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 Tzioufas1,2

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 (sarcoïdosis 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.

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) 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 miR200-3p 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.

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, 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 (DLCLB). The SS-associated NHLs consisted of 28 MALTs, 2 NMZLs, 2 DLCLBs, 1 B cell bronchial associated lymphoid tissue (BALT), 1 lymphoplasacytic (LP) and 1 small lymphocytic (SLL) lymphoma. In 14 cases (11 MALTs, 2 NMZLs and 1 DLCLB), 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 cells, T cells and CD68+ macrophages, in a proportion of patients (six from each SS group) for identifying the most appropriate reference. In these patients, a common reverse transcription using Megaplex RT Primers, Human Pool B V3.0 (Applied Biosystems) was performed in order to exclude variations due to distinct RT reactions. NormFinder analysis 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−ΔΔCT 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 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 |
| Neutropenia | 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 |
| Spleenomegaly | |
| Lymphadenopathy | |
| Presence of B symptoms | |
| Serum lactate dehydrogenases | |
| β2-microglobulin levels | |

**SS, Sjögren’s syndrome.**
Basic and translational research

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.

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 with the Pearson $\chi^2$ or the Fisher’s exact test.

Statistical analyses

The characteristics of the patients studied are shown in Table 2. The general characteristics, including age, sex, and duration of sicca symptoms, were similar among the subgroups. However, the biopsy focus score, a measure of lymphocytic infiltration, was significantly higher in the SSL subgroup compared to the SSwo subgroup. Additionally, a higher proportion of patients in the SSL subgroup had germinal center formation, indicating a more advanced disease state.

The clinical characteristics, such as arthralgias, arthritis, SG enlargement, Raynaud’s phenomenon, parenchymal organ involvement, renal involvement, palpable purpura, vasculitis, and glomerulonephritis, were also compared among the subgroups. The SSL subgroup had a higher proportion of patients with these clinical features, indicating a more severe disease state.

The laboratory characteristics, such as anti-Ro/SSA and/or La/SSB, anti-Ro/SSA, anti-La (SSB), rheumatoid factor, C3 and C4 levels, C4 hypocomplementaemia, cryoglobulinaemia, hypergammaglobulinaemia, leucopenia, and treatment, were also compared among the subgroups. The SSL subgroup had a higher proportion of patients with these laboratory features, indicating a more severe disease state.

Table 2 Characteristics of the patients studied

| Features | Non-SS (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) |
| Germinatal 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) | 0 (0) | 0 (0) | 0 (0) |
| 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) |
| Leucopenia, n (%) | 0 (0) | 2 (11.8) | 1 (5.9) | 4 (11.4) |
| Treatment, n (%) | 0 (0) | 0 (0) | 2 (11.8) | 3 (8.5) |

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.
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≤0.01 and p≤0.0001 for prelymphoma and lymphoma, respectively) or non-SS sialadenitis controls (0.95±0.84, p≤0.01 and p≤0.001, respectively) (figure 1A). Low miR200b-5p levels were also detected in patients with SSL who had no 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≤0.0001 and r=−0.5048, p≤0.0001, respectively) (mean±SD: 0.24±0.28 vs 0.10±0.06 in patients with low/low-intermediate IPI (0–2),

---

**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)                  |
| UP, n (%)                      | 1 (2.8)                  |
| DLCLB, n (%)                   | 2 (5.7)                  |
| SSL, 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)                |
| Spleenomegaly, 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; **DLCLB**, diffuse large B cell lymphoma; **ECOG**, Eastern Cooperative Oncology Group performance status; **EFS**, event-free survival; **IPI**, International Prognostic Index; **LP**, lymphoplasmaacytic; **MALT**, mucosa-associated lymphoid tissue; **MSG**, minor salivary gland; **NHL**, non-Hodgkin’s lymphoma; **NMZL**, nodal marginal zone lymphoma; **OS**, overall survival; **SSL**, 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 was 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,
Basic and translational research

**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 miR-200b-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
**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 DLCLB (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.29,30 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 lymphoma 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 lymphoma 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.31–34 Epithelial cells are key regulators of SS autoimmune responses, whereas preliminary data suggest that they can drive B cell activation and differentiation.35 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.

Acknowledgements The authors gratefully acknowledge Haralampos M Moutsopoulos, MD, FACP, FRCPC(hc), Master ACR, for his contribution in patient selection and manuscript preparation and editing.

Contributors EK, designed the study, performed the experiments, analysed the data and wrote the paper. AP evaluated the clinical data of patients with non-Hodgkin’s lymphoma (NHL) patients, ADP performed statistical analyses, MV evaluated the clinical data of patients with NHL and contributed in data analysis and manuscript preparation and AGT designed the study and wrote the paper. AGT and EK had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Funding The research has been financed by grants of the Research Grant from the Greek Rheumatology Society and Professional Association of Rheumatologists and the European-funded multicentric protocol ‘HARMONization and integrative analysis of regional, national and international Cohorts on primary Sjögren’s Syndrome (pSS) towards improved stratification, treatment and health policy making’ (HARMONICSS; H2020-SC1-2016; grant agreement no: 731944).

Competing interests AGT has received research grants from Novartis, Pfizer, UCBA AbbellVe and GSK pharmaceutical companies, through the National and Kapodistrian University of Athens, outside the submitted work.

Patient consent Not required.

Ethics approval Ethics Committee of School of Medicine, National and Kapodistrian University of Athens, Greece (protocol no: 1516023881).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement There are no additional unpublished data related to the study.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/.

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;42:1763–72.
3. Skopoulis 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 L. 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-Casais M, Bove A, et al. Predicting adverse outcomes in primary Sjögren’s syndrome: identification of prognostic factors. Rheumatology 2007;46:1359–62.
7. Christodoulou MI, Kapsogorgou EK, Moutsopoulos HM. Characteristics of the minor salivary gland infiltrates in Sjögren’s syndrome. J Autoimmun 2010;34:400–7.
8. Theander E, Vasilitis LS, 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, Virene A, Ng WE, 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. Papageorgou E, Ziogas DC, Mavragani CP, et al. Predicting the outcome of the Sjögren’s syndrome-associated non-Hodgkin’s lymphoma patients. PLoS One 2015;10:e0161689.
11. Baima E, Dahabreh IJ, Voulgarelis M, et al. Hematologic manifestations and predictors of lymphoma development in primary Sjögren’s syndrome: clinical and pathophysiological aspects. Medicine (Baltimore) 2009;88:284–93.
12. Humphries B, Yang C. The microRNA-200 family: small molecules with novel roles in cancer development, progression and therapy. Onco Targets Ther 2015;6:6472–98.
13. Senfter D, Madenier S, Krugtja 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. MR152, MR1208, and MR1338, 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
Basic and translational research

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. *Med Mol Rep* 2014;10:1549–54.

18 Gourzi VC, Kakogiorgou 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;182:65–70.

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 Fraikisudaki 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 Kakogiorgou 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 WY, 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 Sjögren’s syndrome: at the interplay between chronic inflammation, autoimmunity and lymphomagenesis. *Curr Pharm Biotechnol* 2012;13:1989–96.

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

31 Seror R, Ravaud P, Bozeman SJ, et al. EULAR Sjögren’s syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjögren’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 Project. A predictive model for aggressive non-Hodgkin’s lymphoma. *The New England journal of medicine* 1993;329:987–94.