EXTENDED REPORT

Low miR200b-5p levels in minor salivary glands: a novel molecular marker predicting lymphoma development in patients with Sjögren's syndrome

Efstathia K Kapsogeorgou,[1,2] Aristea Papageorgiou,[1,2] Athanase D Protogerou,[1,2] Michael Voulgarelis,[1,2] Athanasios G Tzioufas[1,2]

ABSTRACT

Objectives Development of non-Hodgkin's lymphoma (NHL) is the major adverse outcome of Sjögren's syndrome (SS) affecting both morbidity and mortality. Preliminary evidence suggested that, although not deregulated compared with sicca controls, miR200b-5p levels are decreased in the minor salivary glands (MSGs) of SS patients with NHL. The aim of the current study was to evaluate the MSG expression of miR200b-5p in SS-associated NHLs and its potential predictive value for the identification of patients with SS susceptible to develop NHL.

Methods miR200b-5p expression was investigated in MSG tissues of patients with SS who were at: (A) low risk and did not develop NHL during follow-up (n=27; median follow-up time on biopsy performance, range: 8.9 years, 1.33–14 years), (B) high-risk and diagnosed with NHL during follow-up (prelymphoma; n=17; median follow-up to until lymphoma diagnosis, range: 3.67 years, 0.42–8.5 years) and (C) had NHL (n=35), as well as non-SS sialadenitis controls (sarcoidosis and hepatitis C virus (HCV) infection, four each). The differential miR200b-5p expression, correlations with disease features and its discriminative/predictive value, was evaluated by appropriate statistical approaches.

Results The MSG levels of miR200b-5p were significantly downregulated in patients with SS who will develop or have NHL and strongly discriminated (p<0.0001) them from those without lymphoma or non-SS sialadenitis. Furthermore, they were reduced long before clinical onset of lymphoma, did not significantly change on transition to lymphoma and, importantly, were proved strong independent predictors of patients who will develop NHL (p<0.0001).

Conclusions These findings support that miR200b-5p levels in MSGs represent a novel predictive and possibly pathogenetic mechanism-related factor for the development of SS-associated NHL, since its expression is impaired years before lymphoma clinical onset.

score, salivary gland enlargement (SGE), purpura, vasculitis, leucopenia, cryoglobulinaemia, hypocomplementaemia, rheumatoid factor, formation of germinal centres (GCs) in the histopathological lesion and infiltration by certain cell types, such as macrophages, have been associated with the development of lymphoma in SS.[3 4 6–11]

The miR200 micro-RNA (miRNA) family, consisting of miR200a, miR200b, miR429, miR141 and miR200c miRNAs, possesses a central role in oncogenesis, tumour metastasis and drug resistance. The miR200b-3p and miR200b-5p miRNAs are considered powerful regulators of epithelial-to-mesenchymal transition (EMT) and as such they have been implicated in the oncogenesis of solid tumours.[12–17] Recently, in the context of investigating the expression of several miRNAs that are predicted to target the Ro/SSA and La/SSB autoantigens, we examined the expression of miR200b-3p and miR200b-5p in the minor salivary glands (MSGs) of SS patients and sicca controls.[18] Although their expression in the MSGs of patients with SS was not deregulated compared with sicca controls, miR200b-5p levels were significantly reduced in four SS patients with mucosa-associated lymphoid tissue (MALT) lymphomas compared with those without.[18]

Prompted by this finding, we sought to: (A) validate the hypothesis that miR200b-5p levels are decreased in the MSGs of patients with SS-associated NHL by studying its expression in an adequate population of SS patients with or without NHL, as well as in sialadenitis controls; (B) evaluate its predictive value by investigating its expression in MSG samples from both low-risk patients with SS who did not develop lymphoma during follow-up and high-risk patients who developed SS-associated NHL in the future; (C) test its independent predictive utility over that of previously identified adverse predictive factors for the development of SS-associated NHL.

INTRODUCTION

Primary Sjögren's syndrome (SS) is an autoimmune disease with a diverse clinical picture ranging from benign, mild exocrinopathy to severe, systemic, disorder with high prevalence (5%–10%) of B cell non-Hodgkin's lymphoma (NHL).[1 2] NHL is the major adverse outcome of the disease, affecting both morbidity and mortality.[3–5] Several clinical, laboratory and histological features, including high EULAR SS disease activity index (ESSDAI)

MATERIALS AND METHODS
Patients

MSG samples obtained from 79 patients with primary SS and 8 non-SS sialadenitis associated with sarcoidosis and HCV infection (four each) were studied after informed consent. Patients with SS were diagnosed according to the American–European classification criteria.[19] The patients with SS included 27 low risk who did not develop

lymphoma during follow-up (without lymphoma (SSwo); median follow-up time on biopsy performance, range: 8.9 years, 1.33–14 years), 17 high risk who were diagnosed with SS-associated NHL during follow-up (prelymphoma (SSpL); median follow-up time to NHL diagnosis, range: 3.67 years, 0.42–8.5 years) and 35 with SS-associated lymphoma at the time of biopsy (lymphoma (SSL)). The low-risk group consisted of SS patients with low probability to develop lymphoma,[11 20] including 17 without risk factors for lymphoma development, 5 with low serum C4 levels, four with SGE and one with both low serum C4 levels and SGE (median follow-up time of the patients expressing adverse predictive factors: 8.47 years, range: 4.91–12.91 years). The prelymphoma SS group included 14 MALTs, 2 nodal marginal zone lymphomas (NMZLs) and 1 diffuse large B cell lymphoma (DLCBL). The SS-associated NHLs consisted of 28 MALTs, 2 NMZLs, 2 DLCBLs, 1 B cell bronchial associated lymphoid tissue (BALT), 1 lymphoplasmacytic (LP) and 1 small lymphocytic (SLL) lymphoma. In 14 cases (11 MALTs, 2 NMZLs and 1 DLCBL), the SSpL and SSL samples were paired sequential specimens from the same patient (before and on lymphoma onset). All prelymphoma and lymphoma SS samples that had available MSG specimens from a total of 84 SS patients with NHL who were followed up during 1993–2016 in the Department of Pathophysiology, School of Medicine, National and Kapodistrian University of Athens, Greece, were studied.

The medical records were retrospectively evaluated for various clinical, laboratory and histological parameters of SS and lymphoma that are described in table 1. The characteristics of the patients are summarised in table 2, whereas lymphoma features in table 3 and online supplementary table S1. Three SS patients had received corticosteroids, four hydroxychloroquine, one cyclophosphamide and five B cell depletion therapy (anti-CD20) prior to biopsy performance.

Evaluation of miR200b-5p levels by quantitative PCR (qPCR)

MSG tissues were obtained during diagnostic biopsy, fresh-frozen in RNAlater stabilisation reagent (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions and stored at −80°C until RNA isolation. Total RNA including small RNA molecules, such as miRNAs, was isolated from an MSG lobe using the mirVana PARIS kit (Ambion, Applied Biosystems, Carlsbad, California, USA) according to the manufacturer's instructions. The time of storage has not been found to significantly affect the quantity or quality of the isolated RNA (data not shown). Subsequently, 0.25 μg RNA were reverse-transcribed by TaqMan miRNA reverse transcription kit and miRNA-specific primers (TaqMan-MicroRNA Assays, Applied Biosystems), followed by miRNA-specific amplification by qPCR using primers from TaqMan-MicroRNA Assays and TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems). RNU48 small nucleolar RNA (snoRNA) was selected for normalisation after NormFinder analysis.[21] Three snoRNAs, namely U6, RNU44 and RNU48, have been examined in preliminary experiments, including samples from sicca controls, low-risk SS patients without lymphoma and patients with SS-NHL (six in each group) for identifying the most appropriate reference. In these experiments, a common reverse transcription using Megaplex RT Primers, Human Pool B V.3.0 (Applied Biosystems) was performed in order to exclude variations due to distinct RT reactions. NormFinder analysis[21] revealed that RNU48 had the higher stability value (0.006) compared with RNU44 (stability value: 0.008) or U6 miRNA (stability value: 0.016). The relative quantification of miRNA expression was performed by the $2^{-\Delta\Delta CT}$

Table 1 Clinical, laboratory and histological features of the patients with SS that were retrospectively recorded and their definition

Features	Defined as/documented by
SS associated	
Clinical	
EULAR SS disease activity index (ESSDAI)	31
Arthralgias, arthritis	
Raynaud's phenomenon	
Salivary gland enlargement (SGE)	
Lung involvement	Pulmonary function tests and X-ray and/or CT scans
Renal involvement	Persistent proteinuria and verification by renal biopsy
Liver involvement	Liver biopsy indicative of primary biliary cirrhosis
Palpable purpura	
Vasculitis	
Peripheral neuropathy	Nerve conduction studies
Laboratory	
Anti-Ro/SSA and/or anti-La/SSB autoantibodies	
Rheumatoid factor	
Complement C3 and C4 levels	
Hypocomplementaemia	C4 <16 mg/dL and C3 <75 mg/dL
Cryoglobulinaemia	
Hypergammaglobulinaemia	Total gammaglobulins >2 g/L
Anaemia	Haemoglobin <12 g/dL (females) and 13.5 g/dL (males)
Leucopenia	White cell count <4000/mm³
Lymphopaenia	Lymphocyte count <1000/mm³
Neutropaenia	Neutrophil count <1500/mm³
Histological	
Biopsy focus score	Number of lymphocytic foci/4 mm²
Germinal centre formation	
Lymphoma associated	
Non-Hodgkin's lymphoma (NHL) subtype	
Eastern Cooperative Oncology Group performance status	32
Ann Arbor stage (I–IV)	33
Number and type of involved sites	
International Prognostic Index	0–1 points: low risk, 2 points: low-intermediate risk, 3 points: high-intermediate risk, 4–5 points: high risk[34]
Splenomegaly	
Lymphadenopathy	
Presence of B symptoms	
Serum lactate dehydrogenases	
β2-microglobulin levels	

SS, Sjögren's syndrome.

method using non-malignant parotid gland tissue from a patient subjected to parotidectomy as calibrator. The relative occurrence of various cell types, including epithelial cells, B cells, T cells and macrophages, in MSG samples was evaluated by qPCR for keratin-8 (KRT8), CD19, CD3D and CD68, respectively, using TaqMan expression assays. Samples were processed randomly and without grouping. All samples were run in duplicates. Additionally, in a proportion of patients (six from each SS subgroup and four sialadenitis controls; randomly selected) the infiltration by CD20⁺ B cells, CD3⁺ T cells and CD68⁺ macrophages

Table 2 Characteristics of the patients studied

Features	Non-SS	Patients with SS		
	Sialadenitis (n=8)	SSwo (n=27)	SSpL (n=17)	SSL (n=35)
General				
Age (years), median (range)	61.5 (53–74)	55 (30–76)	35 (24–75)	43.5 (26–79)
Men/women	2/6	0/27	1/16	4/31
Duration (years) of sicca symptoms, median (range)	0.65 (0.1–5.0)	3 (0.5–10.0)	5.7 (1.0–13.0)	8.0 (0.5–36.0)
Histological (MSG biopsy)				
Biopsy focus score (number of lymphocytic foci/4mm^2), median (range)	0.42 (0.0–4.4)	1.45 (1.0–4.0)	4.44 (1.0–11.5)	5.14 (1.0–9.23)
Germinal center formation, n (%)	0 (0)	3 (11.1)	8 (47.1)	16 (45.7)
Clinical				
Arthralgias, n (%)	0 (0)	8 (29.6)	5 (29.41)	12 (34.3)
Arthritis, n (%)	0 (0)	3 (11.1)	1 (5.9)	3 (8.6)
SG enlargement, n (%)	0 (0)	5 (18.5)	9 (52.9)	26 (74.3)
Raynaud's phenomenon, n (%)	0 (0)	6 (22.2)	5 (29.4)	6 (17.1)
Parenchymal organ involvement, n (%)	0 (0)	1 (3.7)	1 (5.9)	4 (11.4)
Lung involvement, n (%)	0 (0)	1 (3.7)	1 (5.9)	4 (11.4)
Renal involvement, n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Liver involvement, n (%)	0 (0)	0 (0)	0 (0)	0 (0
Indicative of vasculitic involvement, n (%)	0 (0)	0 (0)	7 (41.2)	18 (51.4)
Palpable purpura, n (%)	0 (0)	0 (0)	7 (41.2)	18 (51.4)
Vasculitis (%), n (%)	0 (0)	0 (0)	3 (17.6)	4 (11.4)
Glomerulonephritis, n (%)	0 (0)	0 (0)	1 (5.9)	4 (11.4)
Peripheral neuropathy, n (%)	0 (0)	0 (0)	2 (11.8)	6 (17.1)
ESSDAI score, median (range)	NA	1 (0–4)	9 (1–14)	10 (4–19)
Laboratory				
Anti-Ro/SSA and/or La/SSB positive, n (%)	0 (0)	17 (62.96)	11 (64.7)	28 (80)
Anti-Ro/SSA positive, n (%)	0 (0)	17 (63.0)	11 (64.7)	28 (80)
Anti-La (SSB) positive, n (%)	0 (0)	3 (11.1)	8 (47.1)	20 (57.1)
Rheumatoid factor positive, n (%)	1 (12.5)	9 (33.3)	12 (70.6)	28 (80)
C3 levels, median (range)	84 (79–118)	105 (74–139)	87.5 (65–191)	98.2 (36–191)
C4 levels, median (range)	24.5 (13–36.0)	22.0 (4.0–47.3)	10.4 (1.0–28.0)	10.4 (1.0–30.0)
C4 hypocomplementaemia, n (%)	1 (12.5)	7 (25.9)	10 (58.8)	24 (68.6)
Cryoglobulinaemia, n (%)	0 (0)	0 (0)	4 (23.5)	11 (31.4)
Hypergammaglobulinaemia, n (%)	0 (0)	2 (7.4)	10 (58.8)	22 (62.9)
Leucopenia, n (%)	0 (0)	2 (7.4)	1 (5.9)	4 (11.4)
Treatment, n (%)				
Corticosteroids	0 (0)	0 (0)	2 (11.8)	3 (8.5)
Hydroxychloroquine	0 (0)	2 (7.4)	2 (11.8)	2 (5.7)
Cyclophosphamide	0 (0)	0 (0)	1 (5.9)	1 (2.9)
Rituximab	0 (0)	0 (0)	1 (5.9)	5 (14.3)

ESSDAI, EULAR SS disease activity index; MSG, minor salivary gland; NA, not applicable; SG, salivary gland; SS, Sjögren's syndrome; SSL, SS-associated lymphoma; SSpL, pre-lymphoma; SSwo, without lymphoma.

(number of infiltrating cells per mm^2 tissue area), as well as its correlation with miR200b-5p levels were analysed immunohistochemically using ImageJ software, as previously.[7 22]

Statistical analyses

The differential expression of miR200b-5p levels among the subgroups of patients with SS and sialadenitis controls was evaluated by the non-parametric Tukey's multiple comparison test. Significant differences in miR200b-5p levels between patients expressing or not various clinical, histological and serological markers were analysed by the non-parametric Mann-Whitney U test, whereas associations with patient features and overtime changes by Spearman's rank correlation and Wilcoxon's matched pairs tests, respectively. Holm-Bonferroni sequential correction was applied for correcting for multiple comparisons.[23]

Analyses regarding the diagnostic/discriminative utility of miR200b-5p were performed in two levels: (A) all patients with SS who did not have lymphoma at the time of biopsy (SSwo and SSpL) were compared with lymphoma patients (SSL) and (B) comparisons among the three SS subgroups. The 14 patients that were common in the prelymphoma and lymphoma SS group were excluded from lymphoma group, thus remaining 21 SSL patients in the analyses. The diagnostic/discriminative ability of miR200b-5p levels was evaluated by receiver operating characteristic (ROC) curve analysis. Categorical variables were compared by the Pearson χ^2 or the Fisher's exact

Table 3 Features of the patients with SS-associated NHLs (SSL)

Features	Patients with SSL (n=35)
Type	
MALT, n (%)	28 (80)
NMZL, n (%)	2 (5.7)
LP, n (%)	1 (2.8)
DLCBL, n (%)	2 (5.7)
SLL, n (%)	1 (2.8)
BALT, n (%)	1 (2.8)
Involved organs	
Nodal, n (%)	8 (22.9)
Extranodal	
MSG, n (%)	26 (74.3)
Parotid gland (PG), n (%)	7 (20.0)
Both MSG and PG, n (%)	4 (11.4)
Stomach, n (%)	3 (8.5)
Lung, n (%)	2 (5.7)
Nodal and extranodal, n (%)	4 (11.4)
Bone marrow infiltration, n (%)	10 (28.6)
Splenomegaly, n (%)	4 (11.4)
Ann Arbor staging	
I, n (%)	17 (48.6)
II, n (%)	0 (0.0)
III, n (%)	3 (8.5)
IV, n (%)	15 (42.9)
IPI score	
0, n (%)	7 (20.0)
1, n (%)	5 (14.3)
2, n (%)	15 (42.9)
3, n (%)	7 (20.0)
4, n (%)	1 (2.8)
ECOG	
0, n (%)	30 (85.7)
1, n (%)	5 (14.3)
EFS (months), median (range)	61.7 (14–206)
OS (months), median (range)	68.4 (14–206)

BALT, B cell bronchial associated lymphoid tissue; DLCBL, diffuse large B cell lymphoma; ECOG, Eastern Cooperative Oncology Group performance status; EFS, event-free survival; IPI, International Prognostic Index; LP, lymphoplasmacytic; MALT, mucosa-associated lymphoid tissuel; MSG, minor salivary gland; NHL, non-Hodgkin's lymphoma; NMZL, nodal marginal zone lymphoma; OS, overall survival; SLL, small lymphocytic lymphoma; SS, Sjögren's syndrome.

test, when appropriate. HRs are provided with a 95% CI. To identify independent risk factors for NHL development in SS, all variables associated with lymphoma with a p value less than 0.1 in univariate analysis were further evaluated by multivariate logistic or Cox regression analysis using a backward stepwise exclusion method. The predictive ability of miR200b-5p levels was evaluated by Kaplan-Meier lymphoma-free survival curves compared by the log-rank test in the prelymphoma and without lymphoma SS patients, who were split in two groups according to low or not miR200b-5p expression, as this defined by ROC discriminative value.Similar analysis was performed for low-risk and high-risk patients according to previously described models.[4 9 11] Descriptive analyses of all data were performed.

GraphPad Prism-5 (GraphPad Software, San Diego, California, USA) and SPSS V.17 software were used. Statistical significance was defined as a p value of less than 0.05 for all comparisons; p values were two tailed. Only the statistically significant differences are reported. Using two-sided 95% CI,

the observed difference of the means was proved to have 96.5% power (OpenEpi, V.3, open source calculator).

RESULTS

The MSG levels of miR200b-5p are reduced in patients with SS who have or will develop NHL and discriminate them from those who will not

The miR200b-5p levels were significantly lower in the MSGs of high-risk patients with SS who were diagnosed with NHL during follow-up (SSpL; mean relative expression±SD: 0.31±0.33) and lymphoma SS patients (SSL; 0.21±0.25) compared with the low risk that did not develop lymphoma during follow-up (SSwo; 0.72±0.37; p≤0.01and p≤0.0001 for prelymphoma and lymphoma, respectively) or non-SS sialadenitis controls (0.95±0.84, p≤0.01and p≤0.001, respectively) (figure 1A). Low miR200b-5p levels were also detected in patients with SSL who had not lymphoma in MSGs. Interestingly, low miR200b-5p levels (0.17) were also detected in an HBV-patient that had MALT lymphoma (patient excluded from the analysis). Additionally, miR200b-5p levels were not found to significantly change on transition to lymphoma, as indicated by the analysis of 14 sequential paired samples from SS patients before and on lymphoma diagnosis (figure 1B). The significantly lower expression of miR200b-5p in the MSGs of SS patients with NHL long before lymphoma diagnosis indicates that it is possibly implicated in the pathogenesis of SS-associated lymphoma. miR200b-5p levels correlate with clinical, laboratory and histologic factors of adverse outcome and/or NHL development, as well as NHL prognosis.

The low levels of miR200b-5p were associated with several clinical, laboratory and histological features indicative of adverse outcome and lymphoma development that are summarised in table 4. Hence, miR200b-5p levels were negatively correlated with ESSDAI, whereas they were positively correlated with serum C4 levels (table 4). Significantly lower miR200b-5p levels were detected in SS patients with SGE, purpura, peripheral neuropathy, cryoglobulinaemia, hypergammaglobulinaemia and rheumatoid factor compared with those without (table 4). Despite the lower age of SSpL and SSL patients compared with SSwo (table 2), miR200b-5p levels were not correlated with patient age (p=0.1), whereas they were negatively correlated with biopsy focus score (table 4); however, this did not affect their higher expression in SSwo, compared with SSpL and SSL patients (supplementary table S3 and supplementary figure S2). Furthermore, it was negatively correlated with CD3D and CD68 mRNA expression in MSGs (r=−0.5613, p<0.0001and r=−0.5048, p<0.0001, respectively), which are indicative of T-lymphocyte and macrophage infiltration, respectively, but not CD19 (B cell; r=−0.3072, p=0.068) or KRT8 (epithelial cell; r=0.1536, p=0.23) expression. Subsequent immunohistochemical evaluation of the number of infiltrating CD3$^+$ T cells, CD20$^+$ B cells and CD68$^+$ macrophages per tissue area (mm^2) in randomly selected samples from each group of patients with SS (six patients/group), as well as sialadenitis controls (n=4) revealed that the levels of miR200b-5p in MSGs were negatively correlated with all these types of infiltrating cell populations (table 4).

The levels of miR200b-5p in MSGs were not found to associate with the type, stage or number of involved sites of NHL, as well as event-free or overall-free survival, whereas there was a trend to associate with high-intermediate/high international prognostic index (IPI: 3–4) (mean±SD: 0.24±0.28 vs 0.10±0.06 in patients with low/low-intermediate IPI (0–2),
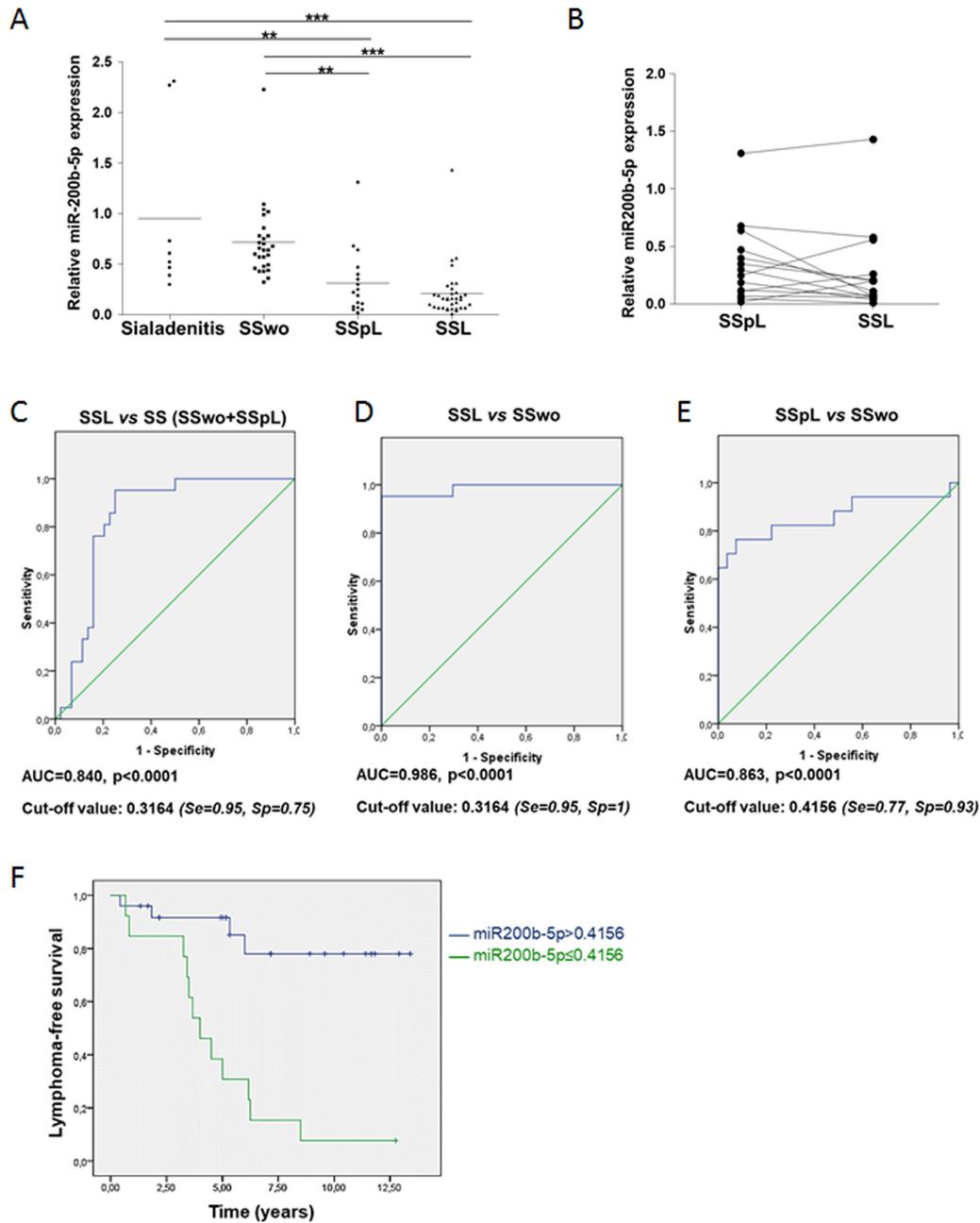
Figure 1 MSG miR-200b-5p levels are downregulated in prelymphoma and lymphoma SS patients, discriminate them from SS patients without lymphoma and predict lymphoma development. (A) Dot plot displaying the expression of miR-200b-5p in the MSG tissues of non-SS sialadenitis controls (sialadenitis), patients with SS who did not develop NHL during follow-up (SSwo), patients with SS who were diagnosed with NHL in the future during follow-up (pre lymphoma; SSpL) and SS patients with NHL (SSL). Comparisons were performed by Tukey's multiple comparison analysis. P values are designated by asterisks (**p<0.01, ***p<0.001), whereas horizontal bars represent the mean value of the group. Only statistically significant associations are indicated. (B) Wilcoxon's matched-pairs analyses of miR-200b-5p expression in sequential MSG-samples from 14 patients with SSpL that transitioned to NHL (SSL) did not reveal any significant changes in miR200b-5p expression before and on lymphoma transition. (C–E) ROC curve analyses of the ability of miR200b-5p to discriminate/diagnose patients with lymphoma SS (SSL) from those without (SSwo and SSpL) at the time of biopsy (C), that were low risk and did not develop lymphoma during follow-up (SSwo) (D), as well as patients with SSpL from those who did not develop lymphoma during follow-up (SSwo) (E). (F) Kaplan-Meier lymphoma-free curves for patients with low miR-200b-5p levels (≤0.4156) (high-risk group; green line) and patients with miR-200b-5p levels (>0.4156) (low-risk group; blue line). AUC, area under the curve; MSG, minor salivary gland; NHL, non-Hodgkin's lymphoma; ROC, receiver operating characteristic; SE, sensitivity; Sp, specificity; SS, Sjögren's syndrome.

p=0.07). miR200b-5p levels in MSGs discriminate patients with SS who have versus those who do not have NHL, as well as those who will develop NHL versus those who will not.

ROC curve analysis revealed that miR200b-5p strongly discriminates prelymphoma and lymphoma SS patients from those without lymphoma. Thus, the lymphoma SS patients were

discriminated from SS patients without lymphoma at the time of biopsy (SSwo and SSpL) with AUC and cut-off values 0.840 (p<0.0001) and 0.3164 (sensitivity=0.952, specificity=0.750), respectively. More importantly, prelymphoma and lymphoma SS patients were discriminated from those that did not develop lymphoma during follow-up (SSwo) with AUC values 0.863 and

Table 4 Correlations between miR200b-5p levels and disease parameters associated with severe systemic disease, SS-associated NHL development or lymphoma prognosis, as analysed by Mann-Whitney and Spearman's rank correlation tests corrected for multiple comparisons by Holm-Bonferroni sequential correction

| Features | Correlation (r) | P values | miR200b-5p levels (mean±SD) | | P values |
			Without	With	
Histological *(MSG biopsy)*					
Biopsy focus score (number of lymphocytic foci/4 mm^2)	−0.6012	<0.0001			
CD3$^+$-T cells (number/mm^2 of tissue)	−0.7535	0.002			
CD20$^+$-B cells (number/mm^2 of tissue)	−0.7182	0.008			
CD68$^+$-macrophages (number/mm^2 of tissue)	−0.7808	0.002			
Clinical					
SG enlargement			0.54±0.34	0.30±0.40	0.003
Palpable purpura			0.49±0.41	0.27±0.32	0.033
Peripheral neuropathy			0.45±0.39	0.11±0.09	0.023
NHL non-Hodgkin's lymphoma			0.72±0.37	0.21±0.25	≤0.0001
Laboratory					
Serum C4 levels (mg/dL)	0.4562	<0.0001			
Cryoglobulinaemia			0.48±0.41	0.15±0.11	0.006
Hypergammaglobulinaemia (total gammaglobulins >2g/L)			0.54±0.41	0.29±0.34	0.033
Rheumatoid factor			0.53±0.31	0.35±0.39	0.033
			Low	High	
Indices	−0.5736	<0.0001			
ESSDAI score (value)					
ESSDAI score (low (≤5) versus high (≥6))			0.62±0.39	0.25±0.30	<0.0001

ESSDAI, EULAR SS disease activity index; NHL, non-Hodgkin's lymphoma; SS, Sjögren's syndrome.

0.986 (p<0.0001), respectively, and cut-off values 0.4156 (sensitivity=0.765, specificity=0.926) and 0.3164 (sensitivity=0.952, specificity=1), respectively (figure 1C–E).

Kaplan-Meier analysis of patients split into two groups according to miR200b-5p expression levels of 0.4156 (as defined by the specificity–sensitivity analysis) revealed that patients with miR200b-5p levels ≤0.4156 had a 4.8-fold (HR: 4.81, 95% CI 3.15 to 6.47, p<0.0001) higher risk to develop lymphoma compared with patients with miR200b-5p levels >0.4156 (figure 1F).

miR200b-5p levels in MSGs predict SS who will develop NHL versus those who will not, independently from other known predicting factors

The known outcome of the NHL development in the patients with SS who did not have lymphoma at the time of MSG biopsy, involving the low-risk ones who did not develop NHL during follow-up and those who evolved to an SS-associated NHL, enabled the evaluation of the utility of miR200b-5p levels in the prediction of lymphoma development. Cox regression analysis was employed to evaluate the value of miR200b-5p in the prediction of lymphoma development along with other disease parameters that were associated with lymphoma development in univariate analysis. These included high ESSDAI (defined as score ≥5, p<0.0001), SGE (p=0.002), purpura (p=0.0001), vasculitis (p=0.024), anaemia (p=0.058), splenomegaly (p=0.024), lymphadenopathy (p=0.005), C4-hypocomplementaemia (p=0.005), RF (p=0.001), GCs (p=0.007), cryoglobulinaemia (p=0.001) and hypergammaglobulinaemia (p<0.0001).

Multivariate analysis confirmed that miR200b-5p was independently associated with development of lymphoma (HR per 1-unit change: 0.10, 95% CI 0.01 to 0.87, p=0.012) along with high ESSDAI (p=0.024), SGE (p=0.012), purpura (p=0.057), vasculitis (p=0.043), splenomegaly (p=0.047), cryoglobulinaemia (p=0.032) and hypergammaglobulinaemia (p=0.055).

Possible role of miR200b-5p levels in monitoring of therapeutic response

Preliminary observations from nine patients with SSL before and after treatment suggest that MSG levels of miR200b-5p may apply in the prediction of therapeutic response. Thus, miR200b-5p levels remained stable or decreased (0.22 to 0.07) in refractory to treatment MALT (n=5) and DLCBL (n=1) lymphomas, respectively, reduced in two MALT-SSLs who relapsed (0.54 and 0.31 to 0.2 and 0.09, respectively) and increased in a MALT-SSL who reached complete remission (0.07 to 0.47).

DISCUSSION

This study supports that the MSG expression levels of miR200b-5p constitute a novel, strong, predictive biomarker for NHL development in SS, since its expression is impaired years before lymphoma clinical onset. Indeed, reduced MSG expression of miR200b-5p characterised patients with SS who have or will develop NHL. Low levels of miR200b-5p were correlated with disease parameters, previously associated with adverse outcome and NHL development. Importantly, miR200b-5p levels strongly discriminated the three groups of patients with SS, namely low-risk, prelymphoma and lymphoma, with high sensitivity and specificity and independently predicted lymphoma development. Furthermore, miR-200b-5p levels in MSGs were downregulated long before the clinical onset of lymphoma, supporting its potential as a predictive biomarker. In addition, our preliminary observations imply that miR200b-5p may also be significant for the therapeutic monitoring of patients with NHL, signifying the need for appropriate prospective studies.

The mechanisms underlying the reduced miR200b-5p expression in MSGs and its role in SS-related lymphomagenesis have not been delineated. The reduced levels of miR200b-5p long before lymphoma development suggest that it is implicated in SS lymphomagenesis, although the pathways that regulate remain unknown and are currently under investigation. Despite

the well-established role of the reduced miR200b-3p expression in EMT and associated oncogenesis, tumour metastasis and invasion,[12–17] little are known for miR200b-5p, possibly because it represents the star strand, which is generally considered to degrade during miRNA biogenesis.[24] Recently, it has been reported that miR200b-5p controls the non-canonical EMT in synergy with miR200b-3p by targeting PRKCA and PIP4K2A molecules in the RHOGDI pathway.[25] Interestingly, miR200b miRNAs have been almost exclusively associated with solid tumours. A recent study suggests that the elevated miR200b expression and subsequent inhibition of zinc finger E-box-binding homeobox 1 transcription factor and increased BCL6 protein expression is associated with the better prognosis of the *Helicobacter pylori*-positive gastric diffuse large B cell lymphomas compared with *H. pylori*-negative ones.[26] To this end, it would be interesting to investigate whether miR200b-5p levels are downregulated in other lymphomas that are not associated with SS.

Despite the clinical progress, the mechanisms underlying SS-related lymphomagenesis have not been delineated. It is considered as a multistep antigen-driven process taking place in the inflammatory MSG lesions that arises from the chronic continuous and/or inappropriate B cell stimulation, which increases the risk of chromosomal translocations, activation of proto-oncogenes and inactivation of tumour-suppression genes resulting in malignant transformation.[27 28] Most likely, the reduced miR200b-5p expression in the MSGs of patients with SS that will develop or have lymphoma does not involve B cells or other types of infiltrating lymphocytes, since we have been previously unable to detect its expression in the peripheral blood B cells.[18 29] Interestingly, miR200b-5p levels were negatively correlated with biopsy focus score and infiltration by T cells, B cells and macrophages, suggesting that the reduction of miR200b-5p in prelymphoma and lymphoma SS patients may result from 'dilution effect' due to increased infiltration by inflammatory cells. However, this reduced expression could be coincidental and reflective of reduced expression in epithelial cells, which are a possible source of miR200b-5p, in severe lesions. This is supported by the higher expression of miR200b-5p in low-risk patients compared with those with prelymphoma and lymphoma and similar MSG infiltration and the lack of correlation with KRT8 expression, which is possibly reflective of downregulated epithelial expression of miR200-5p in MSGs of patients with SS who will develop or have lymphoma and not alteration in epithelial cell number. In contrast to peripheral blood mononuclear cells, cultured salivary gland epithelial cells express miR200b-5p.[18 29] Epithelial cells are key regulators of SS autoimmune responses, whereas preliminary data suggest that they can drive B cell activation and differentiation.[30] Thus, it would be tempting to hypothesise that the reduction of miR200b-5p in MSG epithelia promotes the efficient interaction with B cells, which eventually leads in lymphomagenesis. In this context, the detection of low miR200b-5p levels in NHLs without MSG involvement is not paradoxical. The cellular types expressing miR200b-5p in the MSGs of patients with SS, the effect of its downregulation in their phenotype and the mechanisms underlying its reduction is of high importance for the discovery of novel therapeutic targets and is currently investigated in our lab.

In summary, the expression levels of miR200b-5p in MSG tissues constitute a novel, strong, disease mechanism-related, predictive biomarker for NHL development in SS. Their predictive value will be further validated during the HarmonicSS project (European Union Grant-731944) that includes 21 European patient SS cohorts.

REFERENCES

1. Kassan SS, Thomas TL, Moutsopoulos HM, *et al*. Increased risk of lymphoma in sicca syndrome. *Ann Intern Med* 1978;89:888–92.
2. Voulgarelis M, Dafni UG, Isenberg DA, *et al*. Malignant lymphoma in primary Sjögren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjögren's Syndrome. *Arthritis Rheum* 1999;42:1765–72.
3. Skopouli FN, Dafni U, Ioannidis JP, *et al*. Clinical evolution, and morbidity and mortality of primary Sjögren's syndrome. *Semin Arthritis Rheum* 2000;29:296–304.
4. Ioannidis JP, Vassiliou VA, Moutsopoulos HM. Long-term risk of mortality and lymphoproliferative disease and predictive classification of primary Sjögren's syndrome. *Arthritis Rheum* 2002;46:741–7.
5. Theander E, Manthorpe R, Jacobsson LT. Mortality and causes of death in primary Sjögren's syndrome: a prospective cohort study. *Arthritis Rheum* 2004;50:1262–9.
6. Brito-Zerón P, Ramos-Casals M, Bove A, *et al*. Predicting adverse outcomes in primary Sjogren's syndrome: identification of prognostic factors. *Rheumatology* 2007;46:1359–62.
7. Christodoulou MI, Kapsogeorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren's syndrome. *J Autoimmun* 2010;34:400–7.
8. Theander E, Vasaitis L, Baecklund E, *et al*. Lymphoid organisation in labial salivary gland biopsies is a possible predictor for the development of malignant lymphoma in primary Sjögren's syndrome. *Ann Rheum Dis* 2011;70:1363–8.
9. Nocturne G, Virone A, Ng WF, Wf N, *et al*. Rheumatoid Factor and Disease Activity Are Independent Predictors of Lymphoma in Primary Sjögren's Syndrome. *Arthritis Rheumatol* 2016;68:977–85.
10. Papageorgiou A, Ziogas DC, Mavragani CP, *et al*. Predicting the outcome of Sjogren's syndrome-associated non-hodgkin's lymphoma patients. *PLoS One* 2015;10:e0116189.
11. Baimpa E, Dahabreh IJ, Voulgarelis M, *et al*. Hematologic manifestations and predictors of lymphoma development in primary Sjögren syndrome: clinical and pathophysiologic aspects. *Medicine* 2009;88:284–93.
12. Humphries B, Yang C. The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. *Oncotarget* 2015;6:6472–98.
13. Senfter D, Madlener S, Krupitza G, *et al*. The microRNA-200 family: still much to discover. *Biomol Concepts* 2016;7(5-6):311–9.
14. Ragusa M, Majorana A, Banelli B, *et al*. MIR152, MIR200B, and MIR338, human positional and functional neuroblastoma candidates, are involved in neuroblast differentiation and apoptosis. *J Mol Med* 2010;88:1041–53.
15. Lee TS, Jeon HW, Kim YB, *et al*. Aberrant microRNA expression in endometrial carcinoma using formalin-fixed paraffin-embedded (FFPE) tissues. *PLoS One* 2013;8:e81421.
16. Chen MF, Zeng F, Qi L, *et al*. Transforming growth factor-β1 induces epithelial-mesenchymal transition and increased expression of matrix

metalloproteinase-16 via miR-200b downregulation in bladder cancer cells. *Mol Med Rep* 2014;10:1549–54.

17 Cheng YX, Chen GT, Chen C, *et al*. MicroRNA-200b inhibits epithelial-mesenchymal transition and migration of cervical cancer cells by directly targeting RhoE. *Mol Med Rep* 2016;13:3139–46.

18 Gourzi VC, Kapsogeorgou EK, Kyriakidis NC, *et al*. Study of microRNAs (miRNAs) that are predicted to target the autoantigens Ro/SSA and La/SSB in primary Sjögren's Syndrome. *Clin Exp Immunol* 2015;182:14–22.

19 Vitali C, Bombardieri S, Jonsson R, *et al*. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002;61:554–8.

20 Fragkioudaki S, Mavragani CP, Moutsopoulos HM. Predicting the risk for lymphoma development in Sjogren syndrome: An easy tool for clinical use. *Medicine* 2016;95:e3766.

21 Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004;64:5245–50.

22 Kapsogeorgou EK, Christodoulou MI, Panagiotakos DB, *et al*. Minor salivary gland inflammatory lesions in Sjögren syndrome: do they evolve? *J Rheumatol* 2013;40:1566–71.

23 Holm S. A Simple Sequentially Rejective Multiple Test Procedure. *Scand J Stat* 1979;6:65–70.

24 Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215–33.

25 Rhodes LV, Martin EC, Segar HC, *et al*. Dual regulation by microRNA-200b-3p and microRNA-200b-5p in the inhibition of epithelial-to-mesenchymal transition in triple-negative breast cancer. *Oncotarget* 2015;6:16638–52.

26 Huang WT, Kuo SH, Cheng AL, *et al*. Inhibition of ZEB1 by miR-200 characterizes Helicobacter pylori-positive gastric diffuse large B-cell lymphoma with a less aggressive behavior. *Mod Pathol* 2014;27:1116–25.

27 Papageorgiou A, Voulgarelis M, Tzioufas AG. Clinical picture, outcome and predictive factors of lymphoma in Sjögren syndrome. *Autoimmun Rev* 2015;14:641–9.

28 Bombardieri M, Pitzalis C. Ectopic lymphoid neogenesis and lymphoid chemokines in Sjogren's syndrome: at the interplay between chronic inflammation, autoimmunity and lymphomagenesis. *Curr Pharm Biotechnol* 2012;13:1989–96.

29 Kapsogeorgou EK, Gourzi VC, Manoussakis MN, *et al*. Cellular microRNAs (miRNAs) and Sjögren's syndrome: candidate regulators of autoimmune response and autoantigen expression. *J Autoimmun* 2011;37:129–35.

30 Kapsogeorgou EK, Tzioufas AG. Glandular epithelium: Innocent bystander or leading actor. In: Roberto Gerli EB, Alunno A, eds. *Sjogren's Syndrome: Novel Insights in Pathogenic, Clinical and Therapeutic Aspects: Academic Press*, 2016:189–98.

31 Seror R, Ravaud P, Bowman SJ, *et al*. EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjogren's syndrome. *Ann Rheum Dis* 2010;69:1103–9.

32 Oken MM, Creech RH, Tormey DC, *et al*. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* 1982;5:649–56.

33 Carbone PP, Kaplan HS, Musshoff K, *et al*. Report of the Committee on Hodgkin's Disease Staging Classification. *Cancer Res* 1971;31:1860–1.

34 International Non-Hodgkin's Lymphoma Prognostic Factors P. A predictive model for aggressive non-Hodgkin's lymphoma. *The New England journal of medicine* 1993;329:987–94.