Epigenetic Silencing of MicroRNA-203 Dysregulates ABL1 Expression and Drives Helicobacter-Associated Gastric Lymphomagenesis

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Abstract

Gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) develops in the chronically inflamed mucosa of patients infected with the bacterial pathogen Helicobacter pylori. Here we use patient material, primary gastric lymphoma cell cultures, and a preclinical model of the disease to examine the role of microRNA (miRNA)-mediated posttranscriptional regulation—focusing in particular on miR-203 and its target ABL1—in gastric MALT lymphomagenesis. Microarray-based miRNA expression profiling revealed a strong downregulation of the putative tumor suppressor miRNA miR-203 in human MALT lymphoma samples, which resulted from extensive promoter hypermethylation of the miR-203 locus and coincided with the dysregulation of the miR-203 target ABL1 in lymphoma biopsies compared with matched adjacent normal material from the same patients. Treatment of lymphoma B cells with demethylating agents led to increased miR-203 expression and the concomitant downregulation of ABL1, confirming the epigenetic regulation of this miRNA. Ectopic reexpression of miR-203 by transfection of a human lymphoma cell line or lentiviral transduction of explanted primary MALT lymphoma cells was sufficient to prevent tumor cell proliferation in vitro. Similarly, the treatment of primary MALT lymphoma cells with the ABL inhibitors imatinib and dasatinib prevented tumor cell growth. Finally, we show that the treatment of tumor-bearing mice with imatinib induces MALT lymphoma regression in a preclinical model of the disease, implicating ABL1 in MALT lymphoma progression. In summary, our results show that the transformation from gastritis to MALT lymphoma is epigenetically regulated by miR-203 promoter methylation and identify ABL1 as a novel target for the treatment of this malignancy. Cancer Res; 71(10); 1–9.

Introduction

Mucosa-associated lymphoid tissue (MALT) lymphomas account for 8% of all non-Hodgkin lymphomas (1). The development of gastric MALT lymphoma is tightly linked to chronic infection with the bacterial pathogen Helicobacter pylori (2); consequently, antibiotic eradication therapy is now used as the first line treatment of this malignancy (3–6). MALT lymphomagenesis is initiated by H. pylori-associated chronic inflammation and the subsequent accumulation of gastric organized lymphoid tissue, from which individual neoplastic clones may grow out and invade the adjacent epithelium (2). We have shown recently that MALT lymphoma tumor immunoglobulins (Ig) are clonal, somatically hypermutated, and polyreactive, that is, they bind to a variety of unrelated self- and foreign antigens (7). The same panel of antigens that are recognized by MALT lymphoma surface Ig induce proliferation of explanted tumor cells, supporting the notion that early low-grade gastric MALT lymphoma is an antigen-dependent malignancy (8). Low-grade gastric MALT lymphomas are infiltrated by large numbers of T cells, which are polarized to produce Th2 cytokines such as interleukin-4 (9, 10). The depletion of T cells prevents the proliferation of tumor cells ex vivo and induces tumor regression in a mouse model of gastric MALT lymphoma (8), implying a synergistic role for T-cell-derived growth signals and B-cell receptor-mediated antigen recognition during early MALT lymphoma development.

Low-grade human MALT lymphoma can be modeled by chronic experimental infection of BALB/c mice with Helicobacter species (10–13). In mice, disease progression is reflected by specific gene expression signatures of histologically defined disease stages (13); similar signatures are found in human biopsies of gastritis versus low-grade lymphoma material (14). The number of genes that are differentially expressed between reactive and neoplastic lesions is remarkably low in both species (13, 14), suggesting that the 2 tissues are biologically...
similar. In conjunction with the reversibility of the early tumors upon *Helicobacter* eradication therapy in humans (3, 4, 15) and mice (10, 12), the biologic similarity implies that epigenetic and other regulatory processes rather than genetic events drive MALT lymphoma progression in the early stages.

MicroRNAs (miRNAs) are well conserved, 18–25 nucleotide long noncoding RNAs with pivotal roles in posttranscriptional gene regulation (16). miRNA expression patterns correlate with particular cancer types (17) and are predictive of clinical outcome (18, 19). Over 50% of miRNA genes are located in cancer-associated genomic regions (20). Many miRNAs are known to function as tumor suppressors, regulating the expression of oncoproteins such as RAS (21) and c-MYC (22). Here, we show that miR-203 is specifically downregulated in gastric MALT lymphoma because of promoter hypermethylation. The ectopic reexpression of miR-203 in primary MALT lymphoma cells silences the expression of the miR-203 target ABL1 and blocks tumor cell proliferation *in vitro*. Inhibition of ABL1’s tyrosine kinase activity by imatinib blocks MALT lymphoma cell proliferation *ex vivo* and prevents tumor formation in mice, suggesting for the first time an important oncogenic role for ABL1 in the pathogenesis of a malignancy not harboring the (9;22) chromosomal translocation fusing the BCR and ABL loci.

**Materials and Methods**

**Patient material and cell lines**

Human material was obtained from 8 patients with *H. pylori*-positive, (11;18)(q21;q21)-negative gastric low-grade MALT lymphoma that were part of a previously published study conducted at Philippus-University Hospital Marburg, Germany (14). The whole genome-based miRNA expression analysis was carried out by using fresh frozen cases of *H. pylori*-positive gastric low-grade MALT lymphoma and tonsil material drawn from the surgical pathology files of the Institute of Pathology at the Cantonal Hospital St. Gallen, Switzerland. miR-203 downregulation and promoter methylation was examined by using archived formalin-fixed, paraffin embedded tonsil material, and biopsies of *H. pylori*-positive chronic active gastritis, *H. pylori*-positive gastric low-grade MALT lymphoma and gastric diffuse large B-cell lymphoma (DLBCL) from the Institute of Pathology, Cantonal Hospital St. Gallen. All data were blinded to guarantee patients’ protection and were generated in agreement with the guidelines for use of human material in research issued by the participating Institutions’ Ethics Committees. The Burkitt’s lymphoma cell line BL2 was kindly provided by Prof. Dr. Jean-Claude Weill (Hôpital Necker, Paris, France); BL2 cells are EBV-negative and were generated from the bone marrow of a pediatric nonendemic Burkitt lymphoma patient (23). Cell line authentication was carried out at the onset of the experiments described here and included flow cytometric verification of surface marker expression (CD19, CD20, IgM, HLA-DR) and verification of the IGL-MYC translocation by genomic PCR.

**Animal experimentation, cell culture, nucleoeration, and fluorescence-activated cell sorting**

Female BALB/c mice were infected intragastrically at 6 weeks of age with 5 × 10^7* H. felis* (CS1, ATCC 49179). All procedures were approved by the Zurich cantonal veterinary office. Mice received 75 mg/kg per day imatinib in their drinking water for the final 3 months of an 18-month infection experiment. After 18 months, macroscopically visible gastric tumors were collected, single cell suspensions were generated and cultured for 3 days in RPMI/10% FCS with 10 μg/mL *Helicobacter* lysate and 0.01–30 μmol/L imatinib or dasatinib (generously provided by Novartis and C. Nevado, Institute of Organic Chemistry, University of Zurich, respectively). Tumor cell proliferation was quantified by [3H]-thymidine incorporation. For DNA demethylation, BL2 cells were treated with 5 μmol/L 5'-azacytidine and/or 3 mmol/L 4-phenylbutyric acid (PBA; both from Sigma–Aldrich). For the purpose of miR-203 reexpression, 1 × 10^6 BL2 cells were nucleoporated by using an Amaza Nucleoporator with miR-203 precursor or negative control oligonucleotides (both from Ambion) and harvested 48 hours later for ABL1 expression analysis by quantitative real-time PCR (qRT-PCR) or Western blot. For sorting of pure B-cell populations, splenic B cells were immunomagnetically purified (B-cell isolation kit; R&D Systems), stained with antibodies against CD21 (clone 7G6; BD Pharmingen) and CD23 (clone B3B4; Biolegend) and sorted to more than 95% purity on a fluorescence-activated cell sorting (FACS) Aria (Becton Dickinson) prior to miR-203 qRT-PCR analysis.

**Lentivirus production and infection**

The pre-miR-203 lentiviral expression construct used to reexpress mature miR-203 in murine primary lymphoma cells was purchased from Systems Biosciences. Third-generation lentivector packaging constructs were generously provided by Stefano Ferrari from the Institute of Molecular Cancer Research, University of Zurich, and consisted of the 3 plasmids pMDLg/pRRE, pCMV-G, and pRSVrev. The pre-miR-203 expression vector (2.5 μg) was transfected along with the lentivirus packaging plasmids (8 μg) into HEK293T cells and the supernatant was collected 48 and 72 hours after transfection. Primary lymphoma cells were incubated with the viral particles. Empty lentivirus was used as a control. *H. felis* stimulated primary MALT lymphoma cells were transduced in the presence of polybrevin (8 μg/mL) by spinoculation in a centrifuge at 700 × g for 90 minutes at room temperature in a 96-well plate. Seventy-two hours following infection, tumor cell proliferation was quantified by [3H]-thymidine incorporation assay. Expression of mature miR-203 was validated by qRT-PCR.

**Microarray-based miRNA expression profiling**

Total RNA (including miRNA) was extracted from fresh frozen and FFPE biopsy samples (three 20 μm slices) by using the RecoverAll total RNA Isolation Kit (Ambion). RNA integrity was evaluated by using the Agilent 2100 Bioanalyzer (Agilent Technologies). miRNA microarray experiments were carried out by using the Agilent Human miRNA Microarray Kit version 1.0.0. For each sample, 100 ng total RNA were...
hybridized with the miRNA array and further processed according to Agilent’s miRNA Microarray System protocol. The arrays were scanned with an Agilent Technology G2565B scanner (Agilent Technologies). The scanned images were gridded and analyzed by using Agilent Feature Extraction Software version 9.5. Normalization and statistical analysis was carried out with R/Bioconductor. Specifically, we used the quantile normalization implemented in the package PreprocessCore and ran the statistical test by using the package genefilter. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; ref. 24) and are publicly accessible through GEO Series accession number GSE23877.

miR-203 and ABL1 expression analysis and bisulfite sequencing

Expression of mature miR-203 was analyzed by using the miRCURY locked nucleic acid (LNA) miRNA PCR system following the manufacturers’ protocol (Exiqon). Briefly, 10 ng of total RNA was subjected to cdNA synthesis by using miR-203 or U6 snRNA-specific primers. The cdNA template was diluted 1:10 and RT-PCR reactions were carried out following the manufactures’ recommendations (LightCycler; Roche). Calculations of miRNA expression levels were carried out by using the comparative ΔΔCt method and normalized against U6 snRNA levels. ABL1-specific RT-PCR (LightCycler; Roche) was carried out with the LightCycler 480 SYBR Green I master kit (Roche). GAPDH transcript levels were determined for normalization. Human and murine ABL1 primers were published previously (25, 26). Protein extracts were made by using either BIPA cell lysis buffer [50 mol/L Tris-HCl (pH7.5), 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS, 1 mmol/L PMSF] or 2x Laemml sample buffer [4% SDS, 20% glycerol, 120 mmol/L Tris (pH6.8)]. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with antibodies against ABL1 (BD Biosciences) and α-tubulin (Sigma–Aldrich). Specific oligonucleotides for bisulfite sequencing of the miR-203 Cpg island were described previously (27). Genomic DNA (gDNA) was isolated from FFPE tissue or fresh material by using the RecoverAll total RNA Isolation Kit (Ambion) or the Quiagen DNeasy Blood and Tissue Kit (Qiagen), respectively. Two micrograms of gDNA was converted with sodium bisulfite as previously reported (28). Following amplification of the bisulfite-converted DNA, the methylation status was assessed by sequencing the miR-203 bisulfite converted promoter region.

Results

The expression of miR-203 and of its target ABL1 is dysregulated in MALT lymphoma

To identify miRNAs that are differentially expressed in human Helicobacter-associated MALT lymphoma compared with normal lymphoid tissue, we generated microarray-based expression profiles for 5 low-grade MALT lymphoma and 4 tonsil samples that included all 795 currently annotated human mature miRNAs. Tonsil material was used as a reference because of its comparable cellular composition (B-cells make up ~60%–70% of cells in tonsil tissue and in MALT lymphoma; ref. 8). Of the 157 miRNAs exhibiting high variation across the 9 samples (Supplemental Table S1), 6 were strongly downregulated in all cases of lymphoma, but in none of the tonsil samples (annotated in Fig. 1A). Of these 6 miRNAs, 3 were predicted by miRNA target prediction algorithms (TargetScan, miRDB, miRWalk) to posttranscriptionally regulate the nonreceptor tyrosine kinase ABL1 (c-Ab1) and/or the closely related ABL2 kinase (also called ARG for “Ab1-related gene”). miR-203 (which was downregulated by on an average 10-fold in MALT lymphoma compared with tonsil tissue) binds to position 1074 in the ABL1 3’UTR (27), and is predicted to also target 3 sites in the ABL2 3’UTR (1291, 4963, and 7951). miR141 and miR205 are predicted to target both ABL1 kinases and ABL2 only, respectively. We focused our attention on miR-203 because it has previously been implicated in tumor suppression (27) and has also been identified in a recent screen comparing miRNA expression in the normal, H. pylori-negative gastric mucosa, and H. pylori-positive gastritis (29). Its dysregulation could be confirmed by qPCR in the samples used for expression profiling as well as in an independent set of archived cases of Helicobacter-associated gastritis and gastric MALT lymphoma (Fig. 1B and C). As miR-203 is known to regulate ABL1 (27), we assessed ABL1 expression in 8 cases of gastric MALT lymphoma for which matched gastritis material was available. qPCR analysis revealed that 7 of the 8 lymphoma samples exhibited higher ABL1 expression than the corresponding gastritis (Fig. 1D). Similar patterns of inverse miR-203 and ABL1 expression were revealed that 7 of the 8 lymphoma samples exhibited higher ABL1 expression than the corresponding gastritis (Fig. 1D).

The miR-203 promoter is specifically hypermethylated in MALT lymphoma

The promoter region of the miR-203 genomic locus contains a CpG island flanking the transcriptional start site (Fig. 2A), which has been shown to be hypermethylated in
certain hematologic malignancies and hepatocellular carcinoma (27, 30). We determined the methylation status of the miR-203 promoter in several cases each of normal human tonsil, gastritis, low-grade gastric MALT lymphoma, and gastric DLBCL by sodium bisulfite genomic sequencing. The miR-203 promoter region was heavily methylated in the majority of independent clones analyzed for all MALT lymphoma and gastric DLBCL samples, but was largely unmethylated in the tonsil and gastritis tissues (Fig. 2B). In conclusion, the miR-203 promoter seems to be specifically hypermethylated in MALT lymphoma, which may lead to silencing of the genomic locus and may contribute to MALT lymphomagenesis.

The expression of miR-203 is epigenetically regulated and controls ABL1 levels and lymphoma cell proliferation in vitro

To determine whether promoter methylation indeed affects miR-203 transcription, and as a consequence, ABL1 expression, we took advantage of a non-Hodgkin lymphoma cell line exhibiting a degree of miR-203 promoter methylation that is comparable with that found in human MALT lymphoma (BL2; Fig. 3A). The treatment of BL2 cells with the DNA-demethylating agent 5'-azacytidine (Aza), either alone or in combination with the histone deacetylase inhibitor PBA, resulted in decreased miR-203 promoter methylation and a concomitant rise in miR-203 expression (Fig. 3A, B); the expression of ABL1
was inversely correlated with miR-203 at both the mRNA and protein levels (Fig. 3C, D). The combined treatment with both compounds resulted in a more efficient drop in promoter methylation and a greater increase in miR-203 expression, but did not further reduce ABL1 expression (Fig. 3A-D). To confirm directly that miR-203 targets ABL1, pre-miR-203 pre-cursor molecules were introduced into BL2 cells by electroporation. The ectopic expression of pre-miR-203 led to a significant downregulation of ABL1 expression at both the mRNA and the protein levels as compared with a scrambled negative control pre-miR (Fig. 3E and F) and blocked the proliferation of BL2 cells as determined by [3H]-thymidine incorporation (Fig. 3G). The combined results suggest that miR-203 is epigenetically regulated and acts as a tumor suppressor miRNA through its effects on ABL1 expression.

The proliferation of MALT lymphoma cells is blocked by imatinib in vitro and in vivo

To assess the effects of miR-203 delivery on primary murine MALT lymphoma cells, we generated lentiviral particles carrying a miR-203-encoding expression vector and transduced primary murine gastric MALT lymphoma cells isolated from BALB/c mice that had been infected with H. felis for 18 months. The lentiviral delivery of miR-203 to explanted murine MALT lymphoma cells resulted in increased miR-203 levels, decreased ABL1 expression, and a concomitant block in the Helicobacter antigen-dependent proliferation of the cells (Fig. 4A–C). These observations raised the possibility that ABL inhibitors such as imatinib (Gleevec) might prevent the proliferation of primary MALT lymphoma cells in vitro and in vivo. Indeed, addition of imatinib to the primary cell cultures efficiently blocked their proliferation in a dose-dependent manner (Fig. 4D); similar results were obtained with the ABL inhibitor dasatinib (Fig. 4D). Tumor cells that had spread to the spleen were equally sensitive to ABL inhibition as cells derived from the primary gastric tumor (Fig. 4E). To assess a possible therapeutic effect of imatinib in a preclinical model of Helicobacter-induced MALT lymphoma, BALB/c mice were infected for 15 months with H. felis to allow for lymphoma development. A group of mice received imatinib via the drinking water for the following 3 months whereas all other mice remained untreated. Although between 2 and 15 tumors had formed in the majority of control mice, all imatinib-treated mice were tumor free as assessed macroscopically and histopathologically at the 18-month postinfection endpoint of the study (Fig. 4F and G). Imatinib had no effect on H. felis colonization densities (Fig. 4H), ruling out a direct effect of the treatment on the underlying infection. The combined results suggest that miR-203 reexpression in murine primary MALT lymphoma cells prevents their proliferation and that this effect is mediated by the tyrosine kinase activity of the miR-203 target ABL1 in vitro and in vivo.
Discussion

Our array-based miRNA transcriptome analysis identified miR-203 as one of the most strongly downregulated miRNAs in gastric lymphoma compared with normal lymphoid tissue; its dysregulation could be attributed to extensive promoter hypermethylation. Our results further suggest that the loss of miR-203 expression and the resulting dysregulation of its target ABL1 contribute directly to gastric lymphomagenesis and thus identify ABL1 as a new target in the treatment of this malignancy. Several previous reports have indicated a tumor suppressive role for miR-203. It is encoded on a fragile 7 Mb region of murine chromosome 12 that encodes approximately 12% of all genomic miRNAs and that is frequently deleted in hematopoietic malignancies (27). Its expression is downregulated in cancers of the liver (31), central nervous system (32), and in some types of leukemia (27). Experimental inhibition of miR-203 enhances the growth of lung carcinoma cells (33); conversely, the experimental restoration of miR-203 expression significantly reduces the proliferation of hepatocellular carcinoma (30), of certain leukemias such as chronic myelogenous leukemia (CML; ref. 27) and of head and neck squamous cell carcinoma (34).

MALT lymphomas are negative for the Philadelphia chromosome (Ph°), that is, they do not harbor the t(9;22) reciprocal translocation fusing the BCR and ABL1 gene loci that is a hallmark of CML and of a subset of acute lymphoblastic leukemias (ALL; ref. 35). However, we found that wild type ABL1 is significantly more strongly expressed in human MALT lymphoma biopsies than in corresponding gastritis material from the same patient. The treatment of BL2 cells (which are also Ph° and harbor a comparably methylated miR-203 promoter region) with demethylating agents and the consequent reexpression of miR-203 repressed cellular ABL1 levels and reduced BL2 proliferation, showing that miR-203 can function as a tumor suppressor independently of the t(9;22) translocation.

ABL1 is predominantly expressed in the hematopoietic system, in particular in lymphocytes; the targeted deletion or mutation of ABL1 in mice results in splenic and thymic...
atrophy and lymphopenia as well as increased susceptibility to infections (36). In B-cells, ABL1 functions in B-cell receptor signaling, probably by directly interacting with, and phosphorylating, the BCR coreceptor CD19 (37). ABL1 overexpression has been associated with Ph/C0 hematopoietic malignancies such as chronic lymphocytic leukemia (CLL; ref. 38). In CLL, ABL1 overexpression has been linked to constitutively active BCR signaling and NF-κB activation (38), that is, to a signaling pathway that is known to contribute to MALT lymphomagenesis (2).

We have shown recently that BCR signaling synergizes with T-cell–derived growth signals to drive MALT lymphoma cell proliferation (8). Inhibition of ABL1 expression by miR-203 replacement or inhibition of its tyrosine kinase activity would therefore be predicted to block the Helicobacter-induced proliferation of primary MALT lymphoma cells. Indeed, the lentiviral delivery of miR-203 or treatment of explanted murine MALT lymphoma cells with the tyrosine kinase inhibitor imatinib efficiently prevented their proliferation. The treatment of Helicobacter-infected mice prevented or even reversed MALT lymphomagenesis in vivo, suggesting that ABL inhibition might be a valid strategy for the treatment of patients that are refractory to eradication therapy. In addition to its beneficial effects in patients with CML (39), imatinib has recently shown promise in adult patients with Ph⁺ B-cell ALL (35) and has been proposed for the treatment of Ph⁺ CLL patients overexpressing ABL1 (38).

Figure 4. miR-203 replacement and pharmacologic ABL inhibition block MALT lymphoma growth in vitro and in vivo. A–C, Primary murine MALT lymphoma cells were transduced with lentiviruses carrying a pre-miR-203-expressing or empty vector. miR-203 and ABL1 expression were assessed by qRT-PCR (A and B); the proliferation of transduced cells was determined by [3H]-thymidine incorporation with or without stimulation with H. felis sonicate (C). D and E, [3H]-thymidine incorporation of gastric (D) and splenic (E) primary murine MALT lymphoma cells stimulated with H. felis sonicate and treated with increasing concentrations of imatinib or dasatinib as indicated. Unstimulated cells are included for comparison. Vertical bars indicate standard deviations. The data shown are representative of 2 (A–C) and 3 (D, E) independent experiments. F, gastric MALT lymphoma formation in female BALB/c mice infected for 18 months with H. felis. One group received imatinib through the drinking water for months 16–18 of the experiment. Macroscopically visible tumors greater than 1 mm in diameter are plotted. G, representative micrographs of H&E-stained sections of the mice shown in F. Scale bar indicates 50 μm. H, H. felis colonization as determined by flaB-specific qPCR of the mice shown in F.
In summary, we show here that the progression from Helicobacter-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. Our results identify ABL1 as a promising new target for the treatment of low-grade MALT lymphoma, in particular of approximately 20% of patients who are refractory to eradication therapy as a first line treatment of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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