Serum biomarker analysis in patients with recurrent spontaneous abortion

YING WU[1], JUNQIN HE[1], CHUNYU GUO[1], YING ZHANG[1], WEI YANG[1], MINGWEI XIN[1], XINYUN LIANG[1], XIAODAN YIN[1], JINGSHANG WANG[1] and YANFENG LIU[2]

[1]Department of Traditional Chinese Medicine, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing 100026; [2]Department of Gynecology, Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing 100700, P.R. China

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Abstract. Recurrent spontaneous abortion (RSA) occurs in 1-5% of parturients. The sustained therapy and research for RSA is expensive, which is a serious issue faced by both patients and doctors. The aim of the present study was to detect protein expression profiles in the serum of RSA patients and healthy controls, and to identify potential biomarkers for this disease. A 1,000-protein microarray consisting of a combination of Human L-507 and L-493 was used. The microarray data revealed that eight serum protein expression levels were significantly upregulated and 143 proteins were downregulated in RSA patients compared with the healthy controls. ELISA individually validated 5 of these 151 proteins in a larger cohort of patients and control samples, demonstrating a significant decrease in insulin-like growth factor-binding protein-related protein 1 (IFGBP-rp1)/IGFBP-7, Dickkopf-related protein 3 (Dkk3), receptor for advanced glycation end products (RAGE) and angiopoietin-2 levels in patients with RSA. Sensitivity and specificity analyses were calculated by a receiver operating characteristics curve, and were revealed to be 0.881, 0.823, 0.79 and 0.814, with diagnostic cut-off points of 95.44 ng/ml for IFGBP-rp1, 32.84 ng/ml for Dkk3, 147.27 ng/ml for RAGE and 441.40 ng/ml for angiopoietin-2. The present study indicated that these four proteins were downregulated in RSA samples and may be useful as biomarkers for the prediction and diagnosis of RSA. Subsequent studies in larger-scale cohorts are required to further validate the diagnostic value of these markers.

Correspondence to: Dr Yanfeng Liu, Department of Gynecology, Dongzhimen Hospital, Beijing University of Chinese Medicine, 5 Haiyuncang Hutong, Dongcheng, Beijing 100700, P.R. China
E-mail: 13810123863@163.com

Abbreviations: RSA, recurrent spontaneous abortion; AUC, area under the curve

Key words: recurrent spontaneous abortion, serum biomarker, antibody array

Introduction

Recurrent spontaneous abortion (RSA), also referred to as recurrent miscarriage, habitual abortion or recurrent pregnancy loss, is defined by more than three consecutive miscarriages prior to 20 gestational weeks (1,2). RSA occurs in 1-5% of women during pregnancy (3). The cause of RSA remains unknown; thus, continuing clinical and laboratory investigations are required (4,5). Previous studies have reported that various etiologic factors are involved in certain RSA cases; including chromosome abnormalities, endocrine diseases, uterine abnormalities, placental anomalies, hormonal problems, thrombophilia, infections, nutritional disorders, autoimmune disease and anatomy (6-8). The etiology of RSA remains to be fully elucidated despite numerous studies investigating the above factors. Early prediction of the potential risk of RSA is required to increase live birth rates in patients with RSA (9).

Biomarkers are currently widely used to refine diagnoses, predict disease and monitor the effects of treatment (10). It is established that the human proteome regulates cellular function and determines the phenotype; thus, the identification of relevant proteins is likely to reveal reliable biomarkers for predicting disease (11). A range of potential biomarkers for RSA have been previously reported. Stortoni *et al* (12) reported that expression levels of thrombomodulin were reduced by 45% in patients with RSA compared with healthy individuals, as determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Bao *et al* (13) determined by RT-qPCR, western blot analysis and immunohistochemistry that serum Dickkopf-related protein (Dkk) 1 levels were increased in RSA patients compared with controls. Additional studies are required to validate these potential biomarkers and their prognostic value. Identifying novel RSA biomarkers may improve the diagnosis, safety and efficacy of current therapies for RSA. As one of the most intensely studied protein families in biomedical science, cytokines have been widely investigated as potential disease biomarkers (14). The introduction of high-throughput and high-specificity detection of complex proteins at picomolar and femtomolar quantities, and antibody arrays, are now widely used for mining complex proteomes (15), facilitating

simultaneous screening of numerous secreted signal proteins in complex biological samples (16). However, to the best of our knowledge, no previous study has identified serum RSA biomarkers using antibody array technology. Therefore, the present study used a RayBio® Label-Based (L-Series) Human Antibody Array 1000 Membrane kit (RayBiotech, Inc., Norcross, GA, USA) to identify reliable biomarkers for the prediction of RSA.

Patients and methods

Patients and controls. From January 2014 to March 2015, a total of 60 Chinese patients with a history of RSA were recruited as the patient group from the Department of Traditional Chinese Medicine at the Beijing Obstetrics and Gynecology Hospital. They had normal endocrine levels, and their partners had normal spermatogenesis and sperm function. 'Blood stasis' syndrome (BSS, also known as *Xueyu zheng* in Chinese) is characterized in traditional medicine as 'pain that occurs in a fixed location, dark-purple face or tongue, bleeding, blood spots under the skin, and an astringent pulse' among other features (17). The concept of blood stasis has been interpreted, changed and developed systematically since ancient times (18). All 60 RSA patients exhibited the 'blood stasis' features described above at the time of study. Patient characteristics, including age at diagnosis, gravidities, number of child births and timing of spontaneous abortion, are summarized in Table I. For the control group, 20 Chinese females who had experienced full-term pregnancies were recruited from the Department of Traditional Chinese Medicine at the Beijing Obstetrics and Gynecology Hospital.

Ethical approval and sample collection. All participants signed informed consent forms prior to participation. The present study was approved by the Ethics Committee of the Beijing Obstetrics and Gynecology Hospital, Capital Medical University (Beijing, China; approval no. 2014-KY-001). Whole blood samples were collected from each participant. Serum was collected following blood centrifugation at 550 x g for 10 min at 4˚C, and stored at -80˚C. The sera of 23 RSA patients and 10 healthy subjects were pooled into 6 samples followed by standard processing (19). The samples included those that could be classified as 'blood stasis' 1, 2 and 3, 'non-blood stasis' 1, 2, and 3, and controls 1-6. The order of mixing is presented in Table II. All mixtures were obtained by mixing equal volumes of sera.

Antibody array assay. The 12 samples described above were assayed for the relative expression of 1,000 human proteins. The target proteins included cytokines, chemokines, adipokines, growth factors, angiogenic factors, proteases, soluble receptors and soluble adhesion molecules. A RayBio® Label-Based (L-Series) Human Antibody Array 1,000 Membrane kit (consisting of a combination of Human L-507 and L-493) was used for protein detection in accordance with the manufacturer's protocol. The signals were scanned at a wavelength of 532 nm using an InnoScan 300 Microarray Scanner (Innopsys, Carbonne, France; resolution, 10 μm) and analyzed using RayBio Analysis Tool software (AAH-BLG-1-SW and AAH-BLG-2-SW; RayBiotech, Inc.).

Table I. Characteristics of 60 patients with recurrent spontaneous abortion.

Characteristic	Value
Age at diagnosis[a]	30±2.8
Gravidities[a]	3±0.5
No. of childbirths	
1[b]	5 (8%)
0[b]	55 (92%)
Spontaneous abortions[a]	3±0.5
2[b]	1 (2%)
1[b]	12 (20%)
0[b]	47 (78%)

[a]Data are expressed as the mean ± standard deviation. [b]Data are expressed as the number of patients (% of total).

Table II. Pooling of serum samples.

Pooled serum sample	Original sample
Patients with RSA	
Blood stasis group 1 (thrombus 1)	A1, A2, A3, A4
Blood stasis group 2 (thrombus 2)	A5, A6, A7, A8
Blood stasis group 3 (thrombus 3)	A9, A10, A11, A12
Non-blood stasis group 1 (non-thrombus 1)	C1, C2, C3, C4
Non-blood stasis group 2 (non-thrombus 2)	C5, C6, C7, C8
Non-blood stasis group 3 (non-thrombus 3)	C9, C10, C11
Control	
1	E1, E2, E3
2	E4, E5, E6
3	E7
4	E8
5	E9
6	E10

RSA, recurrent spontaneous abortion.

Detection of protein levels by ELISA. As determined by microarray analysis, serum markers with significant differences in expression levels between patients and healthy individuals were detected in 60 patients and 20 controls using ELISA kits (ELH-TRAPPIN2,ELH-IGFBPRP1,ELH-RAGE,ELH-DKK3 and ELH-Angiopoietin-2; RayBiotech, Inc.) according to the manufacturer's protocol. Trappin-2, insulin-like growth factor-binding protein-related protein 1 (IGFBP-rp1)/IGFBP-7, receptor for advanced glycation end products (RAGE), Dkk3, and angiopoietin-2 levels were detected. Serum samples were incubated at room temperature. Following washing with wash buffer, a prepared biotinylated antibody was added into the microplate to capture the target protein. Following this,

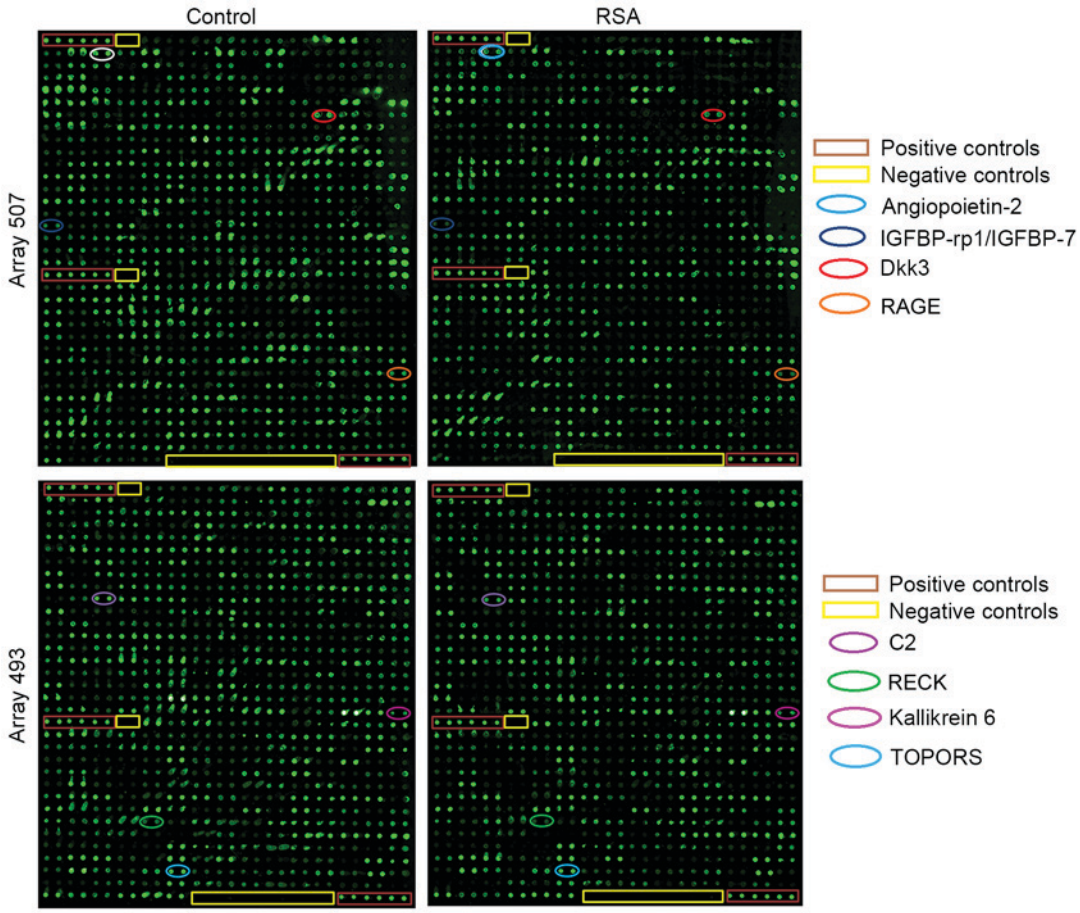

Figure 1. Protein spectra from RayBio L-Series Human 507 (507 proteins) and 493 (493 proteins) antibody arrays. Representative images from human antibody arrays demonstrating the reactivity of pooled serum samples to arrays L series (1,000 proteins) in healthy controls and RSA patients. Each protein was measured in duplicate. A total of eight of significantly different factors on the microarrays are marked in elliptical boxes. IGFBP-rp1/IGFBP-7, insulin-like growth factor-binding protein-related protein 1/insulin-like growth factor-binding protein 7; Dkk3, Dickkopf-related protein 3; RAGE, receptor for advanced glycation end products; RSA, recurrent spontaneous abortion; TOPORS, topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase; C2, complement C2; RECK, reversion-inducing-cysteine rich protein with kazal motifs.

horseradish peroxidase-conjugated streptavidin was used to bind with biotin from the biotinylated antibody. Finally, 1-Step 3,3',5,5'-tetramethylbenzidine-ELISA substrate solution was added followed by stop solution, and absorbance was measured at a wavelength of 450 nm by absorbance microplate reader ELx800 (BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis and bioinformatics. All array data analyses were performed using RayBio Analysis Tool software. Biostatistics and bioinformatics analysis included discriminatory protein analysis and data mining cluster analysis. Statistical differences between two groups were determined by Student's t-test. Fold change values of proteins were used as indicators of relative expression levels. Data mining cluster analysis was used to identify potential biomarkers by clustering all relevant proteins according to the similarity of their expression profiles using Cluster software version 3.0 (http://cluster2.software.informer.com/3.0). ELISA data was analyzed using SigmaPlot software version 12.0 (Systat Software, Inc., San Jose, CA, USA). T- and F-tests were used to analyze ELISA quantification. The receiver operating characteristics curve (ROC) method was used to assess sensitivity and specificity of potential biomarkers using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of antibody microarrays. A total of 1,000 proteins were measured in the serum mixture using the microarray. The spectra of 1,000 proteins from eight samples are presented in Fig. 1. The results demonstrated that 151 proteins had significantly different expressions between the two groups. Of these differential proteins, eight were significantly upregulated, and 143 proteins were downregulated in RSA patients compared with controls (Table III). Fig. 2 presents are boxplots of the fluorescence signal values of eight differential proteins, selected for signal strength, fold changes and clinical significance. Serum mixture samples were arranged by similarities in the abundance of these 151 markers in the sera clustering algorithm, which produced two clusters that contained patients and healthy individuals (Fig. 3).

Validation of microarray data by ELISA. A total of five of the 151 proteins were selected for validation assay

Table III. A total of 151 proteins with significantly different expression levels between patients with RSA and controls.

	RSA		Control		RSA vs. control		
Target	Mean	SD	Mean	SD	F-test	t-test P-value	Fold-change
A2M	4,650.558	373.053	6,374.645	269.687	0.494	0.000	0.730
ADAMTS-10	7,862.456	1,306.692	13,925.383	550.378	0.081	0.000	0.565
ADAMTS-15	2,999.115	479.112	5,705.655	1,924.875	0.008	0.017	0.526
ADAMTS-5	558.209	250.695	1,466.103	483.403	0.176	0.002	0.381
ADAMTS-L2	20,775.755	2,676.424	27,565.213	1,305.789	0.141	0.000	0.754
ALK	7,976.603	1,428.122	13,199.048	2,632.151	0.206	0.002	0.604
Angiopoietin-2	20,789.029	4,292.976	48,570.398	4,977.529	0.753	0.000	0.428
ApoC2	14,305.316	2,066.666	19,754.508	2,309.965	0.813	0.002	0.724
ApoH	4,898.886	462.884	7,760.884	1,065.437	0.091	0.000	0.631
ApoM	1,502.749	273.114	3,924.876	654.067	0.078	0.000	0.383
APP	12,633.899	1,318.521	19,428.245	5,101.616	0.010	0.021	0.650
Axl	1,510.055	1,117.320	4,936.198	495.598	0.099	0.000	0.306
BAF57	1,181.342	265.140	1,906.967	361.301	0.513	0.003	0.619
BAFF R/TNFRSF13C	130.169	152.078	1,362.334	162.415	0.889	0.000	0.096
Bax	15,217.862	3,242.826	38,315.362	7,712.389	0.080	0.000	0.397
BDNF	13,455.044	6,688.395	25,250.436	5,906.534	0.792	0.009	0.533
β 2M	1,320.861	133.263	1,910.310	294.761	0.106	0.001	0.691
BIK	311.407	134.485	1,179.513	269.458	0.153	0.000	0.264
BMP-3	10,702.264	3,851.243	28,110.700	15,617.736	0.008	0.041	0.381
BMP-3b/GDF-10	124.436	41.431	347.417	122.473	0.033	0.005	0.358
BMP-4	901.991	2,56.578	1,767.609	343.897	0.536	0.001	0.510
BMPR-IB/ALK-6	23,780.461	9648.386	62,589.402	6,422.893	0.393	0.000	0.380
BTC	11,944.615	4,921.916	21,760.107	3,620.813	0.517	0.003	0.549
C2	22,604.021	2,719.955	29,647.219	2,700.060	0.988	0.001	0.762
C5/C5a	12,737.991	1,525.086	22,749.974	7,304.236	0.004	0.019	0.560
Calsyntenin-1	1,911.570	607.215	4,505.218	691.477	0.782	0.000	0.424
CD40/TNFRSF5	1,101.031	337.593	3,397.425	678.353	0.152	0.000	0.324
Chordin-Like 1	1,391.942	585.338	3,128.589	566.356	0.944	0.000	0.445
CNTF R α	77,532.324	10,164.182	114,914.734	12,924.497	0.611	0.000	0.675
Contactin-1	3,977.884	438.734	6,093.532	1,458.926	0.020	0.015	0.653
Cripto-1	8,176.343	2,831.476	18,724.736	4,786.781	0.274	0.001	0.437
CRTH-2	34,856.201	6,547.851	55,742.907	8,503.753	0.580	0.001	0.625
CXCR4 (fusin)	3,702.606	1,090.358	11,095.207	6,134.514	0.002	0.031	0.334
Dkk-3	14,387.266	3,452.455	31,716.357	11,437.946	0.020	0.012	0.454
DLL4	1,759.891	629.238	3,330.977	898.310	0.453	0.006	0.528
EDAR	1,240.627	223.958	4,773.756	1,971.378	0.000	0.007	0.260
EGF R/ErbB1	11,026.167	1,733.072	15,758.956	2,371.519	0.508	0.003	0.700
EG-VEGF/PK1	29,916.922	4,877.762	51,349.446	16,211.012	0.020	0.022	0.583
EMAP-II	10,666.342	2,600.714	19,708.795	3,896.837	0.395	0.001	0.541
EphB4	1,962.256	406.898	5,161.814	945.891	0.088	0.000	0.380
ErbB2	4,267.172	1,130.288	15,536.343	9,241.970	0.000	0.030	0.275
ESAM	2,854.186	495.592	5,990.106	2,581.532	0.002	0.030	0.476
FAM3B	1,270.946	447.324	4,455.137	885.200	0.160	0.000	0.285
FGF R4	3,992.796	1,049.190	12,794.810	7,288.004	0.001	0.031	0.312
FGF R5	2,826.176	1,013.809	7,878.562	1,003.601	0.983	0.000	0.359
FGF-19	1,641.777	369.438	4,442.636	778.147	0.128	0.000	0.370
FGF-9	6,413.361	1,443.488	22,180.535	5,185.520	0.014	0.000	0.289
FGFR1	11,978.622	1,527.435	16,683.655	4,343.023	0.039	0.045	0.718
FGFR2	13,703.293	2,914.828	23,307.209	7,653.038	0.054	0.017	0.588

Table III. Continued.

Target	RSA		Control		RSA vs. control		
	Mean	SD	Mean	SD	F-test	t-test P-value	Fold-change
Ficolin-3	3,374.750	486.123	8,120.462	4,212.321	0.000	0.040	0.416
Follistatin-like1	2,481.289	721.297	7,152.976	1,161.224	0.319	0.000	0.347
Galectin-1	1,435.877	634.069	3,694.218	803.285	0.616	0.000	0.389
Galectin-3BP	10,655.461	1,406.056	13,522.242	1,910.307	0.517	0.014	0.788
Gas1	4,530.505	1,493.529	7,110.508	1,228.339	0.678	0.008	0.637
GASP-1/WFIKKNRP	85,406.876	7,073.932	128,207.327	24,054.577	0.018	0.006	0.666
GATA-3	7,625.978	591.277	13,276.727	2,655.282	0.005	0.003	0.574
GCP-2/CXCL6	847.677	514.092	2,243.977	1,012.301	0.163	0.013	0.378
GLO-1	997.491	290.387	2,106.667	501.997	0.255	0.001	0.473
Glucagon	61,025.896	9,853.803	128,506.268	35,031.652	0.015	0.004	0.475
GluT2	11,546.660	704.259	37,514.070	2,261.457	0.023	0.000	0.308
Glypican 3	1,389.140	249.379	2,554.322	649.947	0.056	0.002	0.544
Glypican 5	15,529.644	2,266.574	25,519.026	7,257.053	0.023	0.018	0.609
GPX1	2,008.854	557.930	4,383.148	782.893	0.475	0.000	0.458
GPX3	3,009.541	1,283.488	5,083.531	927.399	0.493	0.009	0.592
GRP78	2,266.940	302.999	4,387.312	1,150.085	0.011	0.005	0.517
Hemopexin	725.265	151.625	3,871.951	913.350	0.001	0.000	0.187
HRG-α	5,321.121	1,385.473	7,078.008	834.906	0.291	0.024	0.752
HSP10	26,971.939	3,802.952	43,781.370	10,058.305	0.052	0.003	0.616
I-309	4,283.951	2,116.728	13,856.485	3,913.389	0.204	0.000	0.309
IBSP	7,712.034	986.133	10,133.874	1,761.061	0.229	0.015	0.761
IGFBP-4	1,453.473	684.730	4,146.757	1,511.595	0.107	0.003	0.351
IGFBP-rp1/IGFBP-7	7,523.924	2,135.058	16,380.247	2,502.068	0.736	0.000	0.459
IGF-II	65,869.138	5,912.640	94,321.230	10,392.485	0.241	0.000	0.698
IL-1 ra	361,872.941	34,398.268	563,878.996	45,414.321	0.011	0.011	0.642
IL-13 R α1	6,360.695	1,012.833	24,083.997	13,484.474	0.000	0.023	0.264
IL-17C	2,345.533	541.033	7,048.395	748.914	0.493	0.000	0.333
IL-18 R β/AcPL	1,935.580	858.981	5,832.686	1,358.705	0.337	0.000	0.332
IL-29	8,733.803	1,800.279	24,866.993	5,813.232	0.022	0.001	0.351
IL-31	1,970.735	687.942	5,958.295	784.836	0.779	0.000	0.331
IL-31 RA	1,182.894	372.281	3,207.266	506.214	0.516	0.000	0.369
IL-33	2,441.589	430.891	3,847.273	1,061.515	0.070	0.013	0.635
IL-6 R	3,540.308	909.773	6,566.421	1,881.755	0.137	0.005	0.539
IL-8	25,002.761	6,204.334	34,829.214	6,706.528	0.869	0.025	0.718
Kallikrein 14	2,979.489	615.746	5,825.747	1,585.646	0.058	0.002	0.511
LBP	3,409.164	463.893	13,342.910	8,052.946	0.000	0.029	0.256
LIF R α	13,797.957	2,293.325	27,604.197	8,923.296	0.010	0.012	0.500
LIF	332.827	242.463	1,628.345	875.661	0.014	0.014	0.204
LIGHT/TNFSF14	2,092.869	917.268	6,360.354	1,227.569	0.538	0.000	0.329
Lipocalin-1	20,832.310	4,151.253	34,570.502	9,172.736	0.107	0.007	0.603
Livin	2,707.067	795.904	4,194.195	745.424	0.889	0.007	0.645
LRG1	11,113.403	863.592	28,581.729	14,531.460	0.000	0.032	0.389
Lymphotoxin β R/TNFRSF3	788.596	380.913	3,291.572	764.370	0.153	0.000	0.240
M-CSF	28,394.735	5,351.774	41,766.017	11,016.348	0.139	0.023	0.680
Midkine	3,267.246	433.600	5,032.121	1,617.032	0.012	0.044	0.649
MIF	3,951.056	885.814	6,986.334	1,120.792	0.618	0.000	0.566
MIP-1α	50,222.493	12,359.813	94,756.426	25,311.008	0.142	0.003	0.530
MMP-11/Stromelysin-3	54,153.970	12,843.620	69,764.345	7,386.564	0.250	0.027	0.776
MMP-16/MT3-MMP	11,330.829	2,524.054	26,764.023	11,916.806	0.004	0.024	0.423

Table III. Continued.

Target	RSA		Control		RSA vs. control		
	Mean	SD	Mean	SD	F-test	t-test P-value	Fold-change
MMP-8	52,067.139	3,933.472	70,211.908	7,728.075	0.165	0.000	0.742
MSP α Chain	13,201.992	2,412.896	25,409.389	4,525.441	0.194	0.000	0.520
NEP	1,581.827	658.480	3,958.249	666.439	0.980	0.000	0.400
NM23-H1/H2	550.675	189.223	2,651.125	1,022.967	0.002	0.004	0.208
Orexin B	43,718.747	7,996.118	74,138.593	18,347.514	0.092	0.004	0.590
Osteoactivin/GPNMB	2,895.689	312.735	5,540.870	1,872.568	0.001	0.017	0.523
PD-1	2,591.490	389.734	4,141.350	1,038.516	0.051	0.007	0.626
PDGF-C	9,264.920	2,881.398	24,377.899	9,157.178	0.024	0.008	0.380
PDGF-D	2,883.630	396.751	5,130.131	907.360	0.093	0.000	0.562
PDX-1	2,270.140	363.742	3,981.057	824.869	0.097	0.001	0.570
PEPSINOGEN I	2,439.074	824.492	6,093.801	1,335.963	0.313	0.000	0.400
Persephin	2,515.448	1,011.303	4,523.807	838.775	0.691	0.004	0.556
PGRP-S	12,316.910	730.024	16,966.564	1,180.649	0.315	0.000	0.726
PIM2	1,865.699	437.191	4,879.191	1,260.593	0.036	0.001	0.382
PKM2	2,655.795	1,099.697	4,528.436	478.749	0.092	0.003	0.586
RAGE	9,020.357	1,947.254	25,325.305	10,739.884	0.002	0.013	0.356
RANK/TNFRSF11A	2,020.058	587.942	4,020.679	833.497	0.462	0.001	0.502
RECK	5,084.724	891.920	11,081.323	3,186.035	0.014	0.005	0.459
RELT/TNFRSF19L	31,871.125	4,886.989	61,866.224	17,568.797	0.014	0.007	0.515
ROBO4	66,732.387	11,925.740	100,118.428	17,349.652	0.430	0.003	0.667
S100A10	1,189.482	653.171	4,146.007	336.156	0.171	0.000	0.287
S100A4	4,024.174	328.979	5,366.717	482.437	0.421	0.000	0.750
S100A6	3,485.252	541.367	6,023.360	1,041.092	0.178	0.000	0.579
Serpin A8	1,755.946	304.447	3,270.617	704.644	0.089	0.001	0.537
Serpin A9	1,258.179	327.453	2,710.239	999.220	0.029	0.015	0.464
Smad 1	1,028.273	540.825	3,720.431	748.020	0.494	0.000	0.276
Smad 7	34,565.690	2,075.517	55,551.432	18,891.474	0.000	0.042	0.622
Smad 8	6,886.663	2,599.832	15,373.512	4,688.229	0.221	0.003	0.448
SOST	3,117.271	461.906	6,218.660	1,701.605	0.012	0.006	0.501
Spinesin	20,626.782	4,848.625	38,828.941	4,444.642	0.853	0.000	0.531
Syndecan-1	1,772.888	379.277	3,778.232	1,215.352	0.023	0.008	0.469
Thrombospondin-4	10,921.569	2,002.073	23,198.652	9,994.362	0.003	0.029	0.471
TIM-1	2,132.294	259.727	4,438.018	697.608	0.049	0.000	0.480
TIMP-3	4,008.775	1,259.727	6,988.724	1,853.036	0.417	0.009	0.574
TRADD	127,030.588	28,309.796	222,266.652	75,285.718	0.051	0.016	0.572
TRAIL R2/DR5/TNFRSF10B	17,190.463	4,094.960	32,861.735	13,104.866	0.023	0.032	0.523
Trappin-2	5,865.048	978.164	19,388.585	11,625.715	0.000	0.036	0.303
TROY/TNFRSF19	2,324.349	786.973	5,012.391	1,945.216	0.069	0.011	0.464
TRPC1	2,436.721	681.450	6,693.017	2,511.517	0.012	0.008	0.364
TSLP	1,853.969	303.884	5,110.938	2,119.578	0.001	0.013	0.363
TSLP R	1,905.470	965.346	6,488.242	1,625.517	0.277	0.000	0.294
Ubiquitin+1	17,529.029	5,002.370	34,561.192	16,122.797	0.023	0.049	0.507
uPA	52,463.526	6,879.542	94,616.706	25,331.681	0.012	0.008	0.554
VEGF-D	24,467.055	5,668.534	52,501.169	6,836.652	0.691	0.000	0.466
WISP-1/CCN4	2,330.112	1,166.842	5,516.779	2,727.490	0.086	0.025	0.422
GASP-2/WFIKKN	71,246.693	3,344.127	31,422.325	19,817.529	0.001	0.004	2.267
IL-1 F5/FIL1δ	30,158.276	6,083.967	13,083.494	8,056.469	0.553	0.002	2.305
IL-28A	71,058.941	7,420.008	30,883.742	18,108.416	0.072	0.001	2.301
Kallikrein 6	18,588.432	1,695.500	10,786.937	3,766.885	0.104	0.001	1.723

Table III. Continued.

Target	RSA		Control		RSA vs. control		
	Mean	SD	Mean	SD	F-test	t-test P-value	Fold-change
NGF R	42,876.389	1,905.084	1,8740.985	13,128.177	0.001	0.006	2.288
NrCAM	73,805.167	6,435.403	5,1415.054	8,782.973	0.511	0.001	1.435
TOPORS	49,264.099	3,557.276	3,4240.228	8,383.960	0.083	0.002	1.439
VEGF R2 (KDR)	37,152.074	4,541.513	1,2904.085	9,743.845	0.119	0.000	2.879

SD, standard deviation; RSA, recurrent spontaneous abortion.

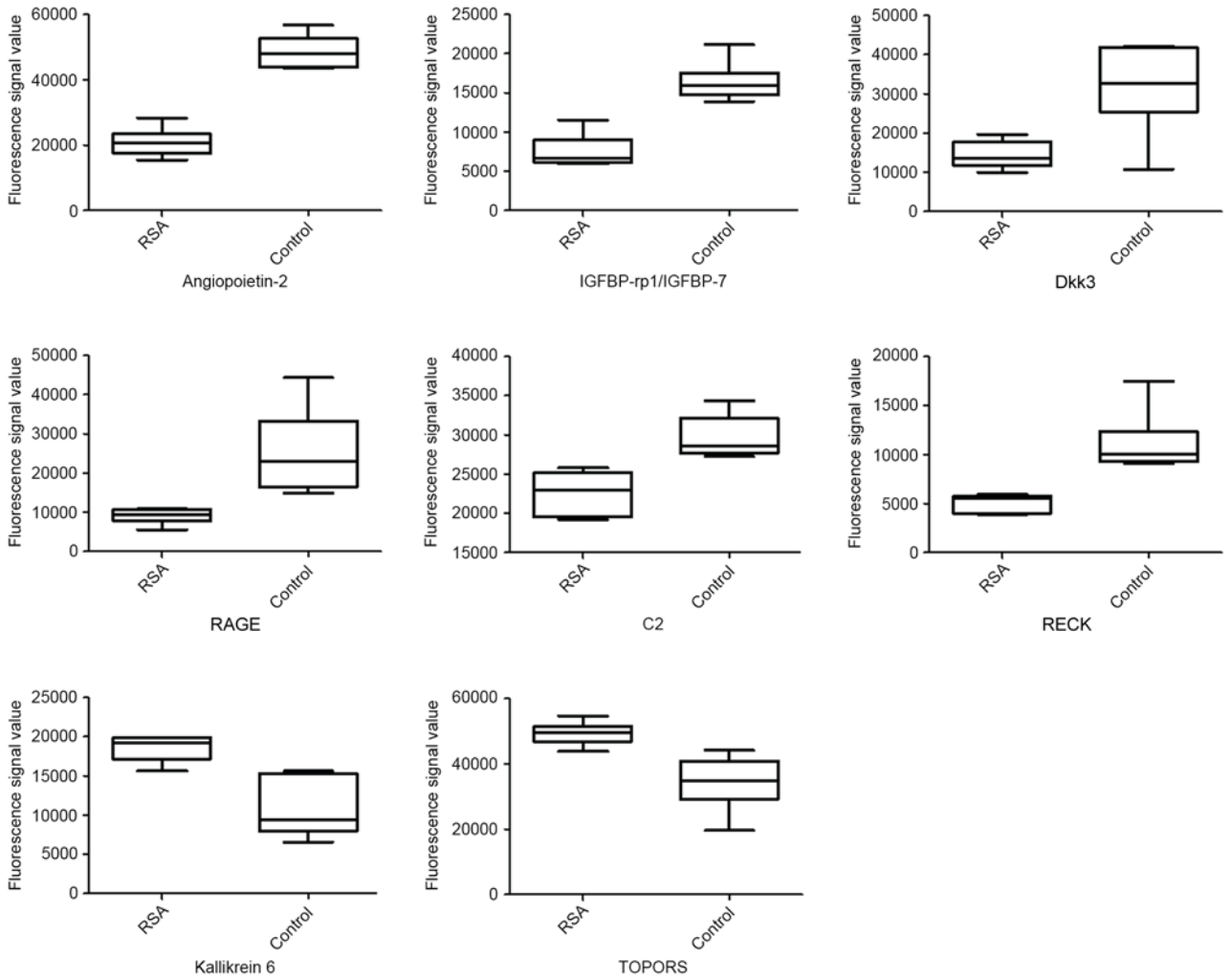

Figure 2. Boxplots of differential serum proteins between RSA patients and controls. P<0.05, RSA vs. control for all presented proteins. ANG-2, angiopoietin 2; IGFBP-rp1/IGFBP-7, insulin-like growth factor-binding protein-related protein 1/insulin-like growth factor-binding protein 7; Dkk3, Dickkopf-related protein 3; RAGE, receptor for advanced glycation end products; RSA, recurrent spontaneous abortion; TOPORS, topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase; C2, complement C2; RECK, reversion-inducing-cysteine rich protein with kazal motifs.

in 60 RSA and 20 control samples. Serum levels of trappin-2, IGFBP-rp1/IGFBP-7, RAGE, Dkk3 and angiopoietin-2 were selected to be measured by ELISA based on the results from the microarray experiments, previous reports on serum biomarkers in RSA and the availability of commercial test kits. Levels ofIGFBP-rp1/IGFBP-7, Dkk3, RAGE and angiopoietin-2 were downregulated in RSA patients compared with healthy controls, which was consistent with the microarray results (P<0.05; Table IV and Fig. 4).

Analysis of sensitivity and specificity of serum biomarkers for RSA. To validate whether IGFBP-rp1/IGFBP-7, Dkk3,

Table IV. ELISA analysis of cytokine levels in the serum of patients with RSA and healthy controls.

			Patients vs. control		
Cytokine	RSA	Control	F-test	t-test P-value	Fold-change
Trappin-2	429.17±125.17	453.26±132.34	0.718	0.473	0.947
IGFBP-rp1/IGFBP-7	86.94±16.49	115.63±20.12	0.246	0.000[a]	0.752
RAGE	91.29±44.28	163.64±76.99	0.001	0.001[a]	0.558
Dkk3	28.16±6.22	38.96±10.05	0.005	0.000	0.723
Angiopoietin-2	461.34±484.38	887.72±576.22	0.312	0.002[a]	0.520

[a]P<0.05, RSA vs. control. Data are expressed as the mean ± standard deviation. IGFBP-rp1/IGFBP-7, insulin-like growth factor-binding protein-related protein 1/insulin-like growth factor-binding protein 7; Dkk3, Dickkopf-related protein 3; RAGE, receptor for advanced glycation end products; RSA, recurrent spontaneous abortion.

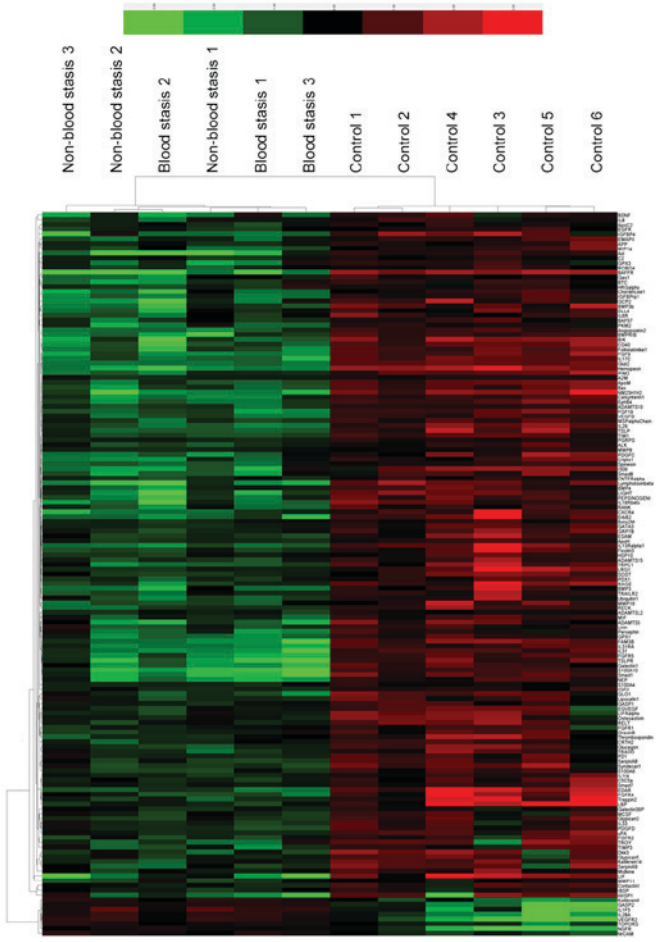

Figure 3. Cluster map of protein expression levels of the 151 proteins in 12 mixed serum samples. The signal values of the 151 proteins from microarray analyses were used to prepare the cluster map. Red shades indicate higher expression levels, green shades indicate lower expression levels, and black shades indicate median expression levels.

RAGE and angiopoietin-2 may be used as biomarkers for predicting RSA, ROC curves were used to analyze sensitivity and specificity. Area-under-ROC-curve values for IGFBP-rp1/IGFBP-7 (Fig. 5A), Dkk3 (Fig. 5B), RAGE (Fig. 5C) and angiopoietin-2 (Fig. 5D) cytokines were 0.881, 0.823, 0.79 and 0.814, respectively. IGFBP-rp1/IGFBP-7 had a sensitivity of 95% and specificity of 78.33%. Dkk3 had a sensitivity of 80% and specificity of 83.33%. RAGE had a sensitivity of 65% and specificity of 86.70%. Angiopoietin-2 had a sensitivity of 90% and specificity of 64.40%. All these were deemed suitable biomarkers for the prediction of RSA.

Discussion

Potential biomarkers of RSA have previously been reported. Khonina *et al* (20) investigated whether mixed lymphocyte reaction blocking factor may be used as an indicator of the efficacy for immunotherapy with paternal lymphocytes in females with RSA. Metwally *et al* (21) performed a proteomic analysis of obese and overweight women with RSA by 2-D gel electrophoresis, principle component analysis and mass spectrometry, and demonstrated that RSA patients exhibit a significant increase in haptoglobin expression. Ibrahim *et al* (22) demonstrated that pentraxin-3 indicates the presence of abnormally exaggerated intrauterine inflammation that may cause pregnancy failure in females with unexplained RSA. Kim *et al* (23) identified RSA-associated factors in human blood samples by 2-D gel electrophoresis, and analyzed spots samples with matrix-assisted laser desorption/ionization-time of flight/mass spectrometry, and reported that in RSA patients, inter-α-trypsin inhibitor heavy chain family member 4 (ITI-H4) expression was low and exhibited a molecular weight of 120 kDa in controls; however, ITI-H4 was expressed at higher levels and at a modified molecular weight of 36 kDa in the RSA patient group. This indicated that ITI-H4 may be used as biomarker of RSA.

The present study used antibody array technology for a primary screening of RSA biomarkers on pooled samples. The array results revealed that the levels of eight cytokines were significantly increased in the RSA patient group compared with controls, and the levels of 143 of the tested 1,000 proteins were significantly reduced in the RSA patient group compared with controls. A total of 5 proteins, trappin-2, IGFBP-rp1/IGFBP-7, Dkk3, RAGE and angiopoietin-2, were selected for ELISA

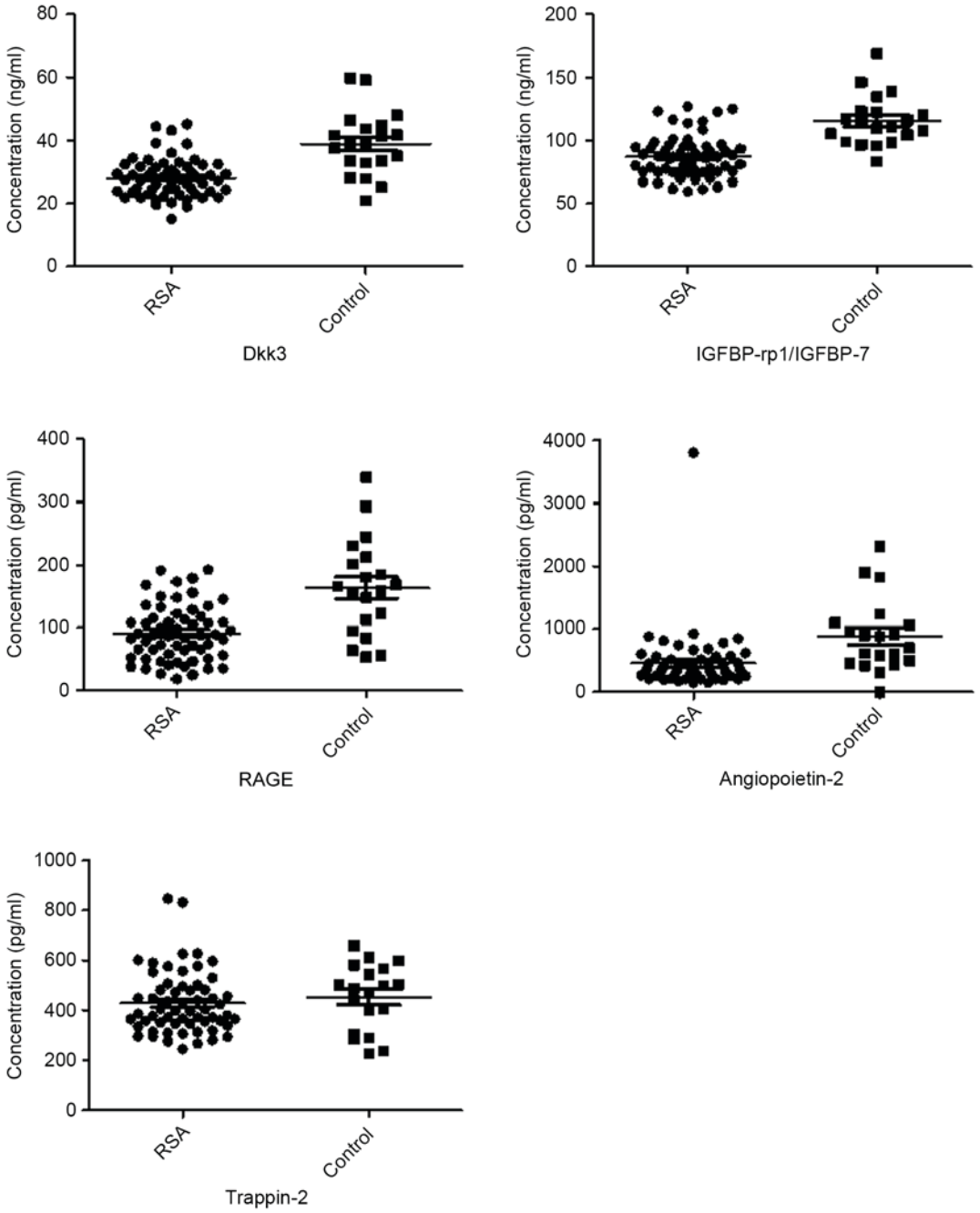

Figure 4. Validation of five differentially expressed proteins obtained from microarray analysis, and validated by ELISA. Concentrations of these factors in serum samples obtained from RSA patients and healthy controls were calculated using the four parameters method. IGFBP-rp1/IGFBP-7, insulin-like growth factor-binding protein-related protein 1/insulin-like growth factor-binding protein 7; Dkk3, Dickkopf-related protein 3; RAGE, receptor for advanced glycation end products; RSA, recurrent spontaneous abortion.

validation assay in a larger cohort of patient and control subjects. ELISA results for these proteins were in accordance with the array results. Sensitivity and specificity analysis by ROC revealed that these four cytokines may be used as biomarkers of RSA.

To the best of our knowledge, the association between IGFBP-rp1/IGFBP-7, Dkk3 and angiopoietin-2, and RSA has not been reported. However, an isoform of the RAGE protein, sRAGE, has been reported to be associated with RSA (24).

IGFBP-rp1/IGFBP-7. IGFs, which have characteristics of tissue growth factors and circulating growth hormones, are potent mitogens and anti-apoptotic agents (25). IGFs include the hormones IGF-I and -II and their corresponding receptors, and the IGFBPs (26). The IGFBP superfamily includes six members (IGFBP-1-6) and 10 associated proteins (IGFBP-rp1-10) (27). IGFBP-7 has been demonstrated to be a tumor suppressor in a variety of cancers. Benatar *et al* (28) reported that treatment with IGFBP-7 may have therapeutic potential for triple-negative breast cancer. Liu *et al* (29) demonstrated that IGFBP-7 was downregulated in gastric cancer, and that it may be used as an indicator of poor prognosis in patients with gastric cancer. IGFBP-7 has additionally been proposed as a novel biomarker for assessing the risk of

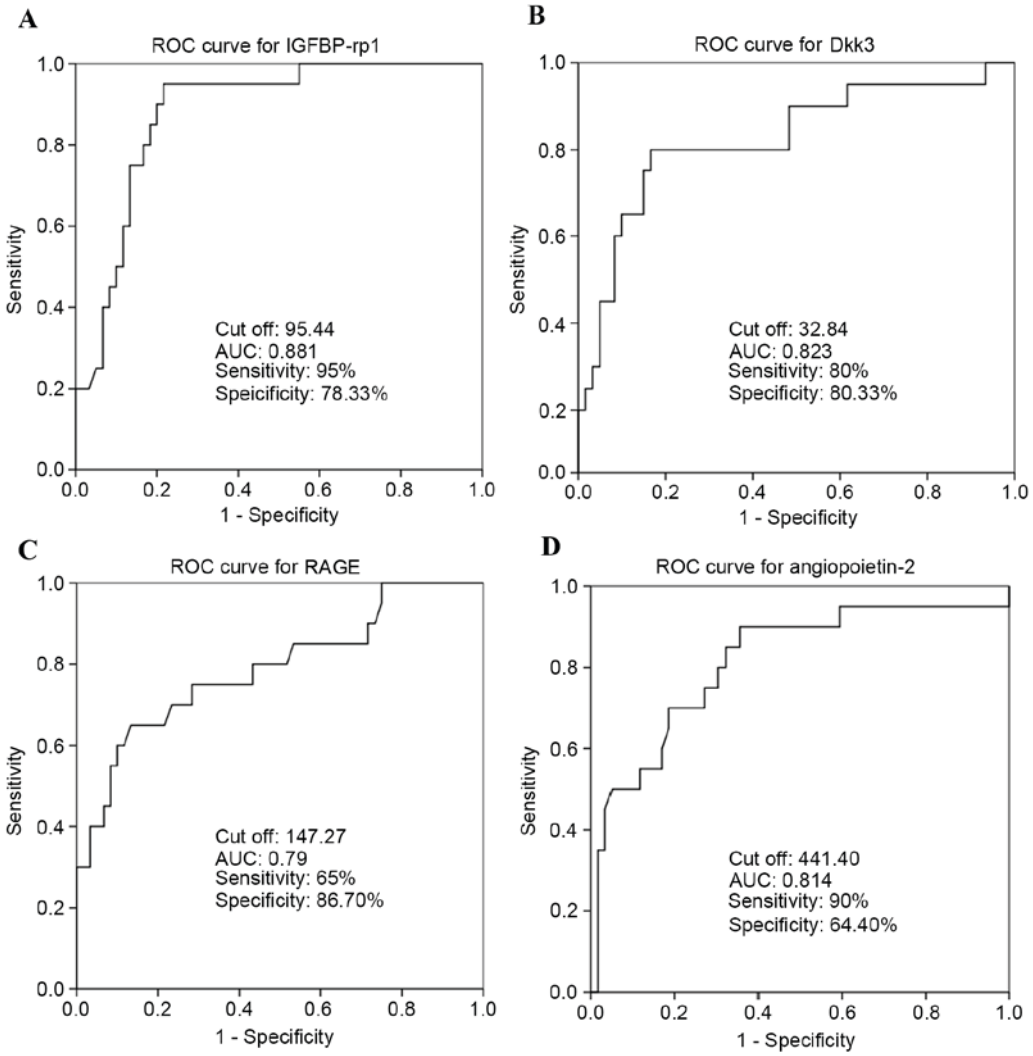

Figure 5. ROC curve analysis for the four upregulated serum cytokines as validated by ELISA. The area under the ROC curve (AUC) indicates the mean sensitivity of the biomarkers (A) IGFBP-rp1, (B) Dkk3, (C) RAGE and (D) angiopoietin-2 (Fig. 5D). 0.5≤AUC≤1, the biomarker is strongly differential between patients and controls; AUC≤0.5, no predictive value. ROC, receiver operating characteristics curve; IGFBP-rp1/IGFBP-7, insulin-like growth factor-binding protein-related protein 1/insulin-like growth factor-binding protein 7; Dkk3, Dickkopf-related protein 3; RAGE, receptor for advanced glycation end products; RSA, recurrent spontaneous abortion.

acute kidney injury (30) and heart failure with reduced ejection fraction, and has been demonstrated to have links to the presence and severity of echocardiographic parameters of abnormal diastolic function (31).

Dkk3. The Wnts are an evolutionarily conserved family of secreted glycoproteins characterized by numerous conserved cysteine residues (32). The Dkk proteins are secreted Wnt inhibitors, inducing removal of the Wnt co-receptor low-density lipoprotein receptor-related protein, and consist of four primary members in vertebrates (Dkk1-4) (33,34). Dkk-3 is downregulated in various types of cancer cells. Loss of Dkk3 protein expression is associated with poor prognoses in patients with gastric cancer, indicating that it may be a biomarker for predicting lymph node involvement in these patients (35). Dkk3 has recently been implicated in clear cell renal cell carcinoma, and may present a novel molecular target for its diagnosis and treatment (36). Additionally, Dkk3 may represent a therapeutic target for the treatment of heart failure following myocardial infarction (37).

RAGE. RAGE is a cell-surface receptor that interacts with AGEs, and is a member of the immunoglobulin superfamily (38). RAGE activation via its multiple ligands, including S100 calcium-binding protein (S100A) 4 (39), high mobility group box 1 protein (40) and amyloid-β protein (41), serves important roles in certain diseases. Dahlmann *et al* (42) demonstrated that the activity of S100A4-RAGE induces RAGE-dependent increases in the migratory and invasive capabilities of colorectal cancer cells. Guo *et al* (43) identified RAGE as a potential prognostic biomarker in renal cell carcinoma. RAGE/S100A7 signaling has been demonstrated to have a functional role in linking inflammation to aggressive breast cancer development; therefore, RAGE expression is

currently regarded as a potential biomarker for triple-negative breast cancer (44). Additionally, overexpression of RAGE may be a useful marker to predict gastric cancer progression (45).

Angiopoietin-2. As a member of the angiopoietin family, angiopoietin-2 has complex and unique roles in regulating angiogenesis, and has additional unconventional functions, including stimulating tumor angiogenesis, invasion and metastasis via Tie2-independent signaling pathways, involving integrin-mediated signaling. Therefore, angiopoietin-2 may have great potential as a therapeutic target, prognostic marker and inhibitor of human cancer (46). Angiopoietin-2 is expressed during vascular remodeling, thus preventing vascular stability (47). A study by Morrissey *et al* (48) demonstrated that angiopoietin-2 inhibition impeded tumor growth of LuCaP 23.1 prostate cancer xenografts, and suggested that angiopoietin-2 inhibition in combination with other treatments is a potential therapy for metastatic disease patients. Calfee *et al* (49) reported that lowering plasma angiopoietin-2 with fluid conservative therapy may be beneficial, in part by decreasing endothelial inflammation. Goede *et al* (50) demonstrated that serum angiopoietin-2 represents a candidate biomarker for the outcome of metastatic colorectal cancer patients treated with bevacizumab-containing therapy. Additionally, angiopoietin-2 has been associated with other diseases, including chronic kidney disease (51) and cerebral malaria (52).

In conclusion, the present study used a microarray platform to detect 1,000 proteins to identify dysregulated serum factors in RSA samples. This method was demonstrated to be effective in investigating dynamic alterations in protein profiles, and to select target proteins for further RSA research. The results indicated that IGFBP-rp1/IGFBP-7, Dkk3, RAGE and angiopoietin-2 expression were downregulated in RSA patients, suggesting that they may be important in the pathological process of RSA. Furthermore, upregulating them may inhibit the development of RSA. Therefore, these biomarkers represent potential predictive and diagnostic markers for RSA due to their high sensitivity and specificity. However, larger-scale studies are required to confirm the diagnostic value of these markers.

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