the study in the residential
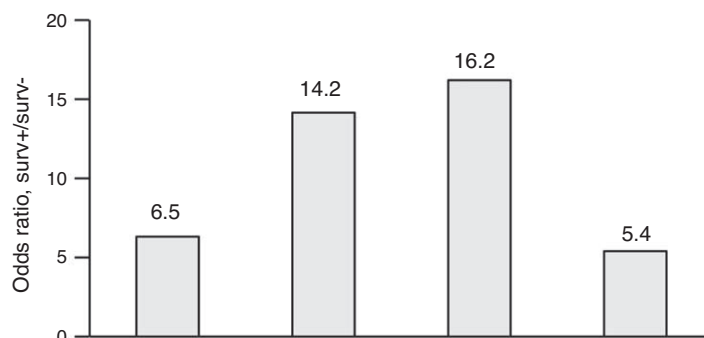
RA/Co	ACPA-SE+	ACPA+SE-	ACPA+SE+	ACPA-SE-
Surv +, n	26/13	265/1	254/3	80/68
Surv -, n	72/235	131/7	141/27	264/1212
95% CI	3.2-13.4	1.7–116	4.8–54.4	3.8–7.7
P-value	*P*<0.0001	*P* = 0.003	*P* <0.0001	*P* <0.0001

FIGURE 3. The added value of high survivin to the relative risk of RA. The added risk of RA was estimated as the OR calculated for the survivin-positive subjects (Surv+) within the ACPA-negative noncarriers of SE (SE−ACPA−), ACPA-positive carriers of SE (SE+ACPA+); ACPA-negative carriers of SE (SE+ACPA−); and ACPA-positive noncarriers of SE (ACPA+SE−). ACPA = antibodies to citrullinated peptides, Co = controls, OR = odds ratio, RA = rheumatoid arthritis, SE = shared epitope.

areas of the patients. Importantly, the ethnic differences of the Malaysian population are represented in the RA cases and in the control group. The obtained results are consistent within all ethnic subgroups. This strengthens the external validity of the study and permits extrapolation of the results to more universal population of RA patients.

In the clinical context, simultaneous presence of SE alleles with survivin and ACPA may reflect common biological pathways in RA. Indeed, survivin-positive RA patients demonstrate common features to ACPA-positive subset of patients. Similar with ACPA, the high levels of survivin may be measured in the preclinical stage of the disease and predicted development of RA.[31] At early stages of RA, survivin predicts joint destructive course of disease,[32] and resistance to antirheumatic treatment.[33] Interestingly, high levels of survivin are frequently found in association with smoking and ACPA, and a combination of these parameters predicts high levels of survivin.[38]

Taken together, the present study demonstrates that survivin is a valuable biomarker specific for RA. Survivin positivity accounts for the increased susceptibility for RA in the subjects negative for SE and ACPA. Survivin is frequently detected in association with SE alleles and ACPA and combination of these parameters increases further the risk of RA and may assist its preclinical diagnosis.

ACKNOWLEDGMENTS

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