Hirosaki Med. J. 63：12—20, 2012

ORIGINAL ARTICLE

IS ANALYSIS OF EXHALED BREATH CONDENSATE AN EQUIVALENT TO BRONCHOALVEOLAR LAVAGE FLUID IN SARCOIDOSIS PATIENTS?

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Abstract Introduction: Although analysis of bronchoalveolar lavage fluid (BALF) is a useful examination to assess airway inflammation, it is an invasive technique with limitation and risk of complications.
Objectives: The aim of this study was to examine molecules in exhaled breath condensate (EBC) in comparison with BALF, and to clarify the clinical usability of EBC.
Methods: EBC was collected from sixteen subjects suspected to have sarcoidosis just before BAL. The 40 different inflammatory molecules in EBC and BALF were analyzed by protein array.
Results: BALF levels of 6 molecules including soluble tumor necrosis factor receptor type II (sTNF-RII) and regulated upon activation, normal T cell expressed and secreted (RANTES), and EBC levels of 13 molecules including sTNF-RII and RANTES were significantly correlated with percentage of lymphocyte in BALF (%Lym). We found significant correlations between levels of EBC and BALF in 16 out of 40 molecules. Levels of macrophage colony-stimulating factor (M-CSF), RANTES, TNF-α and sTNF-RII in EBC were significantly correlated with BALF. Their levels in EBC and BALF also were correlated with %Lym.
Conclusion: Protein array, a highly sensitive approach allowed us to detect inflammatory molecules in EBC. Comprehensive analysis of EBC might be an equivalent to that of BALF.

Hirosaki Med. J. 63：12—20, 2012

Key words: Inflammatory molecules; protein array analysis; sarcoidosis; exhaled breath condensate; bronchoalveolar lavage.

原　著

呼気凝縮液の有用性に関する研究 —気管支肺胞洗浄液との比較検討—

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抄録　【背景】気管支肺胞洗浄(BAL)はびまん性肺疾患の診断に有用だが侵襲的である．呼気凝縮液(EBC)は非侵襲的に採取できる．【目的】EBC，気管支肺胞洗浄液(BALF)中の炎症性分子を比較検討し，EBC の有用性を明らかにする．【方法】サルコイドーシスを疑い BAL を行った16名からEBCを採取した．EBC，BALF中の炎症性分子レベルをプロテインアレイで測定した．【結果】BALF 中の 6 種，EBC 中の13種の炎症性分子が BALF 中リンパ球分画(%Lym)と有意に相関した．16種の炎症性分子が EBC，BALF 間で有意に相関した．その内 M-CSF，RANTES，TNF-α，sTNF-RⅡは EBC 中，BALF 中とも %Lym と有意に相関した．【結論】プロテインアレイにより EBC，BALF 中の炎症性分子を高感度に測定し網羅的に解析することができた．EBC の分析は BAL と同等の有用性を有する可能性がある．

弘前医学　63：12—20, 2012

キーワード：プロテイン・アレイ；サルコイドーシス；呼気凝縮液；気管支肺胞洗浄；炎症性サイトカイン．

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Received for publication, November 28, 2011
Accepted for publication, December 28, 2011

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別刷請求先：中村邦彦
平成23年11月28日受付
平成23年12月28日受理

Table 1 Characteristic of study subjects and results of BAL

Patient	Sex	Age (years)	Smoking habit	BALF differential count				
				Recovery	TCC	%Lym	Lym	CD4/CD8
1	Male	37	Ex	73.3	0.44	47	0.21	2.29
2	Male	25	Current	70.7	0.56	23	0.13	4.56
3	Male	37	No	64.0	1.35	14	0.19	4.56
4	Female	33	Current	70.7	2.06	26	0.54	3.75
5	Female	51	Ex	68.7	1.91	50	0.96	4.05
6	Male	55	Ex	62.0	0.85	9	0.08	3.00
7	Female	28	No	70.0	0.90	31	0.28	3.90
8	Female	71	No	66.7	0.25	20	0.05	5.36
9	Male	71	Ex	53.3	2.44	33	0.81	2.24
10	Female	42	No	68.0	3.49	34	1.19	1.48
11	Male	23	Current	70.0	0.80	1	0.01	1.14
12	Male	24	Current	80.7	0.96	5	0.05	6.54
13	Female	45	No	78.0	1.05	10	0.11	1.00
14	Female	47	Current	59.3	0.67	7	0.05	5.12
15	Male	31	Current	58.7	1.40	9	0.13	0.39
16	Male	29	No	70.7	0.32	15	0.05	1.38

BALF: bronchoalveoral lavage fluid, Recovery: Recovery rate of BALF(%), TCC: total cell count(x10*5/ml), %Lym: percentage of lymphocyte(%), Lym: lymphocyte count(x10*5/ml), CD4/CD8: CD4+/CD8+ ratio

Introduction

Analysis of bronchoalveolar lavage fluid (BALF) is a useful test for diagnosing diffuse lung disease. Increased cell count in BALF, particularly an increased lymphocyte count, and CD4/CD8 ratio are among the parameters assessed for the diagnosis of diffuse lung disease. However, because of the invasiveness of the procedure, BAL cannot be easily repeated. Analysis of exhaled breath condensate (EBC) is a novel technique for analyzing the lining fluid of the lung. EBC contains other constituents such as small molecules and proteins. As EBC samples can be collected noninvasively, the test can be repeated easily[1-3]. Such a test may be of major clinical relevance if they can accurately reflect BAL findings.

In recent years, developments in protein array technology have facilitated the comprehensive analysis of inflammatory molecules[4, 5]. Although there have been numerous studies on inflammatory molecules in BALF, there has been a few study on the comprehensive analysis of inflammatory molecules. Comprehensive analysis to compare cellular components between EBC and BALF would be useful to determine the suitability of EBC in the screening and diagnosis of diffuse lung disease.

The technique of EBC collection is simple, quick, safe, noninvasive, and suitable for patients with any ages. However, analysis of EBC obtained from sarcoidosis patients has not been done sufficiently. In the present study, we collected EBC and BALF samples from patients with sarcoidosis and performed comprehensive analyses of samples using a protein array. Direct comparison of biomarkers using both techniques would clarify the potential of EBC for clinical use.

Materials and Methods

Patients

Sixteen steroid-naïve subjects suspected to have sarcoidosis who underwent bronchoscopic examination were enrolled after giving informed consent. The clinical characteristics of these

subjects are shown in Table 1. Patients with other respiratory disorders, such as asthma, COPD, bronchiectasis, tuberculosis, acute infection (4 weeks), and atopy were excluded. The study was approved by the ethics committee at Hirosaki university school of medicine.

Collection of EBC

All patients underwent EBC collection within 120 minutes prior to bronchoscopy. EBC collection was performed according to a standard protocol [3, 8]. Subjects breathed tidally for 10-15 minutes using a nose-clip into a special chamber of the condenser (EcoScreen, Jaeger, Germany). EBC samples were stored at -80 °C until analyses were performed.

Bronchoschopy

Bronchoscopy was performed with a flexible bronchoscope (Olympus; Tokyo, Japan). BAL was performed according to the international guidelines [29, 30]. BALF was collected from the medial lobe or lingula by the instillation and subsequent withdrawal of 3 x 50 ml portions of 0.9% NaCl solution. The recovered BALF was filtered through nylon gauze and centrifuged (4 °C, 300 x g for 10 min.). The supernatant was collected and frozen at -80 °C until analyses were performed. The pellet was suspended in 0.9% NaCl solution. The total cell count (TCC) was calculated (x10*6). Cytospin slides were stained with Diff-Quik stain. The numbers of lymphocytes were calculated under a light microscope, and were presented as a percentage of the TCC.

Measurement of Inflammatory Molecules

The inflammatory molecules in EBC and BALF were measured by Human Inflammation Antibody Array 3 (RayBiotech, Norcross, GA, USA). This consists of 40 different cytokine or chemokine antibody spots. Briefly, after pre-incubation of membranes with a blocking buffer, 1.0 ml of BALF or EBC was added to the membrane and incubated for 2 hours. After washing with wash buffers, primary biotin-conjugated antibody cocktail (1 ml) was added to the membrane and incubated for 2 hours; this was washed again and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (2 ml). After the final wash, detection buffer (500 µl) was added and intensities were recorded by Kodak X-Omat AR film (Kodak, Tokyo, Japan). The Biotin-Conjugated Ig-G produces positive signals, which can be used to identify the orientation and to compare the relative expression levels throughout between the different membranes. Quantification of signal intensities on recorded images was performed using SCION image (Scion, Maryland, USA).

Statistical analysis

Spearman's correlation coefficients were calculated to determine the correlation between protein levels in EBC and BALF, their levels in BALF and percentage of lymphocyte in BALF (%Lym), and their levels in EBC and %Lym. Statistical differences in their levels of inflammatory molecule concentration between EBC and BALF were assessed by Mann-Whitney U test. PASW Statistics 18.0.0 (IBM corp., Soners, NY, USA) software was used for the analyses. All data were expressed as means ± SE, and significance was defined as $p < 0.05$.

Results

Approximately 1–2 ml of EBC was collected in a 10- to 15-minute period in each patient. In

Table 2 Relative molecule levels in EBC and BALF

Cytokine	EBC(%)	BALF(%)	P value	Cytokine	EBC(%)	BALF(%)	P value
Eotaxin	23.42 ± 0.58	21.87 ± 0.94	0.175	**IL-13**	10.56 ± 1.02	13.06 ± 1.00	0.102
Eotaxin2	29.91 ± 0.65	28.43 ± 0.68	0.097	**IL-15**	22.52 ± 0.55	20.57 ± 0.99	0.158
G-CSF	19.61 ± 1.08	18.39 ± 1.48	0.504	**IL-16**	23.23 ± 0.97	24.09 ± 0.77	0.635
GM-CSF	17.91 ± 0.65	14.39 ± 1.11	0.026*	**IL-17**	12.65 ± 1.23	13.03 ± 0.81	0.827
ICAM-1	21.72 ± 0.83	37.81 ± 1.42	<0.001*	**IP-10**	35.02 ± 1.27	34.74 ± 1.32	0.821
IFN-γ	24.68 ± 0.90	23.95 ± 0.82	0.546	**MCP-1**	24.57 ± 0.51	27.37 ± 1.03	0.035*
I-309	25.35 ± 0.81	26.28 ± 0.66	0.527	**MCP-2**	21.24 ± 0.71	20.23 ± 1.16	0.607
IL-1α	21.83 ± 0.88	19.50 ± 0.84	0.061	**M-CSF**	23.70 ± 0.52	24.41 ± 0.85	0.572
IL-1β	22.51 ± 0.88	18.37 ± 1.31	0.040*	**Mig**	22.27 ± 0.58	20.92 ± 0.61	0.097
IL-2	20.94 ± 0.92	17.16 ± 1.15	0.026*	**MIP-1α**	23.85 ± 0.68	22.18 ± 0.54	0.065
IL-3	18.12 ± 2.04	20.14 ± 2.64	0.526	**MIP-1β**	31.00 ± 0.39	30.93 ± 0.48	0.502
IL-4	15.69 ± 0.99	15.70 ± 1.03	0.982	**MIP-1δ**	18.92 ± 0.98	17.85 ± 0.98	0.596
IL-5	20.48 ± 0.87	19.25 ± 1.32	0.604	**RANTES**	30.73 ± 1.08	29.55 ± 0.56	0.343
IL-6sR	24.98 ± 0.98	26.61 ± 1.04	0.142	**TGF-β1**	22.38 ± 0.84	21.53 ± 0.81	0.604
IL-7	13.68 ± 0.75	14.34 ± 1.27	0.435	**TNF-α**	26.13 ± 0.72	25.93 ± 0.50	0.851
IL-8	26.30 ± 0.74	27.77 ± 0.70	0.158	**TNF-β**	24.97 ± 0.61	25.52 ± 0.68	0.607
IL-10	16.82 ± 0.63	18.01 ± 1.25	0.495	**sTNF-RI**	22.62 ± 0.41	22.53 ± 0.84	0.693
IL-11	11.00 ± 1.14	14.03 ± 1.43	0.063	**sTNF-RII**	24.78 ± 0.46	27.26 ± 0.68	0.013*
IL-12p40	23.16 ± 0.55	23.83 ± 0.73	0.337	**PDGF-BB**	23.14 ± 0.70	24.20 ± 0.71	0.366
IL-12p70	25.25 ± 0.84	23.88 ± 0.89	0.291	**TIMP-2**	21.88 ± 0.91	24.04 ± 0.80	0.083

Relative cytokine levels to positive contorol in EBC and BALF.

IL: Interleukin. IL-6sR: IL-6 soluble receptor. TNF: Tumor Necrosis Factor. GM-CSF: Granulocyte Macrophage colony-stimulating Factor. MCP: Monocyte Chemotactic Protein. IFN: Interferon. ICAM: Intercellular adhesion molecule. IP-10: Interferon-γ-inducible protein-10. Mig: Monokine induced by IFN-γ. MIP: Macrophage inflammatory protein. RANTES: Regulated upon Activation, Normal T cell Expressed and Secreted. sTNF-R: soluble TNF receptor. TGF: transforming growth factor. PDGF: platelet-derived grows factor. TIMP: tissue inhibitor of metalloprotease.

both EBC and BALF, the levels of all 40 types of inflammatory molecules could be measured.

Comparison with the levels of the inflammatory molecules in EBC and BALF

The concentrations of granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, and IL-2 in EBC were significantly higher than those in BALF. Those of intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein (MCP)-1, and soluble tumor necrosis factor receptor type II (sTNF-RII) in EBC were significantly lower than those in BALF (Table 2).

Among the inflammatory molecules, there were significant correlations in the levels of granulocyte colony-stimulating factor (G-CSF), IL-3, IL-4, IL-5, soluble form of IL-6 receptor (IL-6sR), IL-11, IL-12p70, IL-16, interferon-γ-inducible protein-10 (IP-10), MCP-2, macrophage colony-stimulating factor (M-CSF), regulated upon activation, normal T cell expressed and secreted (RANTES), TNF-α, TNF-β, sTNF-RII and tissue inhibitor of metalloprotease (TIMP)-2 between EBC and BALF (Figure 1).

Comparison with BALF levels of inflammatory molecules and percentage of lymphocytes in BALF

BALF levels of IL-8, M-CSF, RANTES, TNF-α, sTNF-RII and TIMP-2 were all significantly correlated with %Lym (Figure 2).

Comparison with EBC levels of inflammatory molecules and percentage of lymphocytes in BALF

EBC levels of ICAM-1, IFN-γ, IL-1β, IL-8, IL-15, IL-16, M-CSF, macrophage inflammatory protein (MIP)-1α, RANTES, transforming growth factor (TGF)-β1, TNF-α, sTNF-RI and

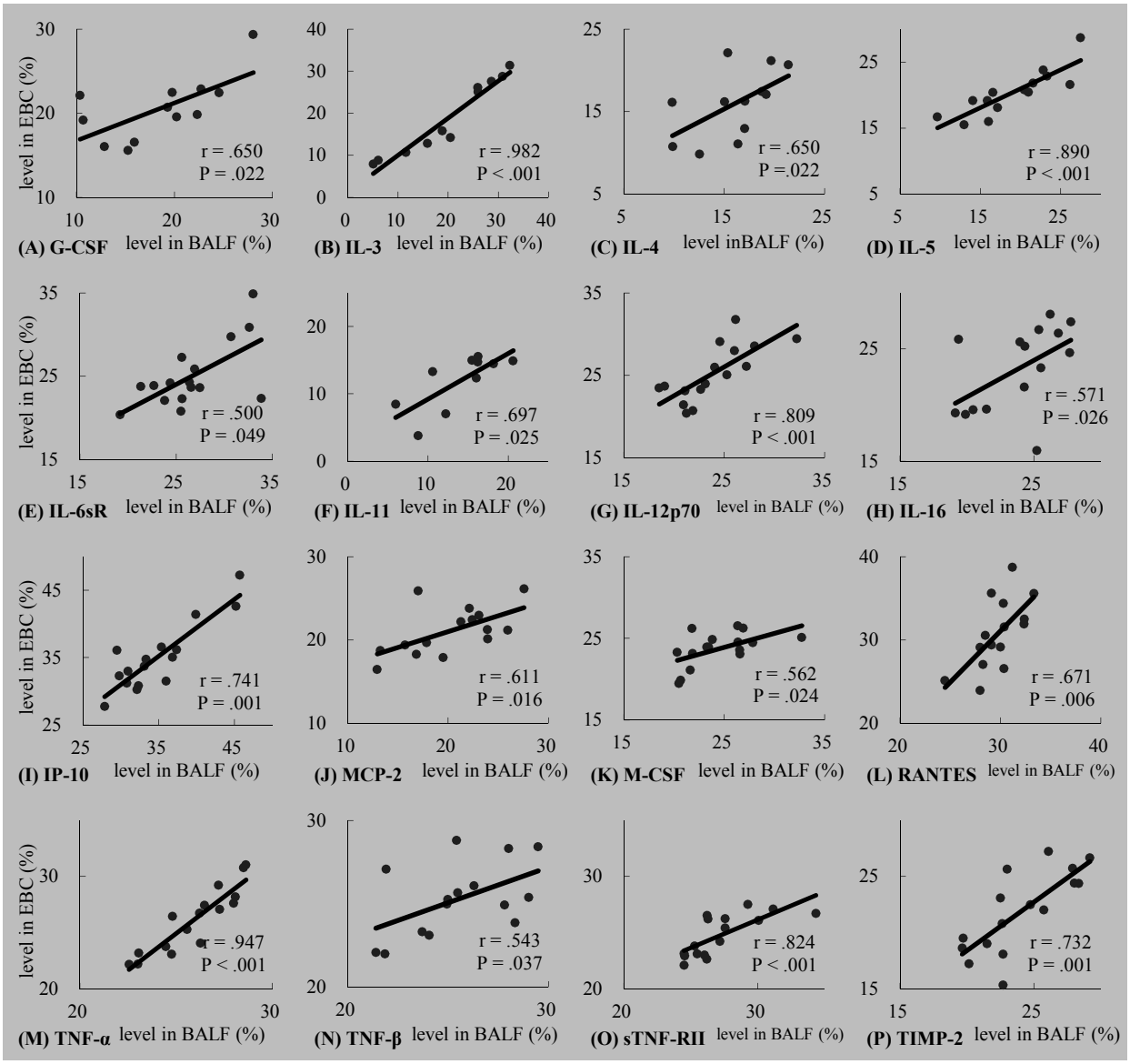

Figure 1 Relationship between relative molecule levels in BALF and EBC. The lines are obtained from the fitted regression equation.

sTNF-RII were all significantly correlated with %Lym (Figure 3).

Discussion

Researches in recent years have shown that many types of inflammatory molecules are found in EBC [6, 7]. The concentrations of these proteins are considered to be extremely low, such that most remain undetectable when standard tests like ELISA are used [3]. The present study revealed that a comprehensive analysis of 40 types of inflammatory molecules was possible using a protein array. Although many studies have analyzed inflammatory molecules in BALF, quantitative measurement of such proteins in BALF is not always performed in the clinical setting. As the mechanism behind inflammation is complex, it is considered difficult to determine the immune response in a disease based on the examination of a single inflammatory

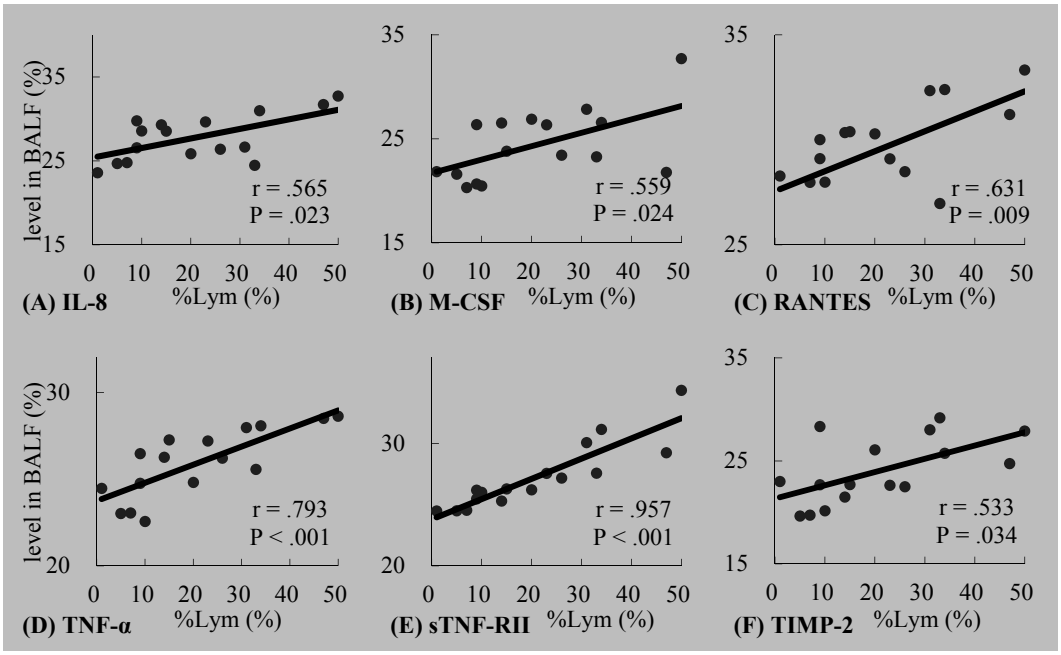

Figure 2 Relationship between relative molecule levels in BALF and percentage of lymphocyte in BALF. The lines are obtained from the fitted regression equation.

molecule. However, as protein arrays have a high sensitivity and can also be performed using small samples, it is a useful method for assessing the state of inflammation.

Comparison of inflammatory molecule levels between EBC and BALF demonstrated variable results; some protein levels in EBC were correlated with BALF, while others were higher in EBC than in BALF or vice versa. Inflammatory molecules in EBC are likely to be derived from the entire lower airways [8]. In contrast, those in BAL are indicators of inflammation at the level of the alveoli and periphery [9]. The difference in the concentration of inflammatory molecules between the EBC and BALF samples may therefore reflect the difference in the locations at which these inflammatory molecules were produced. In diffuse lung disease, BAL of the right middle lobe or left lingula is considered to be representative of the entire lung. However, there is a possibility that the concentration of inflammatory molecules in BALF is variable depending on the location of BAL sampling [10]. BAL enables the evaluation of a specific area of the lung that is sampled. On the other hand, EBC analysis may enable the evaluation of inflammation of the entire lung.

BALF lymphocyte subset values, the most commonly used measurement in the clinical setting, are known to be correlated with levels of inflammatory molecules such as sICAM-1, IL-1, IL-2, IL-6, IL-12, MIP-1α, MIP-1β, RANTES, sTNF-RII, Mig, IP-10 and TNF-α in BALF [11-18]. In the present study, IL-8, M-CSF, RANTES, TNF-α, sTNF-RII and TIMP-2 in BALF were correlated significantly with %Lym. IL-8, M-CSF and TIMP-2, whose levels were demonstrated to be correlated for the first time, are known also to play an important role in sarcoidosis [23-25]. The difference between previous study and ours may be associated with the sensitivity of the testing kit used.

TNF-α is an important cytokine that induces a Th1-type immune response [19, 20], mediated through TNF-R, producing inflammatory molecules such as IP-10, IL-8 and ICAM-1 [21, 22].

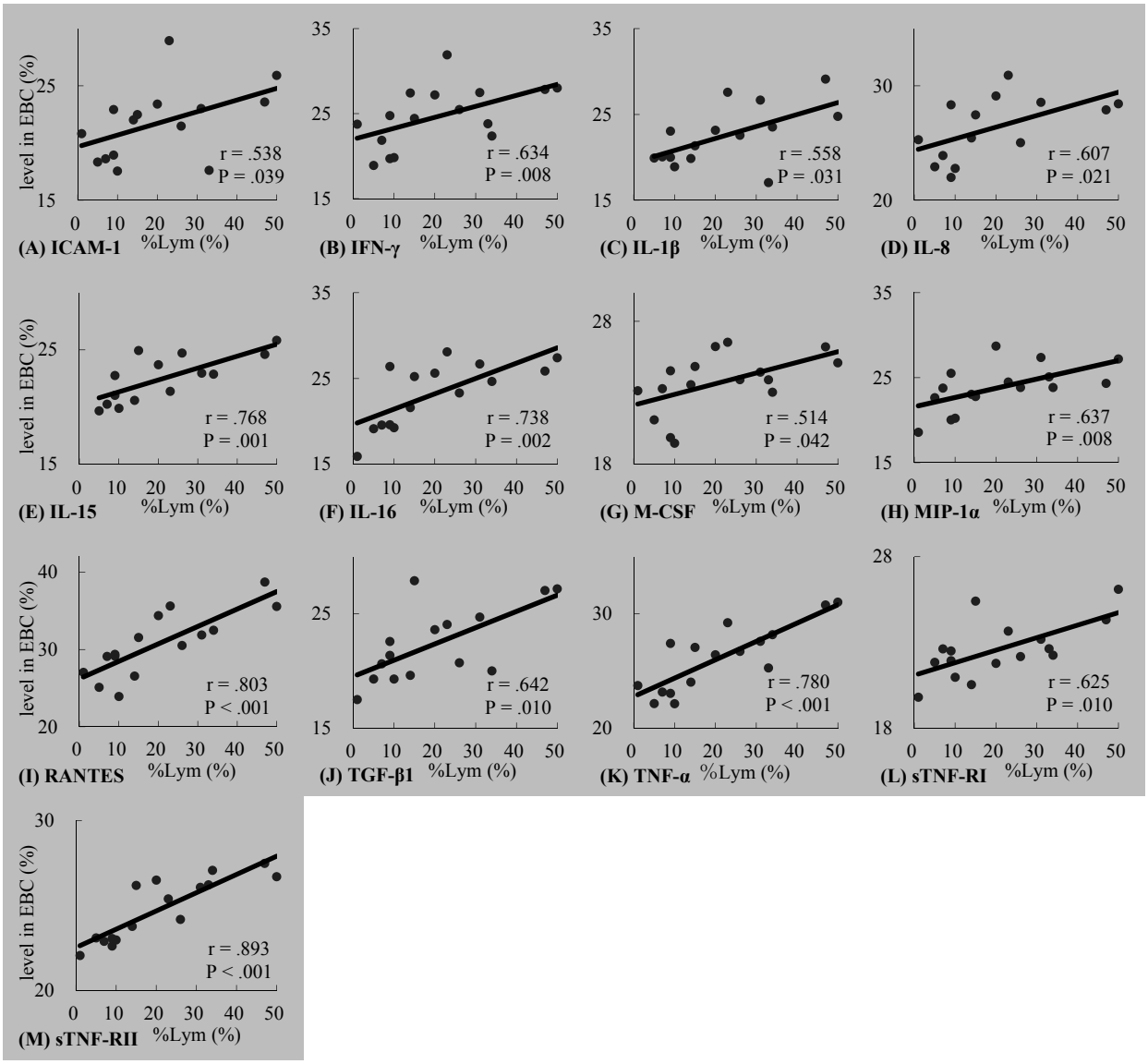

Figure 3 Relationship between relative molecule levels in EBC and percentage of lymphocyte in BALF. The lines are obtained from the fitted regression equation.

TNF inhibitor is known to be effective in the treatment of sarcoidosis [28]. sTNF-R, a soluble form of TNF-R, regulates the Th1-type immune response by inhibiting the activity of TNF-α [26]. Previous studies have suggested that TNF-α and sTNF-RII may have important roles. Only TNF-α is known to have correlation in the level between EBC and BALF, and between BALF level and %Lym in patients with sarcoidosis [17, 27]. In the present study, levels of M-CSF, RANTES, TNF-α and sTNF-RII in EBC were significantly correlated with BALF. Those levels in both EBC and BALF were correlated with %Lym. The analysis of these inflammatory molecules in EBC might be equivalent to that in BALF. Especially, RANTES and sTNF-RII levels in EBC seem to be a good parameter which is related to the necessity of BALF, because they were extremely low in all cases whose %Lym was under 10%. Additional studies are needed to better define their clinical usability.

Finally, protein array, which is a highly

sensitive approach, allows for cytokine detection in EBC. Comprehensive analysis of EBC might be equivalent to that in BALF.

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