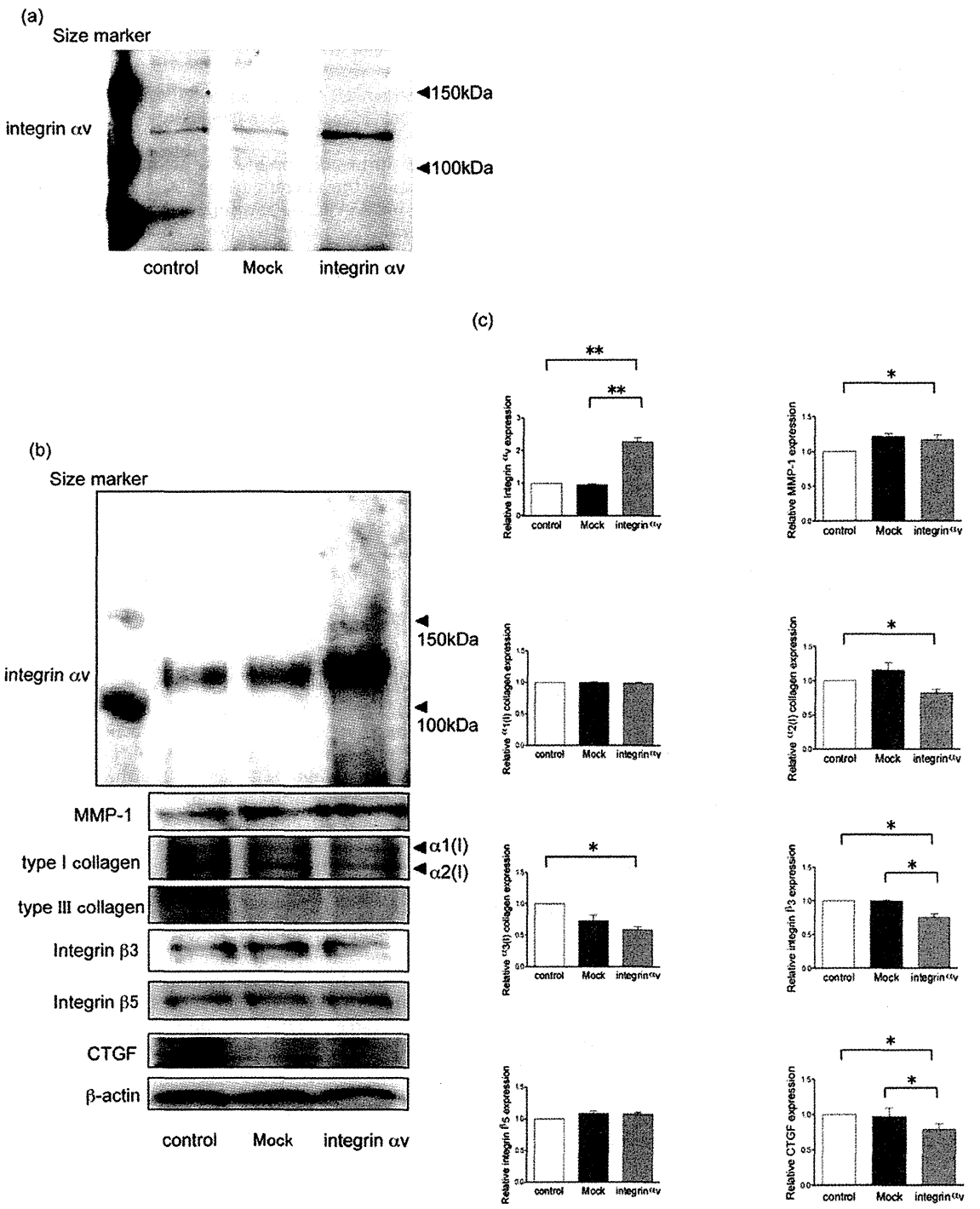

Fig. 5. Protein levels of extracellular matrix-related molecules in NIH3T3 cells in vitro.
(a) Cell surface proteins of untransfected NIH3T3 cells (control), empty vector-transfected cells (Mock) and integrin αv-transfected cells (integrin αv) were labeled with biotin, and cell lysates were immunoprecipitated using anti-integrin αv subunit antibody. One representative of three independent experiments is shown. To show the bands for integrin αv protein are the right size (125kDa), size marker was also included in the blot.
(b, c) Cell lysates obtained from untransfected NIH3T3 cells (control), empty vector-transfected cells (Mock) and integrin αv-transfected cells (integrin αv) were subjected to immunoblotting. To show the bands for integrin αv protein are the right size (125kDa), size marker was also included in the blot. β-actin levels were shown as controls. One representative of three independent experiments is shown (b).
The protein levels of each molecule quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to those in control cells ($n=3$). The values of control in each experiment were set at 1. $^{*}p<0.05$. $^{**}p<0.01$ (c).

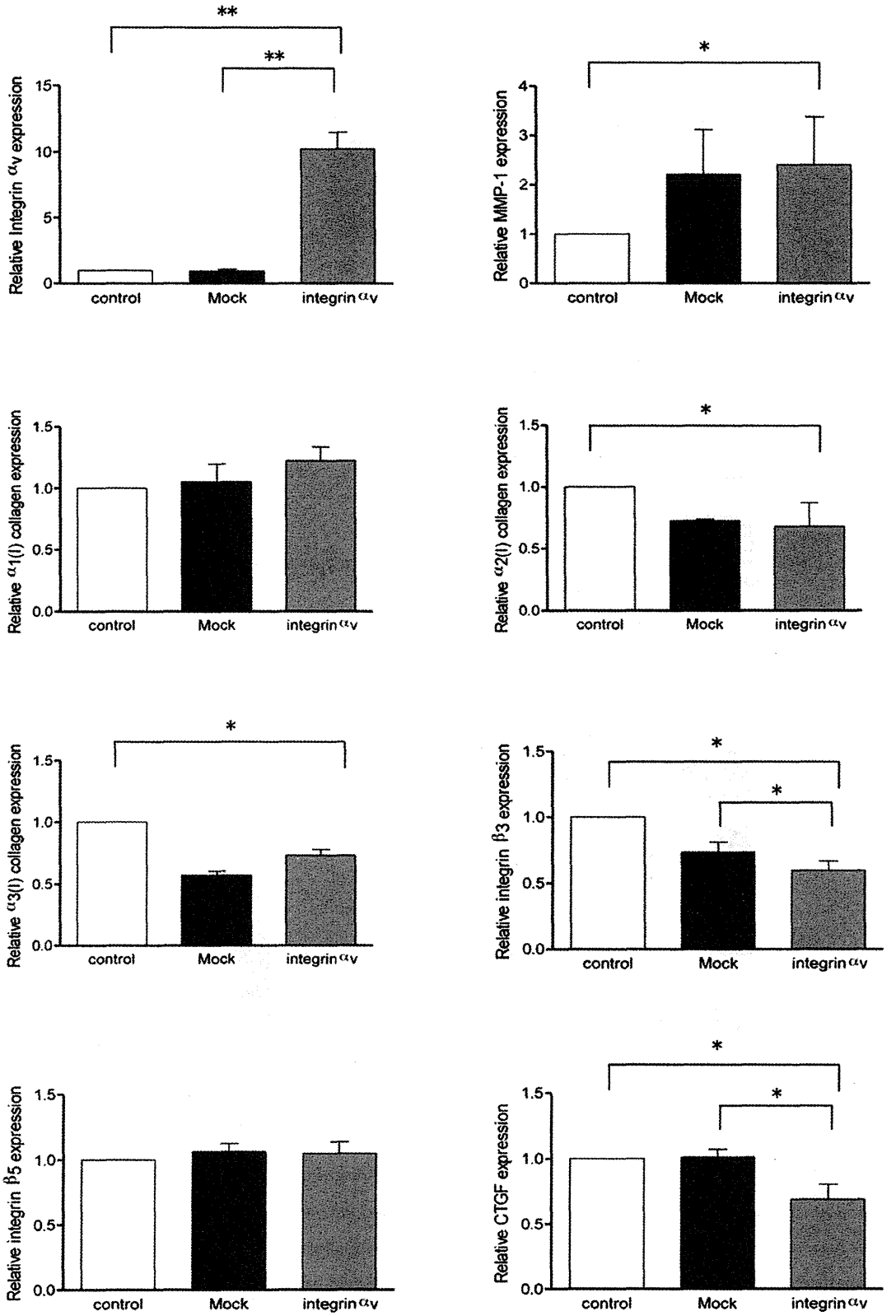

Fig. 6. mRNA levels of extracellular matrix-related molecules in NIH3T3 cells in vitro.
mRNA levels of indicated genes measured by quantitative real-time PCR in untransfected NIH3T3 cells (control), empty vector-transfected cells (Mock) and integrin αv-transfected cells (integrin αv) are shown on the ordinate. Data presented as bar graphs are the means + SE of four independent experiments. The values of control in each experiment were set at 1, and relative expression levels of indicated genes were presented. $^{*}p < 0.05$. $^{**}p < 0.01$.

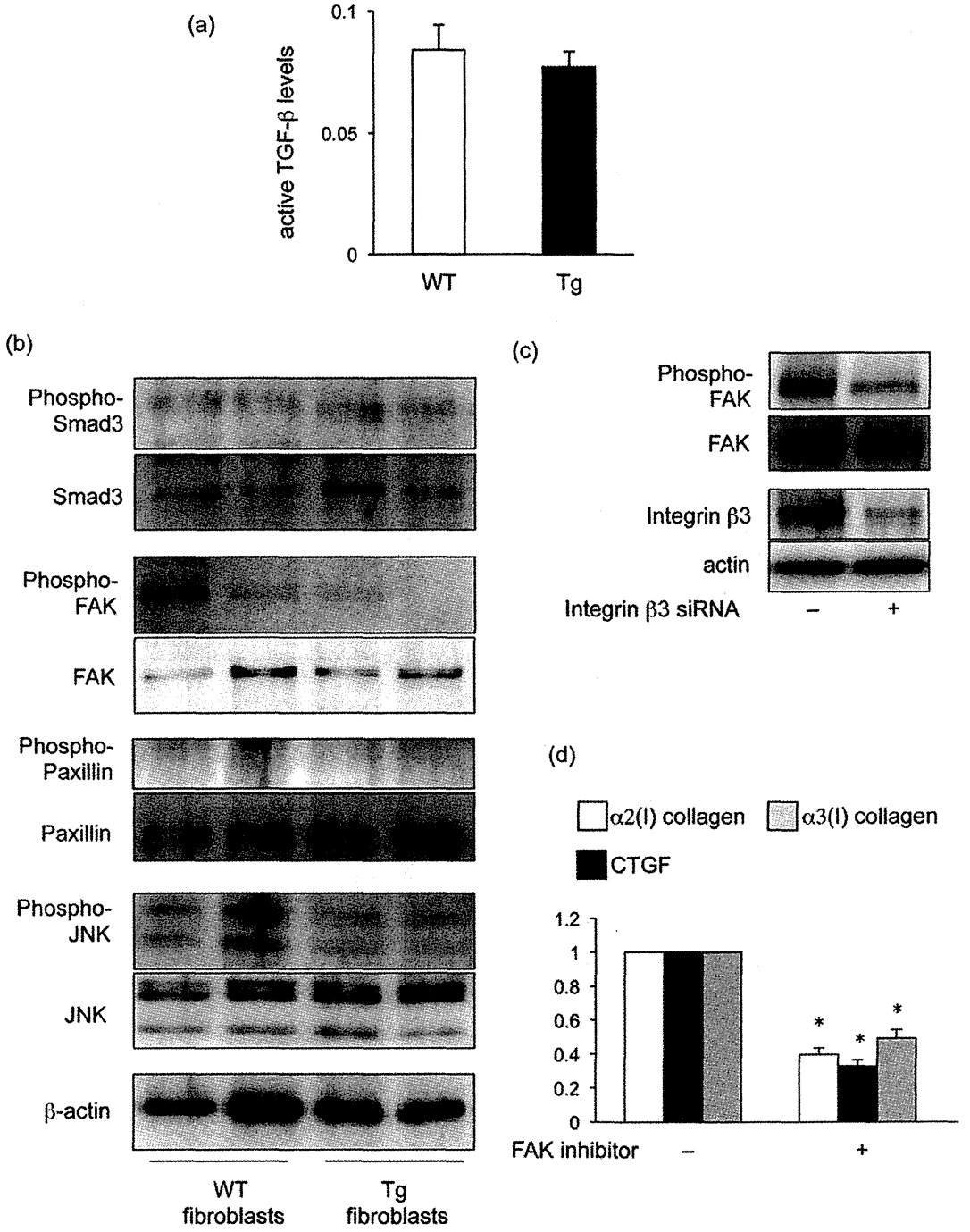

Fig. 7. Integrin-associated signaling pathways in Tg mice.
(a) Active TGF-β levels in the media of fibroblasts cultured from 4 WT or Tg mice skin (male; $n = 2$ and female; $n = 2$) were determined by ELISA. Levels were shown relative to those in cells derived from WT skin (1.0).
(b) Cell lysates of fibroblasts derived from WT or Tg mice were subjected to immunoblotting. β-actin levels were shown as controls. Results are representative of 6 WT or Tg mice (male; $n = 3$ and female; $n = 3$).
(c) NIH3T3 was transfected with control or integrin β3 siRNA for 72 h. Cell lysates were subjected to immunoblotting. One representative of three independent experiments is shown.
(d) NIH3T3 was treated with or without FAK inhibitor for 72 h. mRNAs were subjected to real-time PCR. The bar graphs show the expression of each gene by three independent experiments. The values of control in each experiment were set at 1, and relative expression levels of indicated genes were presented. $^*p < 0.05$.

down-regulated CTGF [23]. Furthermore, it has been reported that the overexpression of ITGAV in human mesenchymal cells is associated with increased MMP-1 transcription [24], which is consistent with our results. Taken together, in this study, we addressed the molecular mechanism by which ITGAV overexpression reduces dermal thickness. As described in the Introduction, we previously showed the overexpression of integrins in cultured human dermal fibroblasts induces collagen expression [7,9,10]. The discrepancy between the present study and the previous reports may be explained by the difference

between in vivo and in vitro. In addition, there may be the difference between mice and human: for example, sequence of COL1A2 promoter is different between them, which may cause the difference in the role of integrins.

There were several limitations in this study. Given that mice overexpressing ITGAV in fibroblasts exhibit dermal thinning contrary to our expectation, we need to confirm that functional ITGAV protein is overexpressed in these mice in the future (e.g. ITGAV-dependent adhesion in ITGAV non-expressing or knockout cells). A possible alternative explanation is that the transgenic ITGAV expressed has a mutation, or is inhibited by the HA tag, leading to effective down-regulation of ITGAV. Likewise, transgenes can insert as serial repeats which can cause expression of antisense transcripts that inhibit expression of the endogenous gene. Furthermore, our study lacks interaction experiments (e.g. co-immunoprecipitation with ITGAV to determine with which β subunit the overexpressed ITGAV pairs, or whether ITGAV competes with other α subunits for binding to integrin β). In addition, NIH3T3 cells overexpressed ITGAV also showed the down-regulation of integrin β3 and CTGF, but did not show the change of collagens and MMP-1. This may be due to the insufficient overexpression levels of ITGAV in these cells, or the lack of other factors in ITGAV-overexpressed NIH3T3. Further studies to clarify these points in the future will give a new insight in the treatment of SSc by balancing integrin expression.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgement

This study was supported in part by a grant for scientific research from the Japanese Ministry of Education, Science, Sports and Culture, and by project research on intractable diseases from the Japanese Ministry of Health, Labour and Welfare. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] A.J. Ablooglu, E. Tkachenko, J. Kang, S.J. Shattil, Integrin αv is necessary for gastrulation movements that regulate vertebrate body asymmetry, Development 137 (2010) 3449–3458.
[2] E.C. LeRoy, E.A. Smith, M.B. Kahaleh, M. Trojanowska, R.M. Silver, A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor β the answer? Arthritis Rheum. 32 (1989) 817–825.
[3] E.C. LeRoy, Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast, J. Clin. Invest. 54 (1974) 880–889.
[4] S.A. Jimenez, G. Feldman, R.I. Bashey, R. Bienkowski, J. Rosenbloom, Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts, Biochem. J. 237 (1986) 837–843.
[5] K. Kikuchi, C.W. Hartl, E.A. Smith, E.C. LeRoy, M. Trojanowska, Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts: insensitivity to TGF β1 stimulation, Biochem. Biophys. Res. Commun. 187 (1992) 45–50.
[6] H. Ihn, The role of TGF-β signaling in the pathogenesis of fibrosis in scleroderma, Arch. Immunol. Ther. Exp. 50 (2002) 325–331.
[7] Y. Asano, H. Ihn, K. Yamane, M. Jinnin, K. Tamaki, Increased expression of integrin αvβ5 induces the myofibroblastic differentiation of dermal fibroblasts, Am. J. Pathol. 168 (2006) 499–510.
[8] H. Ihn, K. Yamane, M. Kubo, K. Tamaki, Blockade of endogenous transforming growth factor β signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor β receptors, Arthritis Rheum. 44 (2001) 474–480.
[9] Y. Asano, H. Ihn, K. Yamane, M. Jinnin, Y. Mimura, K. Tamaki, Involvement of αvβ5 integrin-mediated activation of latent transforming growth factor &βetα;1 in autocrine transforming growth factor β signaling in systemic sclerosis fibroblasts, Arthritis Rheum. 52 (2005) 2897–2905.
[10] Y. Asano, H. Ihn, K. Yamane, M. Jinnin, Y. Mimura, K. Tamaki, Increased expression of integrin αvβ3 contributes to the establishment of autocrine TGF-&βetα; signaling in scleroderma fibroblasts, J. Immunol. 175 (2005) 7708–7718.
[11] B. Maurer, N. Busch, A. Jüngel, M. Pileckyte, R.E. Gay, B.A. Michel, et al., Transcription factor fos-related antigen-2 induces progressive peripheral vasculopathy in mice closely resembling human systemic sclerosis, Circulation 120 (2009) 2367–2376.
[12] R. Higashiyama, T. Moro, S. Nakao, K. Mikami, H. Fukumitsu, Y. Ueda, et al., Negligible contribution of bone marrow-derived cells to collagen production during hepatic fibrogenesis in mice, Gastroenterology 137 (2009) 1459–1466. e1.
[13] Y. Asano, M. Markiewicz, M. Kubo, G. Szalai, D.K. Watson, M. Trojanowska, Transcription factor Fli1 regulates collagen fibrillogenesis in mouse skin, Mol. Cell. Biol. 29 (2009) 425–434.
[14] J.H. Yoon, J. Kim, H. Lee, S.Y. Kim, H.H. Jang, S.H. Ryu, B.J. Kim, T.G. Lee, Laminin peptide YIGSR induces collagen synthesis in Hs27 human dermal fibroblasts, Biochem. Biophys. Res. Commun. 428 (2012) 416–421.
[15] A. van der Flier, K. Badu-Nkansah, C.A. Whittaker, D. Crowley, R.T. Bronson, A. Lacy-Hulbert, et al., Endothelial α5 and αv integrins cooperate in remodeling of the vasculature during development, Development 137 (2010) 2439–2449.
[16] M. Acharya, S. Mukhopadhyay, H. Païdassi, T. Jamil, C. Chow, S. Kissler, et al., αv Integrin expression by DCs is required for Th17 cell differentiation and development of experimental autoimmune encephalomyelitis in mice, J. Clin. Invest. 120 (2010) 4445–4452.
[17] N.C. Henderson, T.D. Arnold, Y. Katamura, M.M. Giacomini, J.D. Rodriguez, J.H. McCarty, et al., Targeting of αv integrin identifies a core molecular pathway that regulates fibrosis in several organs, Nat. Med. 19 (2013) 1617–1624.
[18] A.D. Bradshaw, P. Puolakkainen, J. Dasgupta, J.M. Davidson, T.N. Wight, E. Helene Sage, SPARC-null mice display abnormalities in the dermis characterized by decreased collagen fibril diameter and reduced tensile strength, J. Invest. Dermatol. 120 (2003) 949–955.
[19] M. Sommer, N. Poliak, S. Upadhyay, E. Ratovitski, B.D. Nelkin, L.A. Donehower, et al., ΔNp63α overexpression induces downregulation of Sirt1 and an accelerated aging phenotype in the mouse, Cell Cycle 5 (2006) 2005–2011.
[20] S. Liu, A. Leask, Integrin β1 is required for dermal homeostasis, J. Invest. Dermatol. 133 (2013) 899–906.
[21] C.F. Clemente, T.F. Tornatore, T.H. Theizen, A.C. Deckmann, T.C. Pereira, I. Lopes-Cendes, et al., Targeting focal adhesion kinase with small interfering RNA prevents and reverses load-induced cardiac hypertrophy in mice, Circ. Res. 101 (2007) 1339–1348.
[22] J.H. Yoon, J. Kim, H. Lee, S.Y. Kim, H.H. Jang, S.H. Ryu, et al., Laminin peptide YIGSR induces collagen synthesis in Hs27 human dermal fibroblasts, Biochem. Biophys. Res. Commun. 428 (2012) 416–421.
[23] K. Frazier, S. Williams, D. Kothapalli, H. Klapper, G.R. Grotendorst, Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor, J. Invest. Dermatol. 107 (1996) 404–411.
[24] H. Levinson, A.K. Sil, J.E. Conwell, J.E. Hopper, H.P. Ehrlich, αv integrin prolongs collagenase production through Jun activation binding protein 1, Ann. Plast. Surg. 53 (2004) 155–161.

THE JOURNAL OF DERMATOLOGY

doi: 10.1111/1346-8138.12904

Journal of Dermatology 2015; **42**: 551–558

REVIEW ARTICLE

Recent progress in studies of miRNA and skin diseases

Masatoshi JINNIN

Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

ABSTRACT

miRNA is a family of small non-coding RNA that consists of 22 nucleotides on average. miRNA are implicated in various cellular activities such as cell proliferation or migration via the modulation of gene expression, and also are linked to the pathogenesis of human diseases. This paper reviews recent research progress about the contribution of miRNA to the pathogenesis of various skin diseases, and possible application of miRNA as the disease markers in each disease. For example, downregulated miR-424-5p in psoriatic skin causes the overexpression of MEK1 and cyclin E1 in psoriatic keratinocytes, resulting in the keratinocyte overgrowth and hyperproliferation seen in the disease. Although there was no significant difference in the serum miR-424-5p levels between psoriasis patients and healthy controls, serum miR-1266-5p levels were significantly upregulated in psoriasis patients, and showed weak and inverse correlation with disease activity. Furthermore, combination of serum levels of miR-146a-5p and -203a-3p was more reliable to distinguish psoriasis patients and normal subjects, than each miRNA alone. Hair shaft miR-424-5p levels were significantly higher in psoriasis patients than normal subjects, while hair root miR-19a-3p levels in psoriasis patients were inversely correlated with the duration between symptom onset and the first visit to the hospital. Future researches of miRNA will enable the advances of their clinical applications including the clarification of pathogenesis, disease markers and novel treatments.

Key words: melanoma, miRNA, psoriasis, scleroderma.

INTRODUCTION

miRNA is a family of small non-coding RNA discovered in 1993, which consists of 22 nucleotides on average. A maturation process of miRNA started by the transcription of primary miRNA (pri-miRNA), several hundred to a few thousand nucleotides, from non-coding regions of genome. The pri-miRNA are then degraded by the microprocessor Drosha into precursor-miRNA (pre-miRNA) that have a 60–70 nucleotide double-stranded stem-loop structure. The pre-miRNA were carried from the nucleus to the cytoplasm by Exportin5, and their stem-loop structures are cleaved by the endonuclease Dicer into mature double-stranded miRNA. Both strands from the 5′- and 3′-arms of pre-miRNA can become mature single-stranded miRNA, or can be degraded by Argonautes, the catalytically active RNase family, in the RNA-induced silencing complex (RISC). Mature single-stranded miRNA are incorporated into RISC, which is guided to 3′-untranslated regions of target mRNA.[1] Binding of miRNA in the RISC to the complementary sequences on target mRNA leads to the downregulation of the targets via the repression of mRNA translation or via the degradation of the mRNA itself. The increase of each miRNA usually causes the downregulation of target expression, while its decrease causes the upregulation of targets. To predict the targets of each miRNA, website-based prediction databases are available: micoRNA.org (http://www.microrna.org/), PicTar (http://pictar.mdc-berlin.de/) or TargetScan (http://www.targetscan.org/).

To date, nearly 2500 miRNA have been found in humans. The principle of the nomenclature scheme used in miRBase, one of the most famous miRNA databases (http://www.mirbase.org), is as follows:

1. "miR" usually refers to the mature single-stranded miRNA, while "mir" means the miRNA gene or pre-miRNA with stem-loop structure.
2. Numerical identifiers are given based on sequence similarity (e.g. miR-17, miR-18, miR-19...).
3. miRNA with nearly identical sequences except for one or two nucleotides are differentiated by additional lower case lettered suffixes a, b... (e.g. miR-196**a**; uagguaguuucauguuguuggg, miR-196**b**; uagguaguuuc**c**uguuguuggg).
4. Mature single-stranded miRNA derived from the 5′-arm of pre-miRNA is annotated with an additional suffix -5p, whereas that from the 3′-arm is annotated with -3p.
5. lin-4 and let-7a families, the first-discovered miRNA, are exceptions to the above nomenclature scheme.

This review paper uses the above nomenclature system. miRNA are implicated in various cellular activities such as cell proliferation or migration via the modulation of gene expression, and also are linked to the pathogenesis of human diseases.[2–4]

Correspondence: Masatoshi Jinnin, M.D., Ph.D., Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. Email: mjin@kumamoto-u.ac.jp

Received 11 March 2015; accepted 12 March 2015.

miRNA usually function inside the cells, and have been thought to be destroyed by RNAe in body fluids or extracellular space. However, extracellular miRNA are found to be stable outside the cells, probably because they are packaged and protected from harsh conditions by microvesicles called exosome or shedding vesicles.[5,6] Thus, miRNA can be detected in extracellular body fluids such as serum, urine or saliva,[7,8] and there is a possibility that extracellular miRNA levels, especially serum miRNA levels, become novel biomarkers in human diseases, especially in malignant tumors.

Similarly, because hair shafts lose nuclear components, nucleic acids have been thought to be destroyed except for mitochondrial DNA. However, Lefkowitz *et al.*[9] demonstrated the existence of RNA in both hair roots and shafts. Also, the reproducibility and possible clinical usefulness of the levels of hair miRNA, which are probably protected by exosome or shedding vesicles, have been reported.[10] Because the expression pattern of miRNA in the hair shafts was different from that in sera or hair roots, they can be utilized as independent biomarkers. Hair miRNA may be more accessible than serum miRNA especially in babies or patients with collagen diseases, because blood samples are sometimes difficult to obtain from them due to their narrow vessels.

This paper reviews recent research progress about the contribution of miRNA to the pathogenesis of various skin diseases, and possible application of miRNA as the disease markers in each disease.

MALIGNANT MELANOMA

Malignant melanoma is an aggressive neoplasm with a very poor prognosis. Although several molecular abnormalities or mutations have been implicated in the tumorigenesis, the detailed mechanisms are not completely revealed. Melanoma is one of the skin diseases in which abnormal expression of miRNA was first detected. Zhang *et al.*[11] determined genome-wide miRNA DNA copy number abnormalities in cancers by high-resolution array-based comparative genomic hybridization in 2006. Genes of 283 human miRNA were analyzed in 227 human cancer specimens including malignant melanoma. They identified miRNA commonly dysregulated in several cancer types and those uniquely dysregulated in melanoma. Of note, they also found high frequency copy number abnormalities of miRNA-associated genes including Dicer1 and Argonaute2 in cancers including melanoma. This paper gave clues for the involvement of miRNA in the pathogenesis of melanoma.

miR-221-3p (previous ID: miR-221), encoded on the X chromosome, is overexpressed in various cancer cells such as melanoma as well as glioblastoma, pancreatic cancer and papillary thyroid carcinoma, whereas not detectable in normal human melanocytes.[12] In addition, miR-221-3p expression is increased in accordance with the stepwise melanoma transformation process.[13] miR-222-3p (previous ID: miR-222) is also reported to be increased in several cancer types. Felicetti *et al.*[13] demonstrated that promyelocytic leukemia zinc finger (PLZF) transcription factor is the negative regulator of miR-221-3p and miR-222-3p by directly binding to their putative regulatory region. Lack of PLZF may be the cause of overexpression of miR-221-3p and -222-3p in melanoma. The miRNA targets p27Kip1/CDKN1B and c-Kit receptor, respectively, and the overexpression of miR-221-3p/-222-3p and subsequent suppression of these molecules results in the enhanced proliferation and differentiation blockade of the tumor cells. Therefore, miR-221-3p/-222-3p may play a major role in the tumorigenesis of melanoma, and the functional study using antisense oligonucleotides indicated that inhibition of these miRNA has therapeutic potential (Table 1).

Recently, several studies indicated that MIRSNP, single nucleotide polymorphisms (SNP) associated with miRNA, may alter the function or expression of the miRNA themselves, and may be involved in the pathogenesis of various human diseases.[14,15] For example, miR-146a-5p (previous ID: miR-146a) is one of the miRNA increased in the tumor tissues including melanoma and implicated in the pathogenesis.[16] Double-stranded miR-146a is yielded by Dicer1 from mir-146a pre-miRNA with stem loop structure (CCGAUGUGUAUCCUCAGC UUUGAGAACUGAAUUCCAUGGGUUGUGUCAGUGUCAGACC U**C/G**UGAAAUUCAGUUCUUCAGCUGGGAUAUCUCUGUCAU CGU). Mature functional single-stranded miR-146a-5p (UGAGAACUGAAUUCCAUGGGUU) and miR-146a-3p (previous ID: miR-146a*, CCU**C/G**UGAAAUUCAGUUCUUCAG) are then separated from the double-stranded miR-146a, and MIRSNP rs2910164 is located in miR-146a-3p. The minimum free energy between miR-146a-5p and miR-146a-3p with G allele evaluated based on web bioinformatics (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) is −26.8 kcal/mol while that of C allele was −24.0 kcal/mol.[17] Thus, MIRSNP rs2910164 may be functional, and the change C to G may stabilize mir-146a, leading to the increased expression of miR-146a-5p and -3p. We previously determined the association between malignant melanoma and MIRSNP rs2910164 in miR-146a. The genotype distributions were 0 GG (0.0%), 35 CG (70.0%) and 15 CC (30.0%) in genomic DNA samples of 50 melanoma tissues analyzed by polymerase chain reaction (PCR) restriction fragment length polymorphism method. The frequency of CG genotype was significantly elevated compared with the control DNA, thus indicating that individuals with the CG genotype have an increased risk of melanoma. We could not find significant difference in terms of the relative expression levels of miR-146a-5p between patients with CC and CG genotypes. However, cell proliferation, migration and invasion was significantly increased in human melanoma cell lines with the G allele compared with those with the C allele. Accordingly, these results suggest that MIRSNP rs2910164 play a role in the tumorigenesis of melanoma.[17]

Currently, the serum levels of 5-S-cysteinyl-DOPA (5-S-CD), protein S100b (S100) and melanoma inhibitory activity are tumor markers commonly used for evaluating the progression of melanoma. However, these levels sometimes remain within the normal limits, especially in the early stage, whereas they are significantly elevated in patients with large, invasive or metastatic melanoma.[18] Accordingly, it may be less effective to use 5-S-CD for the early detection of melanoma, and novel biomarkers need to be developed. We determined the possibility

Table 1. Summary of possible roles of miRNA in skin diseases

Disease	miRNA	Previous ID	Target	Tissue	Up/down	Reference
Melanoma	miR-221-3p	miR-221	p27Kip1/CDKN1B	Tumor	Up	13
				Serum	Up	12
				Hair shaft	Up	22
	miR-222-3p	miR-222	c-Kit receptor	Tumor	Up	13
Psoriasis	miR-203-3p	miR-203	SOCS-3	Skin	Up	23
	miR-146a-5p	miR-146a	IRAK-1, TRAF-6	Skin	Up	23
	miR-221-3p	miR-221	TIMP-3	Skin	Up	24
	miR-222-3p	miR-222	TIMP-3	Skin	Up	24
	miR-424-5p	miR-424	MEK1, cyclin E1	Skin	Down	25
				Serum		25
				Hair shaft	Up	29
	miR-1266-5p	miR-1266		Serum	Up	26
	miR-19a-3p	miR-19a	TNF-α	Hair root	Up	30
Drug eruption	miR-18a-5p	miR-18a	BCL2L10	Skin	Up	32
				Serum	Up	32
Dermatomyositis	miR-223-3p	miR-223	PKC-ε	Skin	Down	34
				Serum	Down	34
	miR-7-5p	miR-7		Serum	Down	37
	miR-21-5p	miR-21		Serum	Up	40
	miR-214-3p	miR-214		Hair shaft	Up	41
Scleroderma	miR-29-3p	miR-29	Collagen, elastin	Skin	Down	42
				Hair shaft	Down	51
	let-7a-5p	let-7a	Collagen	Skin	Down	46
				Serum	Down	46
	miR-196a-5p	miR-196a	Collagen	Skin	Down	45
				Hair shaft	Down	10

BCL2L, Bcl-2-like protein; CDKN, cyclin-dependent kinase inhibitor; IRAK, interleukin-1 receptor-associated kinase 1; MEK, mitogen-activated protein kinase kinase; PKC, protein kinase C; SOCS, suppressor of cytokine signaling; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor.

whether serum miR-221-3p levels can be more sensitive marker. Serum samples were collected from 94 melanoma patients and 20 healthy controls.[12] miRNA were extracted from sera, and miR-221-3p levels in sera were quantitated by real-time PCR. We first demonstrated that miR-221-3p was detectable and measurable in serum samples. Serum miR-221-3p levels were significantly higher in melanoma patients than healthy controls, correlating with tumor thickness. Among the melanoma patients, those with stage I, II, III or IV melanoma showed significantly increased miR-221-3p levels compared with those with melanoma *in situ*. In addition, a longitudinal study also indicated a tendency of serum miR-221-3p levels to decrease after surgical excision of the primary tumor, and to increase again at recurrence. Taken together, serum levels of miR-221-3p may be useful for the diagnosis of melanoma, for differentiating melanoma *in situ* from higher stage melanoma, and for monitoring tumor progression or recurrence as a prognostic marker. Furthermore, the sensitivities of serum miR-221-3p for the diagnosis were higher than those of 5-S-CD in stage I–IV patients. We could not find statistically significant correlations between the serum 5-S-CD levels and miR-221-3p levels in the patients, probably because the 5-S-CD level usually starts to increase in metastatic stages whereas miR-221-3p level was increased even at an early stage. Thus, serum miR-221-3p may be a sensitive tumor marker, especially in earlier stages of the tumor. Furthermore, our result also suggested that miR-221-3p contribute to the tumorigenesis of melanoma, especially at the earlier stages.

Since then, many studies have shown that the serum levels of miRNA are useful for the diagnosis or evaluation of activity in melanoma.[19,20] However, it may be less significant to measure the level of only one of more than 2500 miRNA. This idea led us to focus on the interaction among multiple miRNA and to examine the possibility that expression pattern of multiple miRNA in each patient can be utilized as a more reliable tumor marker for melanoma. We selected six miRNA (miR-9-5p, miR-145-5p, miR-150-5p, miR-155-5p, miR-203a-3p and miR-205-5p) that have previously been implicated in the metastatic process of cancers by at least two different papers.[21] The serum levels of six miRNA were determined by real-time PCR in 11 patients with metastatic melanoma and 16 patients without metastasis. The expression pattern of the six miRNA was different between the sera of patients with metastasis and those without it, indicating that the miRNA network may be involved in the pathogenesis of melanoma metastasis. We found strong and significant correlation between miR-9-5p and miR-205-5p or between miR-203a-3p and miR-205-5p, suggesting that expression levels of these miRNA were not independently regulated in melanoma patients. The receiver–operator curve (ROC) analysis for the evaluation of diagnostic performance

indicated that the combination of five miRNA (miR-9-5p, miR-145-5p, miR-150-5p, miR-155-5p and miR-205-5p) was more sensitive than individual miRNA to differentiate melanoma patients with metastasis from those without. This is the first report showing that the expression profiles of multiple miRNA are different among melanoma patients, and useful for the detection of metastasis.

We also determined the possibility that hair shaft miR-221-3p levels could be a marker for melanoma. We first demonstrated that miR-221-3p levels were also quantitated in hair shafts by real-time PCR. As expected, miR-221-3p levels in the hair shafts were significantly elevated in patients with melanoma compared with control subjects.[22] The percentage of patients with higher hair shaft miR-221-3p levels above the cut-off value was similar to that of serum 5-S-CD levels. These data first indicated that the hair miRNA levels have clinical significance in the diagnosis and detection of melanoma.

PSORIASIS

In 2007, Sonkoly *et al.*[23] first demonstrated that miR-203a-3p (previous ID: miR-203) is overexpressed in psoriatic skin, which targets suppressor of cytokine signaling 3, leading to the hyperproliferation and differentiation of keratinocytes. They also described that miR-146a-5p is overexpressed in psoriatic skin, and may be involved in the regulation of innate immune responses and tumor necrosis factor (TNF)-α pathway. Subsequently, Zibert *et al.*[24] showed the upregulation of miR-22-3p, miR-24-3p, miR-498 and miR-551a in both involved and non-involved skin of psoriasis patients compared with skin of normal subjects, and revealed that forced overexpression of miR-221-3p and miR-222-3p result in the reduction of tissue inhibitor of metalloproteinase-3. Of note, miR-146a-5p and miR-221-3p/-222-3p are also thought to play a role in the tumorigenesis of melanoma as described above, indicating that miRNA play different roles in each disease. Furthermore, we previously performed PCR array to identify miRNA up- or downregulate only in psoriatic skin when compared with normal skin or atopic skin.[25] Among them, we focused on miR-424-5p (previous ID: miR-424), because its expression was significantly decreased specifically in psoriasis skin. We also found MEK1 and cyclin E1 as the targets of miR-424-5p. Downregulated miR-424-5p causes the overexpression of MEK1 and cyclin E1 in the psoriatic keratinocytes, resulting in the keratinocyte overgrowth and hyperproliferation seen in the disease.

To date, objective biomarkers that are associated with the diagnosis and disease activity have not been clinically available in psoriasis. Thus, we determined serum miR-424-5p levels of psoriasis patients: there was no significant difference in the serum miR-424-5p levels between psoriasis patients and healthy controls.[25] We then focused on serum levels of miR-1266-5p, a putative controller of interleukin (IL)-17A, in psoriasis patients. We expected its decrease, but serum miR-1266-5p levels turned out to be significantly upregulated in psoriasis patients compared with normal subjects.[26] Furthermore, the miR-1266-5p levels showed weak and inverse correlation with Psoriasis Area Severity Index scores and body surface areas of involved skin. Accordingly, miR-1266-5p may regulate other targets than IL-17A in psoriasis, and our results indicated the possibility of serum miR-1266-5p levels as a new disease marker.

Pivarcsi *et al.*[27] reported up- or downregulation of serum levels of multiple miRNA including miR-128-3p, let-7d-5p, miR-142-3p and miR-181a-5p in psoriasis patients compared with normal subjects. On the other hand, we also focused on the combination of miRNA levels in the sera of psoriasis: Serum levels of six miRNA (miR-21-5p, -24-3p, -125b-5p, -146a-5p, 203a-3p and -205-5p) were determined by real-time PCR in 15 psoriasis patients, 15 atopic dermatitis patients and 15 normal subjects.[28] The order of six miRNA expression levels in the serum of each individual was different among psoriasis patients, atopic dermatitis patients and normal subjects. Combination of serum levels of miR-146a-5p and -203a-3p was more reliable to distinguish psoriasis patients and normal subjects than each miRNA alone, according to the ROC analysis. Thus, we first demonstrated that multiple miRNA play a role cooperatively in the pathogenesis of psoriasis, and the combination of miRNA may be more reliable for the diagnosis of the disease.

In addition, we studied the clinical significance of hair miRNA in psoriasis. A single hair root and five pieces of hair shafts were collected from 26 psoriatic patients.[29] The levels of miR-424-5p in hair roots and hair shafts were determined by quantitative real-time PCR. Samples from atopic dermatitis patients were included as the disease control. Control hair samples were also collected from normal subjects. Hair shaft miR-424-5p levels were significantly higher in psoriasis patients than normal subjects, but not altered in those with atopic dermatitis. By ROC analysis of hair shaft miR-424-5p, the area under the curve (AUC) was 0.77. AUC of more than 0.7 indicates that hair shaft miR-424-5p levels can distinguish psoriasis patients from normal subjects effectively. However, the miR-424-5p levels were not correlated with clinical disease activity markers of psoriasis, including disease duration, body surface area and Psoriasis Area and Severity Index. Hair root levels of miR-424-5p were not useful for evaluating diagnosis or disease severity. These results suggest that hair shaft miR-424-5p levels, not hair root miR-424-5p levels, are useful as a diagnostic marker of psoriasis.

On the other hand, although serum miR-19a-3p levels were not altered in psoriasis patients,[30] given that serum miRNA levels are not always correlated with hair miRNA levels as described above,[10] we also investigated the hair levels of miR-19a-3p, one of the putative regulatory miRNA of TNF-α.[31] Hair root levels of miR-19a-3p were significantly higher in psoriasis than those in normal subjects, but not increased in atopic dermatitis patients or dermatomyositis patients. In ROC analysis to distinguish psoriasis patients from normal subjects, the AUC was 0.87. Furthermore, hair root miR-19a-3p levels in psoriasis patients were inversely correlated with the duration between symptom onset and the first visit to the hospital, suggesting that hair root miR-19a-3p levels reflect the subjective severity of symptoms and are effective as a disease marker.

DRUG ERUPTION

Toxic epidermal necrolysis (TEN) is severer form of drug-induced cutaneous reaction. It is well known that keratinocyte apoptosis is one of the histological characteristics of TEN, but its exact mechanism is still to be clarified. The PCR array analysis using skin-derived miRNA indicated that miR-18a-5p level was increased in the TEN tissues *in vivo*, compared with normal skin or skins of other diseases, which was validated by real-time PCR.[32] Based on the web-based computational miRNA target gene prediction databases, we identified BCL2-like protein 10 (BCL2L10), an anti-intrinsic apoptotic molecule, as the putative direct target of miR-18a-5p. Forced overexpression of the miR-18a-5p mimic into cultured normal keratinocytes increased caspase-9 activity as well as cell apoptosis, which are known to be increased in the skin of TEN patients. Considering that we demonstrated that the protein and mRNA expressions of BCL2L10 were downregulated in involved skin of TEN, the decrease of BCL2L10 may be induced by the miR-18a-5p overexpression in TEN, which leads to the keratinocyte apoptosis. TEN may be caused by the "seed and soil" theory, the inflammatory cells being the "seeds" and the keratinocytes being the "soil", which is modulated by miRNA.

We found that serum miR-18a-5p levels were also significantly increased in TEN patients compared with normal subjects, indicating that the miRNA levels are useful for the diagnosis.[32] Furthermore, because serum miR-18a-5p levels in patients with drug eruption were correlated with body surface areas of skin erythema and erosion, they can also be utilized as a disease activity marker of drug eruption.

POLYMYOSITIS/DERMATOMYOSITIS

Several researches investigated the role of miRNA in the pathogenesis of polymyositis/dermatomyositis. In 2007, Eisenberg *et al.*[33] determined the expression of several miRNA in the muscle tissues of patients with muscular disorders, including polymyositis/dermatomyositis, and found several dysregulated miRNA. We then focused on miRNA expressed in the skin of dermatomyositis and showed that expression of miR-223-3p was decreased in Gottron's papules of patients with dermatomyositis and clinically amyopathic dermatomyositis compared with normal skin or psoriatic skin.[34] Protein kinase C (PKC)-ε is the target of miR-223-3p, and the specific miRNA inhibitor of miR-223-3p induced PKC-ε expression and cell proliferative activity of keratinocytes. Thus, PKC-ε upregulation induced by miR-223-3p downregulation may stimulate keratinocyte proliferation and contribute to the acanthosis in the epidermis of Gottron's papules. Taken together, we demonstrated the significance of novel miRNA target interaction in the pathogenesis of dermatomyositis.

We also tried to clarify whether MIRSNP rs2910164 in miR-146a can be a useful marker for polymyositis/dermatomyositis.[35] Serum and skin DNA samples were obtained from 25 patients with dermatomyositis, 16 with clinically amyopathic dermatomyositis and three with polymyositis. We found that serum miR-146a-5p levels tended to be downregulated in patients with dermatomyositis with CC genotype. On the other hand, there was no significant difference in the frequency of genotype distribution between control subjects and patients. However, patients with CC genotype showed significantly increased frequency of muscle weakness and dysphagia compared with patients with CG or GG genotype. Accordingly, our study implicated miR-146a MIRSNP in the pathogenesis of polymyositis/dermatomyositis and the CC genotype become a risk maker of muscle involvement in patients.

The diagnosis of dermatomyositis, especially amyopathic dermatomyositis, is sometimes difficult.[36] Serum miRNA have also been well investigated in polymyositis/dermatomyositis for the possible application as a disease marker. For example, the serum miR-223-3p levels were significantly lower in patients with polymyositis/dermatomyositis, especially amyopathic dermatomyositis, than healthy controls.[34] When we compared the serum miR-223-3p levels and the clinical features of patients, we found patients with decreased miR-223-3p levels showed significantly shorter disease duration between symptom onset and the first visit to the hospital than those with normal levels, indicating that serum miR-223-3p levels can be a subjective symptom marker. Furthermore, serum miR-7-5p levels were decreased in dermatomyositis patients,[37] but not serum miR-142-3p and miR-92a-3p.[38,39] In addition, we found upregulation of serum miR-21-5p levels in dermatomyositis, correlating with serum immunoglobulin G levels.[40]

We next determined miR-214-3p levels in the hair, because miR-214-3p is a unique miRNA that is detected in the skin but not in serum.[41] Hair root and hair shafts were obtained from nine dermatomyositis patients, 18 scleroderma patients and 15 normal subjects. There were no significant differences in hair root miR-214-3p levels among normal subject, scleroderma patients and dermatomyositis patients. However, we found significantly higher miR-214-3p levels in hair shafts of dermatomyositis patients than in normal subjects and scleroderma patients. ROC analysis of hair shaft miR-214-3p levels indicated that the AUC was 0.90 to distinguish dermatomyositis patients from normal subjects. Furthermore, because patients with elevated hair shaft miR-214-3p levels showed significantly shorter disease duration between symptom onset and the first visit to the hospital, hair shaft miR-214-3p levels may be useful for the diagnosis and evaluating subjective disease severity of dermatomyositis.

SCLERODERMA

miR-29-3p is the first miRNA that is implicated in the pathogenesis of scleroderma. In 2000, Maurer *et al.*[42] described that miR-29-3p is downregulated in scleroderma skin both *in vivo* (e.g. sclerotic skin tissues) and *in vitro* (cultured scleroderma dermal fibroblasts). miR-29 has also been reported to contribute to the pathogenesis of other fibrotic conditions such as myocardial fibrosis or keloid.[3,43,44] miR-29-3p targets several fibrosis-related molecules including multiple collagens, elastin and fibrillins. miR-29-3p supplementation to cultured scleroderma fibroblasts leads to the normalization of these molecules. Thus, the downregulation of miR-29-3p in scleroderma

dermal fibroblasts will result in the increased expression of fibrosis-related molecules and tissue fibrosis. The miR-29-3p downregulation in scleroderma fibroblasts is likely to be caused by the stimulation of fibrosis-related cytokines such as transforming growth factor-β1, IL-4 and platelet-derived growth factor-B.

On the other hand, we identified miR-196a-5p and let-7a-5p as the fibrosis-related miRNA by PCR array, real-time PCR and *in situ* hybridization.[45,46] These miRNA target both the α1(I) and α2(I) chain of type I collagen, and their expression was significantly downregulated in scleroderma dermal fibroblasts compared with normal fibroblasts *in vitro* and *in vivo*. The supplementation of miRNA by miR-196a-5p and let-7a-5p mimics to cultured scleroderma fibroblasts reduced the excess expression of collagens, whereas the miRNA inhibitor of these miRNA induced collagen expression in normal dermal fibroblasts. These results indicate that downregulation of these miRNA also contribute to the collagen overexpression and tissue fibrosis in scleroderma.

In addition, we investigate the link between scleroderma and MIRSNP rs2910164. DNA was obtained from skin samples of 52 Japanese scleroderma patients and 107 control subjects. Genotype distribution of rs2910164 was 44.1% of CC, 53.8% of CG and 1.9% of GG in scleroderma patients, while 47.7% of CC, 49.5% of CG and 2.8% of GG in 107 control DNA. There was no significant difference in the distribution between the two groups. However, when the genotypes and clinical/laboratory features were compared, the prevalence of telangiectasia in patients with CC genotype was significantly higher than those with CG or GG. Because miR-146a-5p has been recently implicated in vascular abnormalities,[47] the MIRSNP may also be involved in vascular involvement of scleroderma and become a risk factor of vascular abnormality.

miRNA levels in the serum or hair have already been determined in scleroderma patients. We could not found a significant difference in the serum miR-29a-3p levels between scleroderma patients and control subjects.[48] However, miR-29a-3p levels in the sera of patients diagnosed with scleroderma spectrum disorder (SSD) is significantly decreased compared with scleroderma patients and healthy controls: SSD is defined as a clinical entity of patients who do not fulfill the criteria of scleroderma but were thought to develop scleroderma in the future.[49] Accordingly, serial measurement of serum miR-29a-3p may be useful for the screening of early scleroderma. Furthermore, our result indicated that expression of skin miRNA are not always correlated with serum levels of the miRNA. On the other hand, serum miR-196a-5p levels were not altered in scleroderma patients, but serum let-7a-5p levels were found to be significantly decreased in scleroderma patients compared with normal subjects.[45,46] By the analysis of correlation between serum let-7a-5p levels and clinical/serological features, we found that scleroderma patients with lower serum let-7a-5p levels had significantly higher modified Rodnan total skin thickness score compared with those without. Thus, serum let-7a-5p levels may be a putative biomarker of the severity of skin sclerosis, reflecting collagen overexpression. In addition, the considering significantly decreased frequency of anticentromere antibody in patients with lower let-7a-5p levels compared with those without, let-7a-5p may also be involved in the autoimmune dysregulation as well as tissue fibrosis in this disease.

We also determined whether the combination of serum levels of multiple miRNA can be a more useful biomarker of scleroderma patients. Serum levels of six miRNA (let-7 g-5p, miR-21-5p, miR-29b-3p, miR-125b-5p, miR-145-5p and miR-206), selected according to the previous studies, were measured using real-time PCR in 15 scleroderma patients and 15 normal subjects.[50] The serum levels of each miRNA did not differ between scleroderma patients and normal subjects. On the other hand, when the six miRNA levels of each individual were ranked, we found significant difference in the ranks among miRNA only in scleroderma patients, suggesting the difference of expression patterns of multiple miRNA in scleroderma patients. We also demonstrated that the combination of miR-206 level with miR-21-5p level improves the AUC of ROC analysis compared with that of either miR-206 or miR-21-5p alone. Taken together, this study suggests that the combination of serum levels of multiple miRNA may be more reliable to diagnose patients of scleroderma.

As described in the Introduction, it is difficult to take blood samples of severe scleroderma patients due to their sclerotic skin and narrowed vessels. Thus, the development of biomarkers in hair samples is more useful for these patients. For example, hair shaft miR-29a-3p levels in scleroderma patients were significantly decreased compared with those in control subjects.[51] Furthermore, there was no significant difference in hair root miR-196a-5p levels between normal subjects and scleroderma patients, while hair shaft miR-196a-5p levels were significantly decreased in scleroderma patients.[10] Although we have been unable to find the correlation of hair miRNA levels with clinical features of scleroderma so far probably because of the small number of samples, there is a possibility that serial measurement of miRNA levels in the hair will lead to the early detection of visceral involvements including pulmonary fibrosis or renal dysfunction in scleroderma patients in the future.

CONCLUSION

In the review, we showed various examples of involvement of miRNA in skin diseases. In 2005, Tamai *et al.* developed nuclear factor-κB decoy DNA ointment for atopic dermatitis. In addition, Yokozeki *et al.* also demonstrated a therapeutic effect of signal transducer and activator of transcription-6 decoy oligodeoxynucleotide ointment for the disease.[52,53] Such small oligodeoxynucleotides are considered new therapeutic approaches against skin diseases. There are also attempts in the clinical application of miRNA for the treatment of intractable diseases. For example, Lanford *et al.*[54] reported that long-lasting suppressive effect of SPC3649, an oligonucleotide targeting miR-122-5p essential for hepatitis C virus (HCV) RNA proliferation, on HCV viremia in chronically HCV-infected chimpanzees. Then, Miravirsen (SPC3649; Santaris Pharma, Copenhagen, Denmark) was tested by the randomized, double-blind, placebo-controlled, ascending multiple-dose phase

2a proof-of-concept study. The clinical trial demonstrated its dose-dependent, continuous and prolonged antiviral activity, or its safety and tolerability in HCV patients as a new antiviral therapy.

For the treatment of each disease, miRNA increased in the disease needs to be downregulated using miRNA inhibitors, whereas miRNA decreased in the disease needs to be supplemented using miRNA mimics. In 2013, we demonstrated that let-7a-5p expression in the mouse skin can be induced by intermittent i.p. let-7a-5p injection with atelocollagen, and bleomycin-induced skin fibrosis in mice can be prevented by let-7a-5p supplementation.[46] This may be the first report to supplement miRNA in the mouse skin. As the advantages of miRNA treatments, because miRNA target multiple molecules, miRNA can normalize multiple factors causing each disease at the same time. Accordingly, in conclusion, future researches of miRNA will enable the advances of their clinical applications including the clarification of pathogenesis, disease markers and novel treatments.

CONFLICT OF INTEREST: None.

REFERENCES

1 Maragkakis M, Alexiou P, Papadopoulos GL *et al.* Accurate microRNA target prediction correlates with protein repression levels. *BMC Bioinformatics* 2009; **10**: 295.
2 Chinnathambi S, Wiechert S, Tomanek-Chalkley A, Winter MC, Bickenbach JR. Treatment with the cancer drugs decitabine and doxorubicin induces human skin keratinocytes to express Oct4 and the OCT4 regulator mir-145. *J Dermatol* 2012; **39**: 617–624.
3 Li P, He QY, Luo CQ. Overexpression of miR-200b inhibits the cell proliferation and promotes apoptosis of human hypertrophic scar fibroblasts *in vitro*. *J Dermatol* 2014; **41**: 903–911.
4 Wang Y, Wang K, Liang J *et al.* Differential expression analysis of miRNA in peripheral blood mononuclear cells of patients with non-segmental vitiligo. *J Dermatol* 2015; **42**: 193–197.
5 van Dommelen SM, Vader P, Lakhal S *et al.* Microvesicles and exosomes: opportunities for cell-derived membrane vesicles in drug delivery. *J Control Release* 2012; **161**: 635–644.
6 Chen X, Liang H, Zhang J, Zen K, Zhang CY. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. *Protein Cell* 2012; **3**: 28–37.
7 Mitchell PS, Parkin RK, Kroh EM *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 2008; **105**: 10513–10518.
8 Hanke M, Hoefig K, Merz H *et al.* A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. *Urol Oncol* 2010; **28**: 655–661.
9 Lefkowitz GK, Mukhopadhyay A, Cowing-Zitron C, Yu BD. The post-apoptotic fate of RNAs identified through high-throughput sequencing of human hair. *PLoS ONE* 2011; **6**: e27603.
10 Wang Z, Jinnin M, Kudo H *et al.* Detection of hair-microRNAs as the novel potent biomarker: evaluation of the usefulness for the diagnosis of scleroderma. *J Dermatol Sci* 2013; **72**: 134–141.
11 Zhang L, Huang J, Yang N *et al.* microRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci USA* 2006; **103**: 9136–9141.
12 Kanemaru H, Fukushima S, Yamashita J *et al.* The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker. *J Dermatol Sci* 2011; **61**: 187–193.
13 Felicetti F, Errico MC, Bottero L *et al.* The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. *Cancer Res* 2008; **68**: 2745–2754.
14 Bertino JR, Banerjee D, Mishra PJ. Pharmacogenomics of microRNA: a miRSNP towards individualized therapy. *Pharmacogenomics* 2007; **8**: 1625–1627.
15 Furer V, Greenberg J, Attur M, Abramson S, Pillinger M. The role of microRNA in rheumatoid arthritis and other autoimmune diseases. *Clin Immunol* 2010; **136**: 1–15.
16 Philippidou D, Schmitt M, Moser D *et al.* Signatures of microRNAs and selected microRNA target genes in human melanoma. *Cancer Res* 2010; **70**: 4163–4173.
17 Yamashita J, Iwakiri T, Fukushima S *et al.* The rs2910164 G>C polymorphism in microRNA-146a is associated with the incidence of malignant melanoma. *Melanoma Res* 2013; **23**: 13–20.
18 Ikuta Y, Nakatsura T, Kageshita T *et al.* Highly sensitive detection of melanoma at an early stage based on the increased serum secreted protein acidic and rich in cysteine and glypican-3 levels. *Clin Cancer Res* 2005; **11**: 8079–8088.
19 Friedman EB, Shang S, de Miera EV *et al.* Serum microRNAs as biomarkers for recurrence in melanoma. *J Transl Med* 2012; **10**: 155.
20 Greenberg E, Besser MJ, Ben-Ami E *et al.* A comparative analysis of total serum miRNA profiles identifies novel signature that is highly indicative of metastatic melanoma: a pilot study. *Biomarkers* 2013; **18**: 502–508.
21 Shiiyama R, Fukushima S, Jinnin M *et al.* Sensitive detection of melanoma metastasis using circulating microRNA expression profiles. *Melanoma Res* 2013; **23**: 366–72.
22 Inada T, Fukushima S, Murai M *et al.* Hair shaft miRNA-221 levels as a new tumor marker of malignant melanoma. *J Dermatol* 2015; **42**: 198–201.
23 Sonkoly E, Wei T, Janson PC *et al.* MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? *PLoS ONE* 2007; **2**: e610.
24 Zibert JR, Løvendorf MB, Litman T, Olsen J, Kaczkowski B, Skov L. MicroRNAs and potential target interactions in psoriasis. *J Dermatol Sci* 2010; **58**: 177–185.
25 Ichihara A, Jinnin M, Yamane K *et al.* microRNA-mediated keratinocyte hyperproliferation in psoriasis vulgaris. *Br J Dermatol* 2011; **165**: 1003–1010.
26 Ichihara A, Jinnin M, Oyama R *et al.* Increased serum levels of miR-1266 in patients with psoriasis vulgaris. *Eur J Dermatol* 2012; **22**: 68–71.
27 Pivarcsi A, Meisgen F, Xu N, Ståhle M, Sonkoly E. Changes in the level of serum microRNAs in patients with psoriasis after antitumour necrosis factor-α therapy. *Br J Dermatol* 2013; **169**: 563–570.
28 Koga Y, Jinnin M, Ichihara A *et al.* Analysis of expression pattern of serum microRNA levels in patients with psoriasis. *J Dermatol Sci* 2014; **74**: 170–171.
29 Tsuru Y, Jinnin M, Ichihara A *et al.* miR-424 levels in hair shaft are increased in psoriatic patients. *J Dermatol* 2014; **41**: 382–5.
30 Oyama R, Jinnin M, Kakimoto A *et al.* Circulating microRNA associated with TNF-α signaling pathway in patients with plaque psoriasis. *J Dermatol Sci* 2011; **61**: 209–211.
31 Hirao H, Jinnin M, Ichihara A *et al.* Detection of hair root miR-19a as a novel diagnostic marker for psoriasis. *Eur J Dermatol* 2013; **23**: 807–811.
32 Ichihara A, Wang Z, Jinnin M *et al.* Upregulation of miR-18a-5p contributes to epidermal necrolysis in severe drug eruptions. *J Allergy Clin Immunol* 2014; **133**: 1065–1074.
33 Eisenberg I, Eran A, Nishino I *et al.* Distinctive patterns of microRNA expression in primary muscular disorders. *Proc Natl Acad Sci USA* 2007; **104**: 17016–17021.
34 Inoue K, Jinnin M, Yamane K *et al.* Down-regulation of miR-223 contributes to the formation of Gottron's papules in dermatomyositis via the induction of PKCε. *Eur J Dermatol* 2013; **23**: 160–167.
35 Okada Y, Jinnin M, Makino T *et al.* MIRSNP rs2910164 of miR-146a is associated with the muscle involvement in polymyositis/dermatomyositis. *Int J Dermatol* 2014; **53**: 300–304.

36 Inoue K, Jinnin M, Hara Y *et al.* Serum levels of tenascin-C in collagen diseases. *J Dermatol* 2013; **40**: 715–719.
37 Oshikawa Y, Jinnin M, Makino T *et al.* Decreased miR-7 expression in the skin and sera of patients with dermatomyositis. *Acta Derm Venereol* 2012; **93**: 273–276.
38 Makino K, Jinnin M, Kajihara I *et al.* Circulating miR-142-3p levels in patients with systemic sclerosis. *Clin Exp Dermatol* 2012; **37**: 34–39.
39 Sing T, Jinnin M, Yamane K *et al.* microRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma. *Rheumatology (Oxford)* 2012; **51**: 1550–1556.
40 Shimada S, Jinnin M, Ogata A *et al.* Serum miR-21 levels in patients with dermatomyositis. *Clin Exp Rheumatol* 2013; **31**: 161–162.
41 Inoue M, Jinnin M, Wang Z *et al.* microRNA level is raised in the hair shafts of the patients with dematomyositis in comparison with the normal subjects and sclerodermic patients. *Int J Dermatol* 2015; [forthcoming].
42 Maurer B, Stanczyk J, Jüngel A *et al.* MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. *Arthritis Rheum* 2010; **62**: 1733–1743.
43 van Rooij E, Sutherland LB, Thatcher JE *et al.* Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA* 2008; **105**: 13027–13032.
44 Cheng J, Wang Y, Wang D, Wu Y. Identification of collagen 1 as a post-transcriptional target of miR-29b in skin fibroblasts: therapeutic implication for scar reduction. *Am J Med Sci* 2013; **346**: 98–103.
45 Honda N, Jinnin M, Kajihara I *et al.* TGF-β-mediated downregulation of microRNA-196a contributes to the constitutive upregulated type I collagen expression in scleroderma dermal fibroblasts. *J Immunol* 2012; **188**: 3323–3331.
46 Makino K, Jinnin M, Hirano A *et al.* The downregulation of microRNA let-7a contributes to the excessive expression of type I collagen in systemic and localized scleroderma. *J Immunol* 2013; **190**: 3905–3915.
47 Nazari-Jahantigh M, Wei Y, Schober A. The role of microRNAs in arterial remodelling. *Thromb Haemost* 2012; **107**: 611–618.
48 Kawashita Y, Jinnin M, Makino T *et al.* Circulating miR-29a levels in patients with scleroderma spectrum disorder. *J Dermatol Sci* 2011; **61**: 67–69.
49 Ihn H, Sato S, Tamaki T *et al.* Clinical evaluation of scleroderma spectrum disorders using a points system. *Arch Dermatol Res* 1992; **284**: 391–395.
50 Koba S, Jinnin M, Inoue K *et al.* Expression analysis of multiple microRNAs in each patient with scleroderma. *Exp Dermatol* 2013; **22**: 489–491.
51 Takemoto R, Jinnin M, Wang Z *et al.* Hair miR-29a levels are decreased in patients with scleroderma. *Exp Dermatol* 2013; **22**: 832–833.
52 Tamai K, Kaneda Y, Nakamura H *et al.* NF-κB decoy DNA for treatment of atopic dermatitis. *Nihon Rinsho* 2005; **63**: 659–663.
53 Igawa K, Satoh T, Yokozeki H. A therapeutic effect of STAT6 decoy oligodeoxynucleotide ointment in atopic dermatitis: a pilot study in adults. *Br J Dermatol* 2009; **160**: 1124–1126.
54 Lanford RE, Hildebrandt-Eriksen ES, Petri A *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010; **327**: 198–201.

International Journal of
Rheumatic Diseases
International Journal of Rheumatic Diseases 2015

ORIGINAL ARTICLE

Risk factors for development of systemic lupus erythematosus among Japanese females: medical history and reproductive factors

Masakazu WASHIO,[1] Hiroki TAKAHASHI,[2] Gen KOBASHI,[3] Chikako KIYOHARA,[4] Yoshifumi TADA,[5] Toyoko ASAMI,[6] Yuichiro IDE,[1] Tatsuya ATSUMI,[7] Takahiko HORIUCHI[8] and the Kyushu Sapporo SLE (KYSS) Study Group*

[1]*Department of Community Health and Clinical Epidemiology, St. Mary's College, Kurume,* [2]*Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University School of Medicine, Sapporo,* [3]*Department of Public Health, Dokkyo Medical University School of Medicine, Tochigi,* [4]*Department of Preventive Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka,* [5]*Department of Internal Medicine, Faculty of Medicine, Saga University, Saga,* [6]*Rehabilitation Center, Saga University Hospital, Saga,* [7]*Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, and* [8]*Department of Internal Medicine, Kyushu University Beppu Hospital, Beppu, Japan*

Abstract

Aim: The aim of the present study was to examine the influence of medical history and reproductive factors on the development of systemic lupus erythematosus (SLE) among Japanese females.

Methods: One hundred and sixty female SLE patients and 660 female volunteers were studied in a case–control study. Unconditional logistic regression was used to compute odds ratios (OR) and 95% confidence intervals (CIs).

Results: The present study demonstrated that medical histories of operations without blood transfusion (OR = 1.64, 95% CI = 1.10–2.44) and operations with blood transfusion (OR = 4.44, 95% CI = 1.93–10.23) increased the risk of SLE with adjustment for age, region, smoking and alcohol drinking. Among 91 SLE patients and 284 control subjects who had the experience of married life, nulliparity (OR = 2.29, 95% CI = 1.05–5.17), increased the risk of SLE, while the risk decreased according to the number of children (one to two *vs.* none, OR = 0.27, 95% CI = 0.10–0.73; three or more *vs.* none, OR = 0.14, 95% CI = 0.04–0.51; P for trend < 0.01).

Conclusions: Several factors are suggested to be associated with the development of SLE among Japanese females.

Key words: case–control study, epidemiology, risk factors, systemic lupus erythematosus.

INTRODUCTION

The Japanese Ministry of Health and Welfare designated systemic lupus erythematosus (SLE) as an intractable disease because there is no established way to prevent or cure it.[1] SLE is a prototypic systemic autoimmune disease characterized by a broad spectrum of clinical manifestations and diverse immunological disorders.[2–4] Although genetic factors play a role in the development of SLE,[2,4,5] environmental factors may also play a role in the development of SLE.[2,4] Thus, interactions between susceptibility genes and environmental factors are responsible for the development of SLE.

Many factors have been proposed as causing or preventing the development of SLE.[6–15] However, there are only a few studies on non-genetic risk/preventive fac-

Correspondence: Professor Masakazu Washio, Department of Community Health and Clinical Epidemiology, St. Mary's College, 422 Tsubuku-honmachi, Kurume, Fukuoka 830–8558, Japan. Email: washio@st-mary.ac.jp
*Members of the Kyushu Sapporo SLE (KYSS) Study Group are given in Appendix.

tors for the development of SLE in a Japanese population.[10–15] Therefore, we continued our case–control study of SLE to increase the number of SLE patients so that we could detect more factors related to the development of SLE among Japanese females in the Kyushu Sapporo SLE (KYSS) study. The present study was conducted to investigate the influence of medical history, and reproductive factors on the development of SLE among Japanese females.

METHODS

Profile of the Kyushu Sapporo SLE (KYSS) study

The KYSS study was a case–control study in order to evaluate risk factors for SLE, which was supported by a Grant for Research on Measures for Intractable Diseases from the Japanese Ministry of Health, Labor and Welfare. The study methods and ethical issues have been described elsewhere.[13] Briefly, SLE patients were recruited from 2002 to 2006 on Kyushu Island (i.e., the south-western edge of mainland Japan with a temperate climate) and from 2004 to 2010 on Hokkaido Island (i.e., the northern edge of mainland Japan with a subarctic climate). The patients had been followed up at the rheumatology clinic of Kyushu University Hospital and Saga University Hospital in Kyushu, and Sapporo Medical University Hospital and Hokkaido University Hospital in Hokkaido. All patients fulfilled the American College of Rheumatology (ACR) 1982 and 1997 revised criteria for SLE.[16] In Japan, an antinuclear antibody (ANA) test is ordered as a routine screening test if there is a reasonable suspicion of SLE from family history and/or clinical features. Therefore, we performed an ANA test for all SLE patients before the diagnosis of SLE, and almost all SLE patients had a positive ANA test result.

Controls were recruited from nursing college students and care workers in nursing homes in Kyushu, while population controls were recruited from participants of a health check-up in a local town in Hokkaido.

A self-administered questionnaire was obtained from SLE patients and controls, along with written informed consent for incorporation into this study. A section of the participants also agreed to donate blood samples, which were stored until use for DNA extraction and genotyping of the candidate genes for SLE.

All questions referred to the subject's status before the diagnosis of SLE in cases and before the interview day for controls. The family medical history included selected chronic diseases occurring among parents and siblings.

The present study was approved by the institutional review boards of Kyushu University Graduate school of Medical Sciences, Saga Medical School, St. Mary's College, Sapporo Medical University, Hokkaido University School of Medicine and each of the other institutions involved.

Subjects and methods in the present study

In Kyushu, 185 out of 343 female SLE patients (53.9%) agreed to participate in this study, while 177 out of 338 female SLE patients (52.4%) agreed in Hokkaido. On the other hand, 256 out of 317 nursing college students (80.8%) and 137 out of 175 care workers (78.3%), who were 393 (79.9%) out of 492 female volunteers, agreed to participate in this study in Kyushu as controls, while 267 out of 346 females (77.2%) did so in Hokkaido. Thus, a self-administered questionnaire was obtained from 362 out of 681 SLE patients (53.2%) and 660 out of 838 control subjects (78.8%). However, in the present study, we excluded 202 patients who had been treated for SLE for more than 10 years in order to obtain accurate information because cases were asked to answer their status before the diagnosis of SLE. Thus, the cases were 160 female patients and the controls were 660 female volunteers. Among 160 cases and 660 control subjects, we excluded unmarried subjects (69 SLE patients [43.1%] and 376 controls [57.9%]) when we evaluated the association between the risk of SLE and reproductive factors. Thus, we used 91 SLE patients and 284 female volunteers (i.e., eight nursing college students, 97 care workers and 179 participants of a health check-up) to evaluate the reproductive risk factors for SLE.

Statistical analysis

Unconditional logistic model was applied to evaluate the odds ratios (ORs) and their 95% confidence intervals (CIs) after adjusting for age, region and other factors. Since smoking increases the risk of SLE[13–15] and alcohol is suggested to prevent SLE[14] in our previous studies, we calculated ORs of SLE with adjustment for age, region, smoking and alcohol drinking. Age was treated as continuous variables, and indicator variables were used for region (i.e., Kyushu or Hokkaido), smoking, alcohol drinking, medical history, family medical history and reproductive factors. We treated current and former smokers as smoking positive while those who drank 1 day/week or more were defined as drinking positive. Ages at the time of interview day (i.e., the time at completion of the questionnaire) was used for controls, whereas the ages at diagnosis were used for SLE

patients. All statistical analyses were conducted by use of a statistical analysis system package (SAS Institute Inc., Cary, NC, USA). All *P*-values for statistical tests were two-tailed, and a *P*-value of < 0.05 was considered to indicate statistical significance.

RESULTS

Table 1 displays the characteristics of SLE patients and control subjects. SLE patients were older than controls at the time of interview day and they were more likely to experience a married life than control subjects. In addition, the positive rates of smoking and drinking were greater among SLE patients than controls (Table 1). However, drinking showed no meaningful association with the risk of SLE (OR = 1.11, 95% CI = 0.72–1.71) after controlling for age, region and smoking, while smoking (OR = 2.60, 95% CI = 1.76–3.85) remained as a risk factor for SLE after controlling for age, region and alcohol drinking (not shown in the table). On the other hand, the mean age of SLE patients at the time of diagnosis did not differ from the mean age of controls on the interview day and there was no significant difference in educational background or in region between SLE patients and controls (Table 1).

In Kyushu, SLE patients were older than controls at the time of interview day (37.1 ± 13.8 *vs.* 28.0 ± 12.1 years old, $P < 0.01$) and the mean age of SLE patients at the diagnosis was older than the mean age of controls on the interview day (31.9 ± 13.7 *vs.* 28.0 ± 12.1 years old, $P < 0.05$) while in Hokkaido, the mean age did not differ between SLE patients and controls on the interview day (38.5 ± 13.7 *vs.* 39.5 ± 14.0 years old, $P = 0.57$) but the mean age of SLE patients at the time of diagnosis was younger than the mean age of controls on the interview day (32.9 ± 14.0 *vs.* 39.5 ± 14.0 years old, $P < 0.01$; not shown in the table).

Table 2 shows the risk of SLE in relation to self-reported medical history. Histories of rheumatoid arthritis (RA; OR = 10.78, 95% CI = 2.59–44.81), and connective tissue diseases other than RA (OR = 9.59, 95% CI = 1.65–55.64) were associated with an increased risk of SLE after controlling for age, region, smoking and alcohol drinking. In addition, a history of operations increased the risk of SLE according to the

Table 1 Characteristics of SLE patients and controls

	SLE patients (%) $n = 160$	Controls (%) $n = 660$	*P*-value
Region			
Kyushu	94 (58.8)	393 (59.6)	n.s.
Hokkaido	66 (41.3)	267 (40.5)	
Age (years)			
Age at the time of interview	37.5 ± 13.5	32.8 ± 14.1	< 0.01
Age at diagnosis of SLE	32.3 ± 13.8	32.8 ± 14.1	n.s.
Educational background			
Junior high school	16 (10.0)	30 (4.6)	n.s.
Senior high school	71 (44.4)	363 (55.0)	
Junior college	53 (33.1)	174 (26.4)	
University or graduate school	20 (12.5)	93 (14.1)	
Marriage status			
Unmarried	69 (43.1)	376 (57.0)	< 0.01
Married	74 (46.3)	246 (37.3)	
Divorced or widowed	17 (10.6)	38 (5.8)	
Smoking habits			
Never-smokers	95 (59.4)	522 (79.1)	< 0.01
Former smokers	10 (6.3)	20 (3.0)	
Current smokers	55 (37.4)	118 (17.9)	
Drinking habits			
Nondrinkers	116 (72.5)	525 (79.5)	< 0.05
Former drinkers	1 (0.6)	6 (0.9)	
Current drinkers	43 (26.9)	129 (19.6)	

n.s., not significant.
Nondrinkers: drink less than once/week, Current drinkers: drink once/week or more.

Table 2 Medical history and the risk of SLE among Japanese females

Self-reported medical history	SLE patients (%) $n = 160$	Controls (%) $n = 660$	Smoking, drinking, age, and region-adjusted odds ratio (95% CI)
Cancer (yes *vs.* no)	3 (1.9)	10 (1.5)	1.02 (0.27–3.90)
Cardiovascular diseases (yes *vs.* no)	1 (0.6)	2 (0.3)	1.94 (0.17–22.39)
Diabetes mellitus (yes *vs.* no)	3 (1.9)	5 (0.8)	4.04 (0.90–18.24
Kidney diseases (yes *vs.* no)	7 (4.4)	18 (2.7)	1.47 (0.59–3.69)
Asthma (yes *vs.* no)	6 (3.8%)	49 (7.4%)	0.46 (0.19–1.11)
Atopic dermatitis (yes *vs.* no)	15 (9.4)	68 (10.3)	0.80 (0.43–1.46)
Allergy (yes *vs.* no)	16 (10.0)	84 (12.7)	0.76 (0.43–1.36)
Urticaria (yes *vs.* no)	20 (12.5)	78 (11.8)	1.10 (0.64–1.87)
Rheumatoid arthritis (yes *vs.* no)	7 (4.4)	3 (0.5)	10.78 (2.59–44.81)*
Connective tissue diseases other than rheumatoid arthritis (yes *vs.* no)	4 (2.5)	2 (0.3)	9.59 (1.65–55.64)*
Operation			
No history of operation	98 (61.3)	447 (67.7)	1.00 (reference)
Operation without blood transfusion	62 (38.8)	213 (32.3)	1.54 (1.05–2.26)*
Operation with blood transfusion	13 (8.1)	18 (2.7)	4.46 (1.99–10.00)*
			P for trend < 0.01

*$P < 0.05$.
Allergy: e.g., allergic rhinitis, food allergy, medication allergy.

impact of operations (operations without blood transfusion: OR = 1.54, 95% CI = 1.05–2.26 and operations with blood transfusion: OR = 4.46, 95% CI = 1.99–10.00, P for trend < 0.01) after controlling for age, region, smoking and alcohol drinking.

In the analyses of all cases and control subjects in Kyushu, histories of RA (OR = 22.68, 95% CI = 2.64–194.48), connective tissue diseases other than RA (OR = 13.45, 95% CI = 1.36–133.19), and a history of operations (operations without blood transfusion: OR = 1.65, 95% CI = 1.00–2.72 and operations with blood transfusion: OR = 5.22, 95% CI = 1.43–19.10, P for trend < 0.01) remained as risk factors for SLE after controlling for age, region, smoking and alcohol drinking (not shown in the table). On the other hand, in the analyses of all cases and control subjects in Hokkaido, histories of operations with and without blood transfusion remained as risk factors for SLE (operations without blood transfusion: OR = 1.38, 95% CI = 0.73–2.58 and operations with blood transfusion: OR = 3.66, 95% CI = 1.14–11.71, P for trend < 0.05) after controlling for age, region, smoking and alcohol drinking (not shown in the table).

Table 3 displays the association between reproductive factors and the risk of SLE among females who had the experience of married life. There were 91 SLE patients and 284 control subjects (i.e., eight nursing college students, 97 care workers and 179 participants of a health check-up) who had the experience of married life. The proportion of residents in Kyushu was greater in SLE patients than in control subjects. Compared with control subjects, SLE patients were younger than control subjects at the time of interview as well as at the time of diagnosis. They were also more likely to smoke than controls. In the analyses to evaluate the association between reproductive factors and SLE risk, nulliparity (OR = 2.29, 95% CI = 1.05–5.17) was positively associated with the risk of SLE while live births (OR = 0.23, 95% CI = 0.09–0.59) were negatively associated with the risk. Furthermore, the risk of SLE decreased according to the number of children (one to two *vs.* none, OR = 0.27, 95% CI = 0.10–0.73; three or more, OR = 0.14, 95% CI = 0.04–0.51; P for trend < 0.01). In contrast, there was no meaningful association between the risk of SLE and either marriage status, age at menarche, miscarriage or use of oral contraceptives.

DISCUSSION

In the present study, a self-reported medical history of RA, and a history of connective tissue diseases other than RA was associated with an increased risk of SLE. This may be partly explained in the following way. First, patients with undifferentiated connective tissue disease (UDCTD) are more likely to progress to SLE than any other connective tissue disease, although the majority of UCTD patients never progress to defined connective tissue diseases but remain undifferentiated.[17] Second,

Table 3 Reproductive factors and the risk of SLE among Japanese females with the experience of married life

Characteristics	SLE patients (%) $n = 91$	Controls (%) $n = 284$	*P*-value
Regions			
Kyushu	52 (57.1)	105 (37.0)	< 0.01
Hokkaido	39 (42.4)	179 (63.0)	
Age (years)			
Age at the time of interview	43.6 ± 13.3	46.4 ± 8.8	< 0.01
Age at diagnosis of SLE	38.5 ± 35.7	46.4 ± 8.8	< 0.01
Educational background			
Junior College or higher	38 (41.8)	90 (31.7)	n.s.
Smoking and drinking habits			
Current and former smokers	45 (49.5)	79 (27.8)	< 0.01
Drinkers (once/week or more)	27 (29.7)	69 (24.3)	n.s.
Reproductive factors	**SLE patients (%) $n = 91$**	**Controls (%) $n = 284$**	**Adjusted odds ratio (95% CI)**
Marriage status			
Married	74 (81.3)	246 (86.6)	1.00 (reference)
Divorced or widowed	17 (18.7)	38 (13.4)	1.15 (0.57–2.33)
Age at menarche			
12 years old or younger	46 (50.5)	106 (37.5)	1.00 (reference)
13 years old or older	45 (49.5)	177 (62.5)	0.76 (0.45–1.27)
Childbirth			
Nulliparity (*vs.* parity)	55 (60.4)	172 (60.6)	2.29 (1.05–5.17)*
Miscarriage (yes *vs.* no)	6 (6.6)	26 (9.2)	0.73 (0.28–1.95)
Live birth (yes *vs.*no)	31 (34.1)	97 (34.2)	0.23 (0.09–0.59)*
Number of living children delivered			
None	60 (65.9)	187 (65.8)	1.00 (reference)
One or two children	24 (26.4)	59 (20.8)	0.27 (0.10–0.73)*
Three or more children	7 (7.7)	38 (13.4)	0.14 (0.04–0.51)*
			P for trend < 0.01
Any use of oral contraceptive (ever *vs.* never)	12 (13.2)	33 (11.6)	0.95 (0.45–1.98)

*$P < 0.05$; n.s., not significant.
Adjusted odds ratio: smoking, drinking, age, and region adjusted odds ratio.

tumor necrosis factor antagonists, which are used for patients with RA and other connective tissue diseases, can induce the production of autoantibodies characteristic to SLE, such as anti-nuclear antibodies or anti-double-stranded DNA antibodies (anti-dsDNA),[18] and patients occasionally develop anti-dsDNA antibodies associated with renal or cerebral lupus.[18] Third, for the medical history of RA, some patients who had suffered from joint pain before the diagnosis of SLE, might have answered that they had RA before the diagnosis of SLE because arthritis and arthralgia are most common initial manifestations of SLE.[19,20] Therefore, we might have overestimated the risk of SLE in relation to a medical history of RA in this study.

There are some reports of a positive association between allergic disorders (i.e., drug allergy,[6] hives and medication allergies[21]) and the risk of SLE, whereas another study found that allergic disorders (i.e., asthma and allergic rhinitis) reduced the risk of SLE.[11] On the other hand, other study reported that SLE patients did not show an increased risk of immunoglobulin E (IgE) medicated allergic disorders (i.e., allergic rhinitis or allergic asthma).[22] In the present study, any self-reported history of allergic disorders showed no association with the risk of SLE.

A medical history of surgery as well as a medical history of blood transfusion was reported to increase the risk of SLE in a Japanese case–control study,[11] whereas in a European case–control study,[6] blood transfusion showed a marginally increased risk of SLE but surgery failed to do so. In the present study, operations, both with and without blood transfusion, were associated

with an increased risk of SLE. In addition, the risk increased with the impact of operations (operations without blood transfusion: OR = 1.54, 95% CI = 1.05–2.26 and operations with blood transfusion: OR = 4.46, 95% CI = 1.99–10.00, *P* for trend < 0.01). Furthermore, a history of operations (operations with and without blood transfusion) remained as risk factors for SLE even in the sub-analysis of each area (i.e., Kyushu and Hokkaido). Since psychological stress[15] and infection[4,6,23] are associated with an increased risk of SLE, we cannot deny that the psychological stress and infection associated with operations may play a role in the development of SLE.

SLE is more common in women, and this female predominance is especially noteworthy in the 15- to 64-year age group, wherein the ratios of age- and sex-incidence ratios show a six- to 10-fold female excess,[23] which suggests that hormonal factors may play an important role in the development of SLE.

Cooper *et al.*[24] reported a non-significant positive association between the age at menarche and the risk of SLE, whereas the Nurses' Health Study[25] showed an inverse relation between the age at menarche and the risk of SLE. In Japan, one study[10] reported a positive relationship between the age at menarche and the risk of SLE, whereas two other studies[11,12] reported no clear association between the age at menarche and the risk of SLE. In the present study, there was no meaningful association between the age at menarche and the risk of SLE.

Ulff-Møller *et al.*[26] reported that females with one or more children were at lower risk of SLE compared with nulliparous females among Danish women, while Cooper *et al.*[24] reported that it was not the number of live births, but the number of babies breast-fed that decreased the risk of SLE among women in the United States. In the present study, nulliparous females showed an increased risk of SLE, while live birth was negatively associated with the risk of SLE. Furthermore, the risk of SLE decreased according to the number of children (*P* for trend < 0.01), which is consistent with the findings of the study by Ulff-Møller *et al.*[26]

The Nurses' Health Study[25] revealed that oral conceptive use increased the risk of SLE, whereas Bernier *et al.*[27] reported that it was not past use but current use of oral contraceptive pills that increased the risk of SLE in the United Kingdom. In the present study, any use of oral contraceptive pills failed to show an increased risk of SLE among Japanese females. Because we did not obtain precise information about the use of oral conceptive pills (e.g., current use or past use, short-term use or long-term use), further studies are needed to evaluate the precise association between the risk of SLE and oral conceptive pills among Japanese females.

Ulff-Møller *et al.*[26] reported that miscarriage was associated with an increased risk of SLE among Danish women. In contrast, Minami *et al.*[12] found no association between miscarriage and the risk of SLE among Japanese females. In the present study, miscarriage showed no relation with the risk of SLE.

Bengtsson *et al.*[6] reported that neither the death of a husband nor divorce increased the risk of SLE in their case–control study in southern Sweden. In Japan, Nagai *et al.*[11] reported that marital status did not show any meaningful association with the risk of SLE. In the present study, divorced or widowed females did not show a greater risk of SLE compared with married females.

There are some limitations to our study. First, cases were not free from selection bias because 319 out of 681 SLE patients (46.8%) did not participate in our study. SLE patients who participated in the study might be healthier than those who did not. Second, our cases were not free from information bias as well as selection bias (i.e., prevalence-incidence bias) because they were not newly diagnosed SLE patients but patients who had been treated for SLE for < 10 years. There must be some patients who had died and could not participate in this study. Therefore, cases might be healthier than those who had died before this study. Third, self-reported data were not verified by medical records in this study. Fourth, although parity depends on many factors (e.g., age, education, occupation, income and personal preference), we could not adjust all of these factors to evaluate the association between parity and the risk of SLE. Last, our controls were not free from selection bias because 178 out of 838 control subjects (21.2%) did not participate in our study. Those who participated in our study might be healthier than those who did not. In addition, our controls were not randomly selected from the general population. In Kyushu, controls were recruited from nursing college students and care workers in nursing homes, who might have somewhat different characteristics from the general population. On the other hand, in Hokkaido, controls were participants in a health check-up in a local town, and who might have had more healthy lifestyles than the general population.

However, our study has strengths as well. Our study clearly demonstrated that a history of operations increased risk of SLE according to the impact of operations (i.e., operations without blood transfusion and

operations with blood transfusion). In addition, this finding was confirmed in two case control studies with other types of control subjects. Although the case–control study is not free from bias,[28] the findings may be considered plausible when it was confirmed in the different areas where our case–control study of SLE was conducted with the same questionnaire but different control subjects, who were nursing college students in Kyushu and participants of health check-up in Hokkaido.

In conclusion, several factors were suggested to be associated with the development of SLE among Japanese females in this study. However, further studies should be recommended to confirm the findings of the present study in other populations.

ACKNOWLEDGMENTS

This work was supported in part by Health and Labor Sciences Research Grants, Research on Intractable Diseases, and the Research Committee on Epidemiology of Intractable Diseases from the Ministry of Health, Labor, and Welfare, Japan (Chief: Yutaka Inaba, 2002–04 and Masaki Nagai, 2004–10). The authors thank Takasu Town, Hokkaido Prefecture, and its town people for their kind cooperation.

CONFLICT OF INTEREST

The authors declare no competing interests.

REFERENCES

1 Nakatani H (2000) The advance and features of intractable diseases control as the health policy of the Japanese Ministry of Health and Welfare (in Japanese). In: Ohno Y, Tanaka H, Nakatani H, Kurokawa K, Saito H (eds) *The Latest Information About Intractable Diseases, Including Epidemiology, Clinical Medicine and Care for Patients, pp 3–27. Nanzando, Tokyo.*

2 Cooper GS, Dooley MA, Treadwell EL, St Clair EW, Parks CG, Gilkeson GS (1998) Hormonal, environmental, and infectious risk factors for developing systemic lupus erythematosus. Arthritis Rheum **41**, 1714–24.

3 Wallace DJ (2002) The clinical presentation of systemic lupus erythematosus. In: Wallace DJ, Hahn BH (eds) *Dubois' Lupus Erythematosus, 6th edn, pp 621–8. Lippincott Williams and Wilkins, Philadelphia.*

4 Mak A, Tay SH (2014) Environmental factors, toxicants and systemic lupus erythematosus. Int J Mol Sci **15**, 16043–56.

5 Harley JB, Kelly JA (2008) Genetics of lupus. In: Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH (eds) *Rheumatology, 4th edn, pp 1237–42. Mosby Elsevier, Philadelphia.*

6 Bengtsson AA, Rylander L, Hagmar L, Nived O, Sturfelt G (2002) Risk factors for developing systemic lupus erythematosus: a case–control study in south Sweden. Rheumatology (Oxford) **41**, 563–71.

7 Hardy CJ, Palmer BP, Muir KR, Sutton AJ, Powell RJ (1998) Smoking history, alcohol consumption, and systemic lupus erythematosus: a case–control study. *Ann Rheum Dis* **57**, 451–5.

8 Ghaussy NO, Sibbitt WL Jr, Qualls CR (2001) Cigarette smoking, alcohol consumption, and the risk of systemic lupus erythematosus: a case–control study. *J Rheumatol* **28**, 2449–53.

9 Formica MK, Palmer JR, Rosenberg L, McAlindon TE (2003) Smoking, alcohol consumption, and risk of systemic lupus erythematosus in the Black women's health study. *J Rheumatol* **30**, 1222–6.

10 Nagata C, Fujita S, Hirotoshi I *et al.* (1995) Systemic lupus erythematosus: a case–control epidemiologic study in Japan. *Int J Dermatol* **34**, 333–7.

11 Nagai M, Hirohata T, Matsumoto Y *et al.* (1993) A case control study of female systemic lupus erythematosus in Japan. *Eur J Public Health* **3**, 172–6.

12 Minami Y, Sasaki T, Komatsu S *et al.* (1993) Female systemic lupus erythematosus in Miyagi prefecture, japan: a case control study of dietary and reproductive factors. *Tohoku J Exp Med* **169**, 245–52.

13 Washio M, Horiuchi T, Kiyohara C *et al.* (2006) Smoking, drinking, sleeping habits, and other lifestyle factors and the risk of systemic lupus erythematosus in Japanese females: findings from the KYSS study. *Mod Rheumatol* **16**, 143–50.

14 Kiyohara C, Washio M, Horiuchi T *et al.* (2012) Cigarette smoking, alcohol consumption, and risk of systemic lupus erythematosus: a case–control study in a Japanese population. *J Rheumatol* **39**, 1363–70.

15 Takahashi H, Washio M, Kiyohara C *et al.* (2014) Psychological stress in a Japanese population with systemic lupus erythematosus: findings from KYSS study. *Mod Rheumatol* **24**, 448–52.

16 Tan EM, Cohen AS, Fries JF *et al.* (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* **25**, 1271–7.

17 Herrick A (2013) Overlap/undifferentiated syndromes. In: Watts RA, Conaghan PG, Denton C, Foster H, Issacs J, Mueller-Lander U (eds) *Oxford Textbook of Rheumatology, 4th edn, pp 1069–78. Oxford University Press, Oxford.*

18 Oestoer AJK (2013) Anti-cytokine biologics. In: Watts RA, Conaghan PG, Denton C, Foster H, Issacs J, Mueller-Lander U (eds) Oxford Textbook of Rheumatology, 4th edn, pp 636–41. Oxford University Press, Oxford.

19 Hinojosa-Azaola A, Sanchez-Guerrero J (2013) Overview and clinical presentation. In: Wallace DJ, Hahn BH (eds) *Dubois' Lupus Erythematosus and Related Syndromes, 8th edn, pp 304–9. Elsevier Saunders, Philadelphia.*

20 Ozbek S, Sert M, Paydas S, Soy M (2003) Delay in the diagnosis of SLE: the importance of arthritis/arthralgia as the initial symptoms. Acta Med Okayama 57, 187–90.
21 Strom BL, Reidenberg MM, West S, Snyder ES, Freundlich B, Stolley PD (1994) Shingles, allergies, family medical history, oral contraceptives, and other potential risk factors for systemic lupus erythematosus. *Am J Epidemiol* **140**, 632–42.
22 Morton S, Palmer B, Muir K, Powell RJ (1988) IgE and non-IgE mediated allergic disorders in systemic lupus erythematosus. *Ann Rheum Dis* **57**, 660–3.
23 Rus V, Hochberg MC (2002) The epidemiology of systemic lupus erythematosus. In: Wallace DJ, Hahn BH (eds) *Dubois' Lupus Erythematosus, 6th edn, pp 65–83. Lippincott Williams and Wilkins, Philadelphia.*
24 Cooper GS, Dooley MA, Treadwell EL, St. Clair EW, Gilkeson GS (2002) Hormonal and reproductive risk factors for development of systemic lupus erythematosus: results of a population-based, case–control study. Arthritis Rheum **46**, 1830–9.
25 Costenbader KH, Fekanich D, Stampfer MJ, Karlson EW (2007) Reproductive and menopausal factors and risk of systemic lupus erythematosus in women. *Arthritis Rheum* **56**, 1251–62.
26 Ulff-Møller CJ, Jørgensen KT, Pedersen BV, Nielsen NM, Frisch M (2009) Reproductive factors and risk of systemic lupus erythematosus: nationwide cohort study in Denmark. *J Rheumatol* **36**, 1903–9.
27 Bernier MO, Mikaeloff Y, Hudson M, Suissa S (2009) Combined oral contraceptive use and the risk of systemic lupus erythematosus. *Arthritis Rheum* **61**, 476–81.
28 Fletcher RH, Fletcher SW, Wagner EH (1996) *Clinical Epidemiology, the Essential, 3rd edn. Lippincott Williams and Wilkins, Philadelphia.*

APPENDIX

Members of the Kyushu Sapporo SLE (KYSS) Study Group listed in alphabetical order for each affiliation.

Saburo Ide, Yuichiro Ide, Hiroko Kodama, Masakazu Washio [principal investigator] (St. Mary's College); Takahiko Horiuchi [co-principal investigator] (Kyushu University Beppu Hospital); Koichi Akashi, Mine Harada, Chikako Kiyohara [co-principal investigators], Hiroshi Tsukamoto (Graduate School of Medical Sciences, Kyushu University); Toyoko Asami, Takao Hotokebuchi, Kohei Nagasawa, Yoshifumi Tada, Osamu Ushiyama (Faculty of Medicine, Saga University); Mitsuru Mori, Asae Oura, Yasuhisa Sinomura, Hiromu Suzuki, Hiroki Takahashi, Motohisa Yamamoto, (Sapporo Medical University School of Medicine,), Gen Kobashi (Research Center for Charged Particle Therapy, National Institute of Radiological Science); Takashi Abe (Kushiro City General Hospital); Hisato Tanaka (Tanaka Hospital); Tatsuya Atsumi, Tetsuya Horita, Shinsuke Yasuda (Hokkaido University Graduate School of Medicine), Norihiko Nogami (Wakakusuryouikuen Hospital); Kazushi Okamoto (Aichi Prefectural College of Nursing and Health); Naomasa Sakamoto (Hyogo College of Medicine); Satoshi Sasaki (School of Public Health, the University of Tokyo); Yoshihiro Miyake (Faculty of Medicine, Fukuoka University), Tetsuji Yokoyama (National Institute of Public Health); Masayo Oumi (Nakamura Gakuen University); Yoshio Hirota(Osaka City University); Yutaka Inaba (Juntendo University School of Medicine); Masaki Nagai (Saitama Medical School).