

MicroRNAs 142-3p, miR-155 and miR-203 Are Deregulated in Gastric MALT Lymphomas Compared to Chronic Gastritis

CONCEPCIÓN FERNÁNDEZ^{1,2,3}, BEATRIZ BELLOSILLO^{1,2,4*}, MARIANA FERRARO^{3,5},
AGUSTÍN SEOANE⁶, BLANCA SÁNCHEZ-GONZÁLEZ^{2,5}, SILVIA PAIRET^{1,2}, AINA PONS²,
LUIS BARRANCO⁶, MARÍA CARMEN VELA¹, EVA GIMENO^{2,5}, LLUÍS COLOMO¹,
CARLES BESSES^{2,5}, ALFONS NAVARRO^{7*} and ANTONIO SALAR^{2,3,5,*}

¹Pathology Department, Hospital del Mar, Barcelona, Spain;

²IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain;

³Universitat Autònoma de Barcelona, Barcelona, Spain;

⁴Universitat Pompeu Fabra, Barcelona, Spain;

⁵Hematology Department, Hospital del Mar, Barcelona, Spain;

⁶Digestive Department, Hospital del Mar, Barcelona, Spain;

⁷Molecular Oncology and Embryology Laboratory, Human Anatomy Unit,
School of Medicine, University of Barcelona, IDIBAPS, Barcelona, Spain

Abstract. *Background:* Over the last years, our knowledge on pathogenesis of gastric MALT lymphoma has greatly improved, but its morphological diagnosis is still hampered by overlapping histological features with advanced chronic gastritis. *MicroRNAs* are deregulated in lymphomas, but their role and usefulness in gastric MALT lymphoma has not been extensively investigated. *Materials and Methods:* We analyzed the expression of 384 miRNAs using TaqMan microRNA assay in a training series of 10 gastric MALT lymphomas, 3 chronic gastritis and 2 reactive lymph nodes. Then, significantly deregulated miRNAs were individually assessed by real-time PCR in a validation series of 16 gastric MALT lymphomas and 12 chronic gastritis. *Results:* Gastric MALT lymphoma is characterized by a specific miRNA expression profile. Among the differentially expressed miRNAs, a significant overexpression of miR-142-3p and miR-155 and down-regulation of miR-203 was observed in gastric MALT lymphoma when compared to chronic gastritis. *Conclusion:* miR-142-3p, miR-155 and miR-203 expression levels might be helpful biomarkers for the differential diagnosis between gastric MALT lymphomas and chronic gastritis.

Extranodal marginal-zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) is a rare form of indolent non-Hodgkin's lymphoma. These tumors can arise at any extranodal site and the stomach is the most common location, accounting for almost half of all MALT lymphoma cases (1, 2). Chronic antigen-dependent immune stimulation by *Helicobacter pylori* infection is under the pathogenesis of gastric MALT lymphomas (3, 4), although the mechanisms underlying the transformation from chronic gastritis to gastric MALT lymphoma are insufficiently characterized. Several chromosomal abnormalities have been described in gastric MALT lymphoma, with translocation t(11;18)(q21;q21) being the most frequent (5). Histopathological diagnosis of gastric MALT lymphoma is often difficult due to the overlapping histological features observed in chronic gastritis during progressive transformation to overt gastric MALT lymphoma (6). The score-based classification system, as proposed by Wotherspoon and colleagues, supplemented by molecular clonality assessment of the B-cell population has certainly improved the diagnostic accuracy (7). However, the correct diagnosis of some cases remains challenging in the daily practice.

MicroRNAs are small non-coding RNAs that modulate gene expression on a post-transcriptional level. MicroRNAs can function as endogenous silencers of target genes and play critical roles in cellular proliferation, apoptosis and differentiation (8, 9). MicroRNAs have been described to have important roles as tumor suppressor genes and oncogenes in human malignancies, including lymphomas (10, 11).

Several oncogenic pathways are involved in gastric MALT lymphomagenesis (1, 6, 12). Interestingly, the number of genes that are differentially expressed between chronic

This article is freely accessible online.

*These Authors contributed equally to this study.

Correspondence to: Beatriz Bellosillo, Department of Pathology, Hospital del Mar-IMIM, Passeig Marítim 25-29, 08003, Barcelona, Spain. Tel: 0034 93 2483561, e-mail: bbelosillo@parcdesalutmar.cat

Key Words: MicroRNAs, miR-155, miR-203, gastric MALT lymphoma, chronic gastritis.

gastritis *versus* MALT lymphoma is remarkably low (13), indicating that these two disorders are biologically related. Recently, several microRNAs have been identified to be involved in the process of neoplastic transformation in non-Hodgkin's lymphomas (10, 11, 14-17). Only a few studies have analyzed the role of microRNAs in gastric MALT lymphomas, as diagnostic or prognostic tools (18, 19). This study aimed to identify aberrantly expressed microRNAs that can be helpful in the accurate classification of these entities during the diagnostic process.

Materials and Methods

Patient samples. Biopsy samples from 32 patients diagnosed with gastric MALT lymphoma according to the World Health Organization (WHO) classification (20) and the criteria of Wotherspoon *et al.* (21) were reviewed and included in the study. All gastric MALT lymphomas had scores 3-5 and were clonal. *H. pylori* status was determined by histology and/or urea breath test, or serology in negative cases. All patients underwent disease extension study that included medical history, physical examination, laboratory, computed tomography scans, unilateral bone marrow biopsy and upper endoscopy with multiple gastric biopsies. Stage was determined according to Lugano system, as previously reported (22). From the 32 patients, 3 cases were discarded due to insufficient material and 3 cases due to technical reasons. The 26 remaining cases were divided in 2 different groups according to availability of fresh-frozen material. (1) Training series: 10 patients with available frozen material; (2) Validation series: 16 independent patients with available formalin-fixed paraffin-embedded (FFPE) tissue material.

Cryopreserved tissue specimens from 3 chronic gastritis and 2 reactive lymph nodes were used in the training series as control samples. In addition, 12 paraffin-embedded tissue samples diagnosed of chronic gastritis (associated or not to *H. pylori* infection) were drawn from the MarBiobanc and were used in the validation series. Gastritis was considered in scores 1-2 which were not clonal.

All samples were obtained from the tissue bank of MarBiobanc Hospital del Mar, Barcelona, Spain. The study was approved by the institution's medical ethics committee, and tissue and clinical data were retrieved according to the regulations of the institutional review board (Comitè Ètic d'Investigació Clínica del Parc de Salut Mar CEIC-PSMAR) and data protection laws.

Immunohistochemistry, cytogenetic and clonality studies. A series of immunohistochemical stains were performed in consecutive sections including CD20 (L26)(DAKO), CD3 (F7.2.38) (Novocastra), Bcl-10 (clone 151.1, MBL Medical and Biological Laboratories, Nagoya, Japan) and Ki67(MIB1)(DAKO), as previously reported (23). The t(11;18)(q21;q21) was analyzed on all diagnostic specimens using fluorescent in situ hybridization (FISH) with the commercially available break-apart LSI-MALT1 probe (Vysis, Downers Grove, IL, USA) or by reverse transcriptase-PCR (RT-PCR) for API2/MALT1 fusion product, as previously described (22). Immunoglobulin gene rearrangements were analyzed according to the BIOMED2 protocol (24) as previously described (22).

RNA extraction. Total RNA was extracted from the fresh-frozen samples using Trizol (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. Total RNA was extracted from formalin-fixed paraffin-embedded samples using RecoverAll Total Nucleic Acid Isolation (Ambion, Life Technologies, Carlsbad, CA, USA) as per the manufacturer's protocol. The concentration was quantified using a NanoDrop 1000 Spectrophotometer.

miRNA expression analysis. The miRNA expression profile was analyzed in 10 samples from gastric MALT lymphoma, 3 chronic gastritis and 2 reactive lymph nodes using TaqMan Human MicroRNA Arrays v2.0 (Life Technologies), as described previously (25). Briefly, reverse transcription (RT) reaction was performed on Veriti 96-well thermal cycler for 2 min at 16°C, 1 min at 42°C and 1 s at 50°C for 40 cycles, and 5 min at 85°C, and then held at 4°C. The RT reaction contained: 0.8 µl of 10 × RT buffer (Life Technologies), 0.2 µl dNTPs (100 mM each), 1.5 µl MultiScribe Reverse Transcriptase (50 U/µl), 0.10 µl RNase Inhibitor (20 U/µl), 0.80 µl Megaplex RT primers (10X), 0.90 µl of MgCl₂ (20 U/µl) and 500 ng of total RNA. Real-time PCR reaction was performed on an ABI 7900 HT Sequence Detection System (Life Technologies) and contained 450 µl of TaqMan Universal PCR Master Mix No Amperase (2X) (Life Technologies), 6 µl Megaplex RT product and 444 µl nuclease-free water. The relative miRNA expression was calculated using the 2-ΔΔCt method. Normalization was performed with RNU6B. All miRNAs expressed in <10% of samples were excluded from further analysis, leaving a working set of 234 miRNAs.

Selected miRNAs identified in the profile study were analyzed in 16 independent gastric MALT lymphoma and 12 chronic gastritis using TaqMan microRNA assays. Briefly, cDNA was synthesized from total RNA using gene-specific primers according to the TaqMan MicroRNA Assay protocol. Reverse transcriptase (RT) reactions contained 10 ng of RNA samples, 50 nM stem-loop RT primer, 1 × RT buffer, 0.25 mM each of dNTPs, 3.33 U/µl MultiScribe reverse transcriptase and 0.25 U/µl RNase Inhibitor. The 7.5-µl reactions were incubated in an Applied Biosystems (Foster City, CA, USA) Veriti Thermal Cycler in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Real-time PCR was performed using an Applied Biosystems 7500 Fast System. The 10-µl PCR included 0.67 µl RT product, 1 × TaqMan Universal PCR master mix and 1 µl of primers and probe mix. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. RNU6B was used as endogen control. All assays were performed in triplicate.

Statistical analysis. MicroRNA profiling data were analyzed using TIGR Multiexperiment viewer version 4.0 software (Dana-Farber Cancer Institute, Boston, MA, USA). Hierarchical clustering was performed using Euclidean distance and complete linkage. To identify miRNAs with significant differential expression between subgroups Student's *t*-test based on multivariate permutation (with random variance model) was used. Differences between miRNAs were considered statistically significant if corrected $p < 0.05$. To compare expression values of microRNAs in the validation series between groups *t*-test was performed in GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). Differences at $p < 0.05$ were considered significant.

Results

miRNA expression characterizes gastric MALT lymphoma versus chronic gastritis. We performed miRNA profiling of 10 gastric MALT lymphoma cases, 3 chronic gastritis and 2 reactive lymph nodes by real-time PCR using arrays that allow simultaneous analysis of 384 miRNAs. After filtering and normalization, all miRNAs expressed in <10% of samples were excluded from further analysis, leaving a working set of 234 miRNAs.

The unsupervised hierarchical clustering analysis showed that gastric MALT lymphoma is characterized by a specific miRNA profile since all the lymphoma samples clustered together. Moreover, the 3 chronic gastritis samples and the 2 reactive lymph nodes formed 2 additional groups (Figure 1A). The supervised analysis identified 15 deregulated miRNAs, thirteen of them showing decreased expression levels in the gastric MALT lymphoma samples (Figure 1B) when compared to control samples.

We selected four of the fifteen miRNAs for validation in an independent set of samples. The miRNAs analyzed were the two overexpressed miRNAs, miR-142-3p and miR-155, and the two most significantly down-regulated miRNAs miR-203 and miR-378. We then performed individual miRNA expression assays with the 16 gastric MALT lymphomas (baseline characteristics summarized in Table I) and 12 chronic gastritis. These expression assays confirmed the overexpression of miR-142-3p ($p=0.0001$) and miR-155 ($p<0.0001$) and the downregulation of miR-203 ($p=0.0299$) in gastric MALT lymphomas compared to chronic gastritis. The differential expression of miR-378 could not be confirmed in the validation series (Figure 1C).

Gastric MALT lymphomas with t(11;18)(q21;q21) harbors a specific miRNA profile. We next analyzed whether the miRNA expression profile was related to the presence of the t(11;18)(q21;q21) translocation. Although the limited number of patients with the t(11;18)(q21;q21) included in the initial cohort ($n=5$), the supervised analysis identified seventeen differentially expressed miRNAs between patients carrying or not t(11;18)(q21;q21) (Figure 2A). Interestingly, two of these miRNAs, miR-100 and miR-187, were encoded at chromosomes 11(chr11: 122152229-122152308 [-]) and 18 (chr18: 35904818-35904926 [-]), respectively. We selected miR-100 and miR-187 for validation in the independent set of patients, which included 3 patients with t(11;18)(q21;q21). We observed an increased expression of both miR-100 and miR-187 in cases carrying the translocation, although differences did not reach statistical significance ($p=0.077$ and $p=0.066$, respectively; Figure 2B), probably due to the limited number of cases available with the t(11;18) translocation ($n=3$).

Overexpression of miR-155 in multifocal gastric MALT lymphoma. Finally, we evaluated all previously selected

Table I. Patient's characteristics in training and validation series.

	Training series (n=10) N (%)	Validation series (n=16) N (%)
Age median, years (range)	63 (32-85)	70.5 (36-88)
Gender, male	7 (70%)	10 (62.5%)
Lugano stage		
I-II	8 (80%)	13 (81.2%)
III-IV	2 (20%)	3 (18.8%)
Multifocal	2 (20%)	3 (18.8%)
<i>H. pylori</i> -positive	5 (50%)	10 (62.5%)
t(11;18)-positive	5 (50%)	3 (18.8%)

miRNAs according to dissemination (localized *vs.* multifocal) only in the validation series, since in the training cohort only 2 patients had multifocal stage. At the validation series, 13 out of 16 patients were in Lugano stage I (localized) and 3 were in stage IV (multifocal). Only miR-155 was found to be significantly up-regulated in multifocal gastric MALT lymphoma ($p=0.0099$).

Discussion

In this study, we examined the expression profile of 384 selected miRNAs in gastric MALT lymphomas and our results showed that these tumors have a miRNA expression profile different from chronic gastritis samples or normal lymphoid tissue. Several miRNAs have been described to be differentially expressed in *H. pylori* infection (*e.g.* LET7f, miR-155) (26-29) and in the process of neoplastic transformation from *H. pylori*-related chronic gastritis to overt gastric MALT lymphoma (miR-150, miR-550, miR-124a, miR-518b and miR-539) (26, 30, 31).

In the current study, we found an up-regulation of miR-155 and miR-142-3p in gastric MALT lymphoma supporting data of several recently published observations attributing to these miRNAs a role in MALT lymphomagenesis (26, 28, 30, 31). MiR-155, encoded by a non-coding RNA BIC, is one of the most extensively studied miRNAs in B cells, being strongly implicated in lymphocyte development and aberrantly up-regulated in several B-cell malignancies including diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (32-34). Very recently, Due *et al.* and Wan *et al.* conducted two comprehensive reviews elucidating the potential of miR-155 as a biomarker in B-cell malignancies and in gastrointestinal tract diseases, respectively. While elevated expression of miR-155 shows potential as a diagnostic and prognostic biomarker as well as an efficient therapeutic target in DLBCL and CLL (35), there are few recent studies focusing on gastric MALT lymphomas (35, 36).

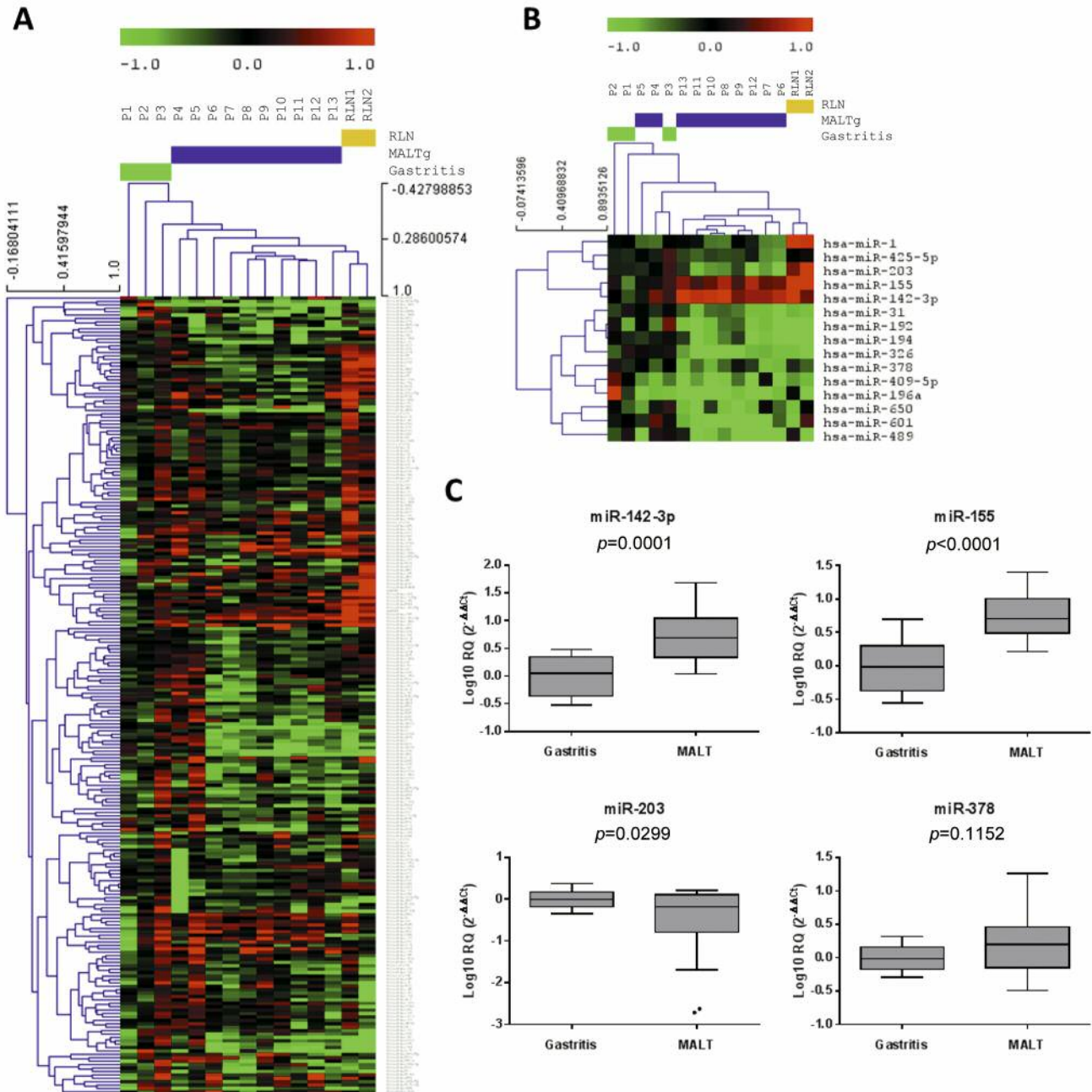


Figure 1. Hierarchical clustering analysis of gastric MALT lymphomas, gastritis and reactive lymph nodes. (A) Unsupervised hierarchical clustering analysis showing the separation of reactive lymph nodes and chronic gastritis from gastric MALT lymphomas. (B) A subset of miRNAs with different expression in gastric MALT lymphoma in comparison to chronic gastritis. (C) Quantitative RT-PCR analysis in the validation cohort of miR-142-3p, miR-155, miR-203 and miR-378 in chronic gastritis and gastric MALT lymphomas.

In gastric MALT lymphomas, up-regulation of miR-155 is observed during *H. pylori* infection and may be used as a potential predictor of resistance to *H. pylori* eradication therapy, independently of the predictive value of the API2/MALT1 fusion gene (30). This may be related to the

negative inflammatory response against *H. pylori* infection induced by miR-155 (28). In addition, expression of miR-155 in B cells is directly activated by NF- κ B and MYB, leading to negative down-regulation of the PI3K/AKT pathway through SHIP-1, and this increased miR-155

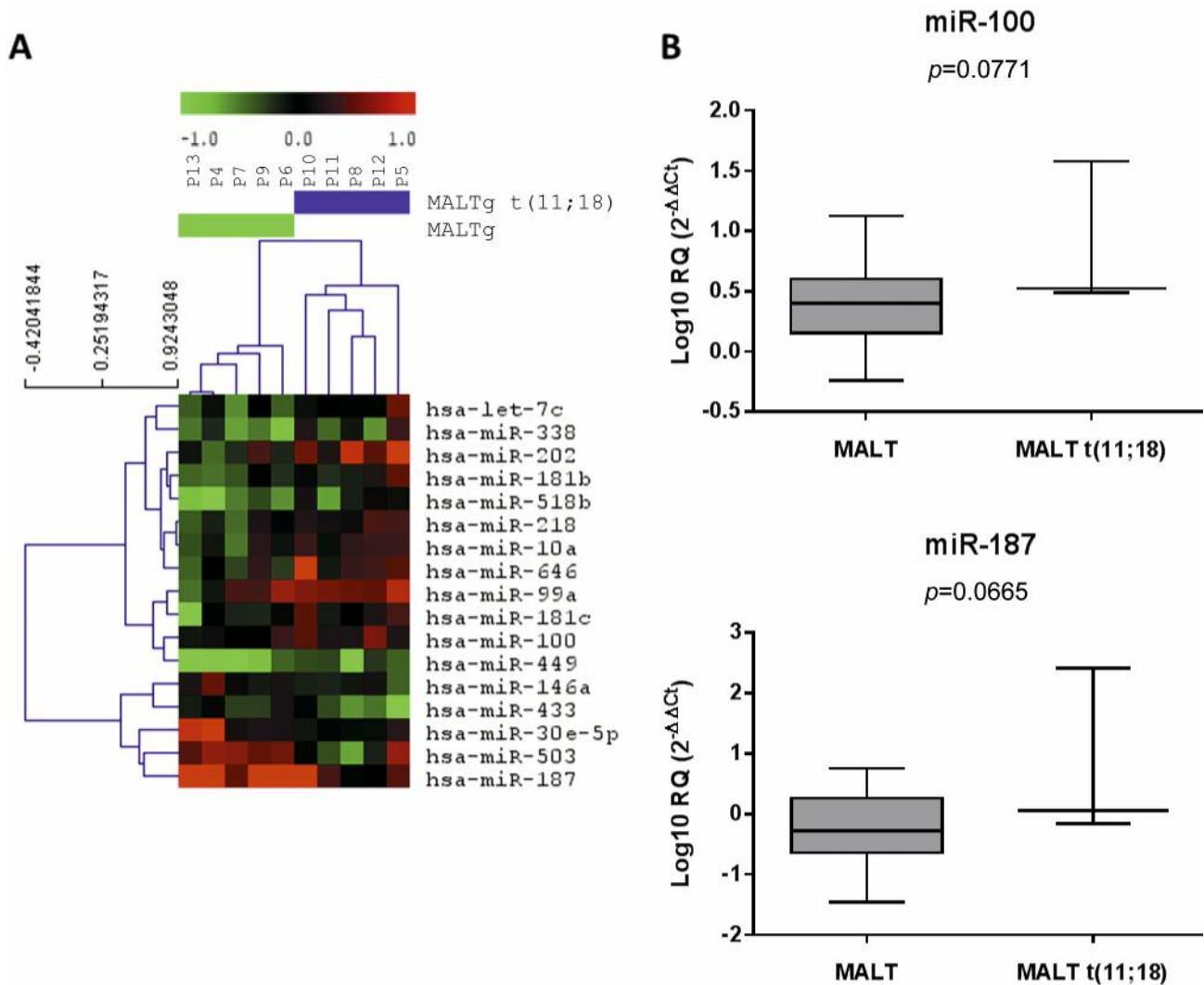


Figure 2. miRNA differentially expressed in gastric MALT lymphomas with $t(11;18)(q21;q21)$. (A) Supervised hierarchical clustering showing differentially expressed miRNAs in the training cohort. (B) Quantitative real-time-PCR analysis in the validation cohort of miR-100 and miR-187.

expression in B cells unblocks AKT activity and affects BCR signaling (32). It is well-known that both NF- κ B pathway and BCR activation are activated and play a critical role in MALT lymphomagenesis.

Our study also revealed an up-regulation of miR-142-3p in gastric MALT lymphoma compared to chronic gastritis. MiR-142-3p is a member of the miR-142 family, and was first discovered to be involved in an aggressive B-cell leukemia being highly specific for hematopoietic cells (37-39). Our finding of higher miR-142-3p expression in gastric MALT cells, a mature B-cell neoplasia, is in line with published expression data showing a higher expression in mature cells compared to immature hematopoietic cells (39) and also in gastric MALT lymphomas (30, 31). Indeed, Saito has

suggested that both miR-142 and miR-155 suppress the pro-apoptotic gene *TP53INP1*, possibly leading to inhibition of apoptosis and acceleration of MALT lymphoma cell proliferation. Interestingly, inhibition of miRNAs with chemically-engineered oligonucleotides, termed “antagomirs”, can work as specific inhibitors of endogenous miRNAs in mice, and might be potentially applicable to silence miR-142 and miR-155 for the treatment of gastric MALT lymphomas that are resistant to *H. pylori* eradication therapy (40-42).

Our results also showed that miR-203 was down-regulated in gastric MALT lymphoma, like other recent published data (26, 43). Craig *et al.* have described that the progression from *H. pylori*-associated gastritis to low-grade MALT lymphoma is epigenetically regulated by methylation of the

miR-203 promoter region. Transcriptional repression of miR-203 results in dysregulation of ABL1, which in turn drives MALT lymphoma proliferation. Then, expression of miR-203 is induced in the course of B-cell maturation, and is secondarily lost as marginal zone B-cells undergo neoplastic transformation during MALT lymphomagenesis (43).

Histopathological diagnosis of gastric MALT lymphoma in the context of morphological overlap between reactive or inflammatory changes during progressive malignant transformation is difficult. The score-based classification system as proposed by Wotherspoon and the use of standardized PCR protocols like the BIOMED2 protocol has certainly improved the diagnostic accuracy. According to our results, discrimination between chronic gastritis and gastric MALT lymphomas on the basis of miRNA expression profiles is feasible. Moreover, we propose that quantitative expression of miR-142-3p and miR-155 may be an additional tool that can help in the challenging process of histologic diagnosis to better differentiate between chronic gastritis and overt gastric MALT lymphoma.

Apart from regulation mechanisms of miRNA expression, cytogenetic aberrations might play a role in miRNA deregulations. In this study, we examined the expression profile of miRNAs according to the presence or absence of t(11;18)(q21;q21), because patients carrying this translocation have a poor response to antibiotic eradication therapy and to alkylating agents like chlorambucil or cyclophosphamide (44, 45). We found 17 miRNAs differentially expressed in the array series and, subsequently, we investigated a series of miRNAs that included some miRNAs mapped to chromosomal regions altered in patients carrying *API2-MALT1* translocation. Overexpression of miR-100 (chromosome 11) and miR-187 (chromosome 18) was found in cases with t(11;18)(q21;q21) but not of miR-155, as previously reported by Saito, although the number of patients carrying t(11;18)(q21;q21) in both studies is limited.

Finally, we also investigated the contribution of miRNA expression in the dissemination trend of gastric MALT lymphomas. We found 23 miRNAs to be differentially expressed according to the dissemination presentation of the disease, with miR-142-3p, miR-155 and miR-222 being the most significantly down-regulated miRNAs. Gastric MALT lymphomas with higher expression of miR-223, a memory B-cell-associated miRNA, have been described to have a tendency to involve perigastric lymph nodes (18). Unfortunately, we did not have stage II cases in the validation series to corroborate this observation.

In summary, gastric MALT lymphomas have differentially expressed miRNAs when compared to chronic gastritis. We validated the overexpression of miR-155 and miR-124-3p and down-regulation of miR-203, which could be used as novel biomarkers for improving the often difficult process of gastric MALT lymphomas diagnosis.

Conflicts of Interest

The Authors declare no conflict of interest.

Acknowledgements

This work was supported by Grants from Instituto de Salud Carlos III FEDER (FIS 07/0586, FIS 15/0459, PT13/0010/0005), 2014 SGR 567, and the "Xarxa de Bancs de tumors" sponsored by Pla Director d'Oncologia de Catalunya (XBTC). Concepción Fernández-Rodríguez received a fellowship from the Ministry of Economy and Competitiveness of Spain (PFIS grant FI11/00353).

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Received November 15, 2016

Revised December 9, 2016

Accepted December 15, 2016