Genetic and Epigenetic Silencing of MicroRNA-203 Enhances ABL1 and BCR-ABL1 Oncogene Expression

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SUMMARY

The mammalian genome contains several hundred microRNAs that regulate gene expression through modulation of target mRNAs. Here, we report a fragile chromosomal region lost in specific hematopoietic malignancies. This 7 Mb region encodes about 12% of all genomic microRNAs, including miR-203. This microRNA is additionally hypermethylated in several hematopoietic tumors, including chronic myelogenous leukemias and some acute lymphoblastic leukemias. A putative miR-203 target, ABL1, is specifically activated in these hematopoietic malignancies in some cases as a BCR-ABL1 fusion protein (Philadelphia chromosome). Reexpression of miR-203 reduces ABL1 and BCR-ABL1 fusion protein levels and inhibits tumor cell proliferation in an ABL1-dependent manner. Thus, miR-203 functions as a tumor suppressor, and re-expression of this microRNA might have therapeutic benefits in specific hematopoietic malignancies.

INTRODUCTION

MicroRNAs (miRNAs) are noncoding RNAs 18–25 nt in length that regulate a variety of biological processes by silencing specific target genes (Ambros, 2004). miRNAs are well conserved during evolution, and it has been estimated that about 250–600 miRNAs have been evolutionarily conserved in vertebrates (Bentwich et al., 2005). Additional nonconserved miRNAs have also been characterized in primates, and humans are reported to contain about 800–1000 miRNAs (Bentwich et al., 2005; Zamore and Haley, 2005). Little is known regarding how miRNA expression is regulated in mammalian cells. Primary miRNA transcripts are generated by polymerases II and III, and they are usually capped and polyadenylated (Borchert et al., 2006; Kim and Nam, 2006). Some miRNAs are clustered and transcribed as multicistronic primary transcripts, but many others are not clustered and are transcribed independently (Ambros, 2004; He and Hannon, 2004; Landgraf et al., 2007). These miRNAs can downregulate various gene products by translational repression when partially complementary sequences are present in the 3′ untranslated regions (3′UTR) of the target mRNAs or by directing mRNA degradation. Using these posttranscriptional control mechanisms, mammalian miRNAs appear to target a diversity of cellular functions, including cell proliferation and differentiation (He and Hannon, 2004).

In the last few years, it has become evident that miRNA expression is deregulated in human cancer, resulting in specific oncogenic events reviewed in Calin and Croce (2006) and Esquela-Kerscher and Slack (2006). Specific over- or underexpression has been shown to correlate with particular tumor types (Lu et al., 2005; Volinia et al., 2006). These changes in expression might modulate known oncogenes or tumor suppressors. For
example, let-7, a microRNA that inhibits RAS expression, is downregulated in lung cancer, leading to increased RAS protein levels (Johnson et al., 2005). On the other hand, miR-17-5p and miR-20a modulate the balance between cell death and proliferation in response to the c-Myc oncogene (O'Donnell et al., 2005). In some cases, it has been demonstrated that miRNAs are inactivated by specific genetic or epigenetic alterations. Thus, miR-15a and miR-16-1 are inactivated by deletions or specific translocations in their chromosomal regions (Calin et al., 2002). In fact, microRNAs exhibit a high frequency of DNA copy changes that correlate with altered levels of expression in various human malignancies (Zhang et al., 2006). Other microRNAs are inactivated by epigenetic mechanisms, and their re-expression by epigenetic drugs can lead to downregulation of target oncogenes (Brueckner et al., 2007; Fazi et al., 2007; Lujambio et al., 2007; Saito et al., 2006). Altered expression of miRNAs, moreover, can predict clinical outcome (Calin et al., 2005; Takamizawa et al., 2006), suggesting that miRNAs play an important role in human cancer.

In this study, we have identified a region of mouse chromosome 12 (F2 region, conserved in human chromosome 14q32) that is frequently lost in T cell malignancies. This chromosomal region is especially rich in microRNAs, since it contains several clusters of microRNAs and expresses 52 mature microRNAs (about 12% of the mammalian miRNA genome: miRBase v 10.0). We have narrowed down the fragile site to a region of about 7 Mb that contains 51 out of these 52 miRNAs. After miRNA expression profiling, we have detected significant silencing of one of these miRNAs, miR-203. Since affected tumors usually lose only one copy of this DNA region, we have investigated whether miR-203 expression is also downregulated by epigenetic mechanisms. We demonstrate here that miR-203 is silenced by the loss of one allele and promoter CpG hypermethylation in the remaining DNA copy. We have further identified ABL1 as one of the targets of miR-203, indicating that both genetic and epigenetic mechanisms participate in transcriptional silencing of miR-203, leading to upregulation of the ABL1 and BCR-ABL1 oncogenes in various murine and human hematopoietic malignancies. Restoration of miR-203 by exogenous transfection or epigenetic drugs results in ABL1 and BCR-ABL1 downregulation (similar to that elicited with RNA interference) and decreased proliferation of tumor cells. This antiproliferative effect is partially rescued by overexpression of BCR-ABL1 and fully rescued by a BCR-ABL1 cDNA without the endogenous 3'UTR, suggesting that ABL1 is a crucial target of the tumor suppressor activity of miR-203.

RESULTS

**A Chromosomal Region Enriched in MicroRNAs Is Frequently Lost in Irradiated T Cell Lymphomas**

In a search for genomic alterations throughout the tumor genome, we performed comparative genome hybridization (CGH) of 12 γ-radiation-induced lymphomas versus wild-type tissues from C57BL/6J mice. We identified consistent DNA losses in the telomeric region of chromosome 12 (about 50% of samples; n = 12). CGH analysis of this fragment (Figure 1A) did not provide resolution enough to analyze putative candidate tumor suppressor genes. We therefore further refined this region using specific single-strand conformation polymorphism (SSCP) analysis in 44 additional tumor samples using F1 mice generated from crosses between pure C57BL/6J and RF/J inbred mice. These studies demonstrated that the DNA fragment most frequently lost in these tumors corresponds to the chromosome region between markers D12Mit132 and D12Mit18 (chromosome 12 positions 107.4 to 113.7 Mb) (Figure 1B). This region contains about 80 protein-coding genes, including Bcl11b, a potential tumor suppressor candidate for this region (Wakabayashi et al., 2003). Interestingly, in addition to these genes, this chromosomal region also contains 52 microRNAs (Figure 1C), accounting for about 12% of the known microRNAs in the mouse genome (Sanger miRBase repository, Release 10.0; August 2007) (Griffiths-Jones et al., 2006). These microRNAs include several clusters located at the callipyge locus, a chromosomal region known to be regulated by genomic imprinting (Davis et al., 2005) and other nonclustered microRNAs.
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Genetic and Epigenetic Alteration of Mouse and Human miR-203

To analyze whether some of these miRNAs were silenced in T cell malignancies, we performed expression profiling using miRNA microarrays (Figure 2). High-stringency analysis of these tumors indicated that only one of these miRNAs, miR-203, was significantly silenced in murine T cell lymphomas (FDR < 0.001; Δ = 1.4; Figure 2B).

Since these murine T cell lymphomas lost expression of miR-203, despite maintaining a normal allele, we analyzed whether this miRNA could be silenced by epigenetic mechanisms. miR-203 presents a clear CpG island in its upstream chromosomal region flanking the transcriptional start site (TSS) similar to that present in many tumor suppressor genes (Figure 3A). As a control, we used miR-345, since it also presents a clear CpG island in its upstream chromosomal region and both are located in the chromosome 12 region deleted in these T cell malignancies. After bisulfite conversion of genomic DNA from these samples, we could not detect any methylation at the miR-345 CpG island, in agreement with previous results (Figure S1A available online). Similarly to miR-345, miR-203 was not methylated in normal lymphocytes. However, it was significantly methylated in seven out of eight murine T cell lymphoma tumor samples (Figure 3C). Detailed analysis by bisulfite sequencing indicated that most of the CpG dinucleotides in this CpG island were methylated in some tumors but not in normal lymphocytes (Figure 3C).

The chromosome 12 region identified in mouse T cell lymphomas is syntenic to a telomeric region in human chromosome 14. Interestingly, all microRNA clusters and genes located at this region in the mouse are conserved in the human genome. In fact, both the miR-203 pre-miR and its putative TSS are highly conserved between human and mouse genomes (Figure 3B). To test whether this epigenetic silencing of miR-203 was also present in human tumors, we analyzed four human T cell tumor cell lines (KARPAS-45, PEER, JURKAT and MOLT-4) and three additional primary peripheral T cell leukemias by methylation-specific PCR (MS-PCR) analysis. In all these human samples, miR-203 was dramatically hypermethylated as detected by MS-PCR (Figure S1B) and bisulfite sequencing (Figure 3D), whereas this sequence was not methylated in human normal T-lymphocytes, suggesting that miR-203 may control ABL1 levels in a variety of organisms.

To experimentally validate that miR-203 can target ABL1, we analyzed ABL1 protein levels in human T cell tumor cell lines after re-expression of miR-203. KARPAS-45 or PEER cells—both expressing GFP and miR-203. GFP-positive cells were isolated by cytometry and analyzed by immunoblot. As indicated in Figure 5, re-expression of miR-203 results in decelerated growth (doubling time of about 56 hr in both cell lines), compared to control cells infected with the empty retrovirus (doubling time of about 24 hr in both control cultures). This antiproliferative response is accompanied by a dramatic reduction (of about 70%) in ABL1 levels in these tumor lymphocytes in both KARPAS-45 and PEER cell lines (Figure 5 and Figure S4). Interestingly, the antiproliferative effect elicited by miR-203 is similar to that observed after expressing a pool of four different small-hairpin interfering RNAs specific for the human ABL1 gene (Figure S5B and S5A), suggesting a specific requirement for ABL1 in these T cell lymphomas.

miR-203 Directly Regulates ABL1 Expression

Computational prediction of both human and mouse miRNA targets (TargetScanS and PicTar [Rajewsky, 2006]) suggests that miR-203 can modulate more than 300 genes. To further select a functional miR-203 target, we performed massive mRNA expression analysis of mouse T cell lymphomas with silenced miR-203 to identify possible targets upregulated in these tumors. Twenty-two of the putative miR-203 targets are significantly overexpressed in these T cell lymphomas (Figure 4A), including Abt1, the murine homolog of ABL1, a known oncogene in hematopoietic malignancies (Ren, 2005). In fact, Abt1 is overexpressed in these T cell tumors as detected by protein analysis, indicating a correlation between Abt1 overexpression (Figure 4B) and loss of miR-203 in these primary tumors (Figure 4B and Figure S2). Both the murine and human 3’UTR of ABL1 genes contain miR-203 target sequences with a computed free energy of −26.8 kcal/mol and −21.0 kcal/mol, respectively (Figure 4C). This target site is well conserved in other vertebrates (Figure S3), indicating a correlation between Abl1 overexpression (Figure 4B) and loss of miR-203 in these primary tumors (Figure 4B and Figure S2).
Next, we subcloned the ABL1 3′UTR downstream of a luciferase reporter vector to analyze whether miR-203 directly targets ABL1 as predicted from the alignment. In 293 cells, miR-203, but not GFP, is able to reduce luciferase activity in this construct by 30% (Figure 5C) indicating that this microRNA may directly target the ABL1 3′UTR. This reduction is abolished by a 3′UTR that contains specific point mutations in the seed region of the miR-203 target sequence (Figure 5C), indicating that miR-203 can directly influence ABL1 protein levels through specific binding to its 3′UTR.

Specific Methylation of miR-203 in Philadelphia-Positive Human Leukemias

To further investigate the relevance of miR-203 in the oncogenic activity of ABL1, we next analyzed the epigenetic silencing of miR-203 in human tumors carrying a Philadelphia (Ph) chromosome. This alteration produces a BCR-ABL1 fusion protein that drives tumor development in several malignancies including some B cell acute lymphoblastic leukemias (B cell ALL) in children and chronic myelogenous leukemias (CMLs). As shown in Figure 6 and Figure S5 the upstream region of the human miR-203 is heavily hypermethylated in most Ph-positive tumors, including B cell ALLs and both primary CMLs and cultured CML cell lines. This methylation correlates with decrease expression in these Ph-positive tumors (Figure S2B). Interestingly, the miR-203 promoter region is not methylated in other hematopoietic tumors that do not carry ABL1 alterations. Thus, miR-203 is not significantly methylated in acute myelogenous leukemias (AMLs), chronic myeloproliferative diseases (CMPDs), or additional B cell ALLs that do not express ABL1 fusion proteins (Figure 6 and Figure S5). These results suggest a specific pressure to downregulate miR-203 in Ph-positive tumors (CMLs and some B cell ALL) or tumors that overexpress ABL1 (such as T cell lymphomas).

Modulation of ABL1 and BCR-ABL1 Expression by miR-203 and Epigenetic Drugs

Reintroduction of miR-203 in the CML cell lines K562 and KCL-22 results in restoration of miR-203 expression and concomitant reduction of ABL1 protein levels (Figure S2B). These cell lines also express the BCR-ABL1 fusion protein resulting from the Ph chromosome translocation. Importantly, re-expression of miR-203 also results in reduced BCR-ABL1 levels (Figure 7A and Figure S4B), likely due to the presence of the ABL1 3′UTR in the BCR-ABL1 fusion transcript. This reduction in
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Abl1 and BCR-ABL1 protein levels is accompanied by a dramatic decrease in the proliferation rate in these CML cells (Figure 7B). As in T cells, downregulation of ABL1 and BCR-ABL1 by specific shRNAs results in a similar proliferation arrest. This arrest is accompanied by apoptotic cell death induced by both miR-203 and, more dramatically, ABL1 shRNAs (Figure 7C). To directly analyze whether the translocated ABL1 is a critical target of miR-203 in this antiproliferative activity in CML cells, we exogenously expressed the p210 BCR-ABL1 fusion gene containing its endogenous 3′ UTR downstream of the cDNA. This protein has little effect on K562 or KCL-22 cells (about 10% increase in cell numbers 3 days after transfection; data not shown) but results in a significant rescue from miR-203-induced cell arrest (Figure 7D). As this exogenous BCR-ABL1-3′ UTR construct contains the miR-203 target site, it probably competes with the endogenous BCR-ABL1 transcript, reducing the titer of miR-203. We also tested two additional BCR-ABL1 translocation variants (p210 and p190) devoid of their 3′ UTR and therefore not containing miR-203 target sites. As shown in Figure 7D, expression of these miR-203-resistant BCR-ABL1 cDNAs in K562 and KCL-22 tumor cells fully overcomes the antiproliferative effect of miR-203, resulting in a significant increase in cell proliferation.

Finally, since promoter methylation can be reversed using specific epigenetic drugs, we treated two T cell leukemias, KARPAS-45 and PEER, and two Ph-positive CML cell lines, K562 and KCL-22, with 5′-azacytidine (Aza), 4-phenylbutyrate (PBA), or a mixture of both drugs. Whereas single treatments with Aza alone or PBA alone have a minor effect on miR-203 demethylation in all these cell lines (Figures S6 and S7), the combined treatment results in a partial but efficient demethylation of the miR-203 upstream region both in T cell leukemias (Figure S6) and CML cell lines (Figure 8). This combined treatment restores miR-203 expression, and re-expression of this miRNA strongly correlates with a significant reduction in both ABL1 and BCR-ABL1 protein levels (Figure 8 and Figures S6 and S7). These results suggest that epigenetic drugs, in addition to re-expressing other methylated cDNAs, can result in oncogene downregulation mediated by the chemical restoration of miRNA function.

**DISCUSSION**

It has been recently reported that miRNA loci are frequently located at fragile sites and suffer frequent genomic alterations in human cancer (Calin et al., 2004; Zhang et al., 2006). As we have described here, loss of heterozygosity (LOH) is relatively frequent in a 7 Mb region surrounding the callipyge locus in γ-radiation-induced T cell lymphomas, a model of neoplasia that resembles human peripheral T cell lymphomas (Melendez et al., 2003). This region, located at chromosome 12 in mice and chromosome 14 in humans, contains some imprinted genes involved in the regulation of muscle and fat biology (Georges et al., 2003). Apart from these genes, this region contains a surprisingly high density of miRNAs, including about 12% of the known mammalian miRNome. Most of these miRNAs are clustered into several transcripts, including six miRNAs at the anti-Peg11 transcript that participate in the trans regulation of the Rtl1/Peg11 gene (Davis et al., 2005). About 30 additional miRNAs are expressed in about four different transcripts downstream of the callipyge region, although all of these transcripts seem to be maternally expressed and controlled by genomic imprinting (Seitz et al., 2003, 2004). In fact, normal lymphocytes display diverse methylation of a CpG island flanking miR-134, likely as a consequence of the genomic imprinting at this locus (M.J.B. and M.M., unpublished data). The presence of this large number of miRNAs might confer specific susceptibility to chromosomal breaks, and large miRNA clusters have been previously suggested to produce fragile sites (Calin et al., 2004).

Among the miRNAs encoded in this area, miR-203 (located about 2 Mb downstream of the callipyge region) is the only sequence significantly downregulated in these T cell lymphomas. Recent results have indicated that some other microRNAs
in this area, such as miR-127, are also deregulated in human tumors by promoter methylation (Saito et al., 2006). We have demonstrated in this report that specific deletions in this region are selected in T cell tumor development where miR-203 is specifically silenced by epigenetic mechanisms. Thus, genetic and epigenetic mechanisms coordinately inactivate some specific miRNAs such as miR-203 in these neoplasias.

Expression of miR-203 seems to be restricted to specific cell types. Thus, miR-203 is significantly expressed in the epithemis but not in the hair follicles of the skin (Yi et al., 2006). In these cells, conditional deletion of the miRNA processing enzyme Dicer results in decreased expression of miR-203 along with other miRNAs and provokes developmental perturbations in epidermal organization (Yi et al., 2006). miR-203 is not expressed either in primary fibroblasts or in many other established cell lines (M.J.B. and M.M., unpublished data). Moreover, inhibition of miR-203 expression induces increased cell proliferation (Cheng et al., 2005), suggesting an antiproliferative function for this miRNA.

Bioinformatics analyses predict that conserved vertebrate miRNAs target more than 100 to 400 regulatory genes. Among the putative targets of miR-203, we have validated ABL1 as a relevant target, at least in hematopoietic cells. ABL1 is a nonreceptor tyrosine kinase that is expressed in most tissues and participates in the transduction of signals from cell-surface growth factor and adhesion receptors to regulate cytoskeleton structure (Ren, 2005). ABL1 is involved in the development of the hematopoietic system, and its overexpression is associated with the development of diverse hematopoietic malignancies (Lin et al., 2006; Ren, 2005). ABL1 plays an important role in T cell signaling (Zipfel et al., 2004), mediates resistance to apoptosis in T-lymphocytes (Fuchs et al., 1995), and it is overexpressed in T cell tumors, in some cases as a result of chromosomal translocations with amplification as reported in the NUP214-ABL1 fusion (Graux et al., 2004, 2006). As reported in this article, RNAi-mediated downregulation of ABL1 in T cell leukemia cells reduces proliferation of these tumor cultures (Figure 5), suggesting putative therapeutic uses of ABL1 inhibitors in these malignancies.

The BCR-ABL1 fusion is a landmark of CML and it is also present in a percentage of B cell ALL with poor prognosis. All of these diseases, but not other Ph-negative leukemias, are accompanied by promoter methylation and decreased expression of miR-203 (Figures 6 and Supplemental Data), suggesting a proliferative advantage in silencing miR-203 in these tumor cells. It is also interesting to note that the 14q32 region of human chromosome 14 where this miRNA resides is frequently lost in lymphoid blast crisis of CML patients (Dastugue et al., 1986; Sercan et al., 2000). It is therefore tempting to speculate that acute transformation and progression of the disease may be accompanied by inactivation of tumor suppressor genes in this region, including miRNAs such as miR-203.

The recent success of some ABL1-targeted small-molecule kinase inhibitors in CML and other diseases (Baselga, 2006; Ren, 2005) has suggested that inhibition of ABL is critical for tumor treatment in these patients. In fact, CML patients with decreased levels of BCR-ABL1 transcripts have a significantly lower risk of disease progression (Druker et al., 2006). The fact that some miRNAs such as miR-203 can modulate ABL1 levels suggests that restoration of the expression of these miRNA can be beneficial to ALL or CML patients carrying the NUP214-ABL1 or BCR-ABL1 fusions. In addition, T cell lymphoma/leukemia patients with high ABL1 protein levels could also benefit from these treatments. Kinase inhibitors such as imatinib (Gleevec) do not completely eradicate ABL1- or BCR-ABL1-expressing cells from the body, and resistance in some cases emerges as a consequence of point mutations that render resistant isoforms of ABL1 or by amplification and overexpression of the BCR-ABL1 fusion (Shannon, 2002). Since the oncogenic transcripts in these translocations contain the target site for miR-203, our results suggest that restoration of miRNA function
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Cancer Cell


Figure 6. Specific Methylation of miR-203 in Philadelphia-Positive Human Leukemias

Methylation map of the human miR-203 upstream region in Philadelphia-positive (Ph+) tumors, including chronic myelogenous leukemias (CML) and CML tumor cell lines, and B cell acute lymphoblastic leukemias (B cell ALL). Additional leukemias that do not contain rearranged ABL1 loci such as Philadelphia-negative (Ph−) B cell ALL, acute myeloid leukemias (AML) and other chronic myeloproliferative diseases (CMPD) are also shown. In these Ph− tumors, miR-203 is not significantly methylated. Three representative tumor samples are shown for each group, and three independent clones were sequenced per tumor sample. Normal blood cells were used as a control (NBC).

might provide some beneficial effects for imatinib-resistant patients. Our data are also in agreement with the finding that epigenetic drugs such as decitabine display some therapeutic benefits, even in patients resistant to imatinib (Issa et al., 2005).

EXPERIMENTAL PROCEDURES

Mouse Colony and Induction of Tumors

C57BL/6J and RF/J F1 hybrid mice and pure C57BL/6J animals were maintained in our animal facilities following the appropriate ethical recommendations of our institutions. For tumor induction, 4-week-old mice of both sexes were exposed to four weekly doses of 1.75 Gy/dose of ionizing gamma radiation from our institutions. For tumor induction, 4-week-old mice of both sexes were exposed to four weekly doses of 1.75 Gy/dose of ionizing gamma radiation from our institutions. For tumor induction, 4-week-old mice of both sexes were exposed to four weekly doses of 1.75 Gy/dose of ionizing gamma radiation from our institutions. For tumor induction, 4-week-old mice of both sexes were exposed to four weekly doses of 1.75 Gy/dose of ionizing gamma radiation from our institutions.

Acute lymphoblastic leukemia (Ph−)

B-ALL primary tumors

Acute lymphoblastic leukemia (Ph−)

B-ALL primary tumors

Acute myeloid leukemia (Ph−)

AML primary tumors

Chronic myeloproliferative disease (Ph−)

CMPD primary tumors

LOH Analysis

LOH analysis was carried out by comparing electrophoresis patterns of tumor and control DNA. The following chromosome 12 markers were used according to sequences deposited in the Gene Expression Omnibus (GEO) database under accession number GSE10858.

MicroRNA and cDNA Expression Analysis

MicroRNA expression profiles were performed essentially as described previously (Liu et al., 2004). Briefly, tumor RNA was isolated using Trizol (Invitrogen), dissolved in an equal volume of isopropanol and dissolved in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA).

Comparative Genome Hybridization

For DNA preparation thymic lymphomas and normal tissues were cut, minced in small pieces, and placed in a microfuge tube containing 0.5 ml lysis buffer (20 mM Tris-HCL [pH 8.0], 25 mM EDTA, 0.5% SDS, 0.1 M NaCl). Proteinase K was added at a final concentration of 100 mg/ml and incubated at 55°C overnight. The lysate was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) (GIBCO-BRL). DNA was precipitated by addition of an equal volume of isopropanol and dissolved in TE buffer (10 mM Tris-HCl [pH 8.0]) and 1 mM EDTA).

About 4000 mouse BAC clones with an average spacing of 1 Mb across mouse chromosomes 1 to 19 and the X chromosome (Chung et al., 2004) were printed on hydrophobic glass slide surfaces using an Omni Grid 100 array printer (BioRobotics). The average spacing between the linear map positions of this 3K collection of BAC clones (calculated as the distance between the midpoint positions of two consecutive BACs) is about 0.9 Mb (Chung et al., 2004). For comparative genome hybridization on these BAC microarrays, 1 μg of tumor or normal DNA was first amplified and labeled with aminoallyl-dUTP (Sigma, St Louis, USA) using the Bioprime random priming kit (GIBCO) and then further labeled with Cy3 or Cy5 mono-reactive NHS esters (Amersham, Piscataway, NJ). DNA samples from six nontreated C57BL/6J mice were pooled and used as a normal control. Tumor samples from male animals were hybridized to female pools and vice versa to have an internal control on DNA gains and losses in chromosomes X and Y. In all, 1 μg of labeled tumor DNA and an equal amount of control DNA labeled differentially were mixed with 200 μg mouse Cot I DNA (GIBCO), dissolved in a formamide-based hybridization buffer containing 50% formamide, 2× SSC, 2% SDS, and 10% dextran sulfate, heat denatured and annealed at 37°C to block repeats, and then hybridized to BAC arrays. Hybridization was carried out in a shaker incubator at 37°C for 16–20 hr. Arrays were washed in 2× SSC, 0.1% SDS at 45°C for 60 min and immediately scanned after rinsing with deionized water. Array images were quantified in a Laser Microarray Scanner G2565BA (Agilent). Array data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE10858.
and 5 μg of RNA was used for hybridization. MicroRNA microarrays have been described previously (Volinia et al., 2006). These microarrays were hybridized in 6x SSPE (0.9 M NaCl/60 mM NaH$_2$PO$_4$/H$_2$O/8 mM EDTA [pH 7.4]/30% formamide at 25°C for 18 hr, washed in 0.75x TNT (Tris·HCl/NaCl/Tween 20) at 37°C for 40 min, and processed by using a method of direct detection of the biotin-containing transcripts by streptavidin-Alexa Fluor 647 conjugate. Processed slides were scanned using a microarray scanner, with the laser set to 635 nm, at fixed PMT setting, and a scan resolution of 10 mm. The SAM (Statistical Analysis of Microarray) tool included in the TM4 package (http://www.tm4.org/mev.html) was used to analyze the statistical significance of microRNA expression data. MicroRNA array data have been deposited in the GEO database under accession number GSE10891.

Figure 7. Restoration of miR-203 Expression in Ph-Positive Tumor Cells Downregulates ABL1 and BCR-ABL1
(A) K562 cells were transfected with GFP or miR-203 vectors. Overexpression of miR-203 results in decreased ABL1 and BCR-ABL1 protein levels as detected by immunoblotting. miR-203 transcript levels were quantified by real-time quantitative PCR and normalized versus the expression levels in non-transfected K562 cells (relative value = 1). α-tubulin was used as a protein loading control. ABL1, BCR-ABL1, and α-tubulin signals were quantified, and their ratios were normalized to BCR-ABL1 protein levels in GFP-expressing cells.
(B) Growth rate of K562 and KCL-22 cells expressing miR-203 (filled circles), a pool of four shRNAs against ABL1 (gray circles), or the empty vector (open circles).
(C) Apoptotic cell death after transfection with miR-203 or ABL1 shRNAs in K562 or KCL-22 cell lines. These vectors were cotransfected with a GFP-expressing plasmid, and apoptosis was monitored with Annexin V staining 5 days after transfection.
(D) K562 or KCL-22 cells were transfected with an empty vector, a vector expressing miR-203, or a combination of vectors expressing miR-203 and BCR-ABL1-3'UTR construct or miR-203 and the p210 or p190 forms of BCR-ABL1 that do not contain the endogenous 3'UTR. The overexpression of BCR-ABL1-3'UTR partially rescues the effect of miR-203, whereas the exogenous expression of miR-203-resistant BCR-ABL1 cDNAs fully rescues miR-203-mediated cell proliferation arrest. Three days after transfection, the number of cells was scored and normalized to the original number of cells transfected (fold increase). Error bars represent SD.

Figure 8. Restoration of miR-203 Expression and Downregulation of BCR and BCR-ABL1 by Epigenetic Drugs
(A) The CML cell lines K562 and KCL-22 were treated with the epigenetic drugs Aza + PBA, resulting in significant demethylation of the miR-203 upstream region. Three independent clones were sequenced per treated cell line.
(B) Upregulation of miR-203 and concomitant downregulation of ABL1 and BCR-ABL1 protein levels after 5-aza + PBA treatment in K562 and KCL-22 cells. miR-203 transcript levels were quantified by real-time quantitative PCR and normalized versus the expression levels in nontreated K562 or KCL-22 cells (relative value in untreated cells = 1). ABL1, BCR-ABL1, and α-tubulin signals were quantified, and their ratios were normalized to BCR-ABL1 protein levels in nontreated cells. Histograms in (B) represent mean ± SD.

The quality of the RNA was evaluated using the BioAnalyzer system (Agilent). Twenty-five micrograms of the test or reference amplified RNAs were labeled with fluorescent Cy5 and Cy3 (Amhersam, Sunnyvale), respectively, using the SuperScript II RNase H Reverse Transcriptase Kit (Invitrogen, USA).
Utilization of specific transcripts, total RNA from tumors and cell lines was isolated using TriZol (Invitrogen). Expression of miR-203 in mouse tumors was analyzed by northern (NorthernMax, Ambion) or RNA protection (mirVana, Ambion) assays using a miR-203-specific probe (see Supplemental Experimental Procedures). 6UsnRNA and miR-16-5p were used as controls in northern hybridizations and RNA protection assays, respectively. The expression levels of individual microRNAs were quantified using the mirVana qRT-PCR miRNA detection kit following the manufacturer’s recommendations (Ambion). GAPDH served as normalization control. In addition, real-time quantitative amplification of miR-203 was performed in triplicate with the TaqMan MicroRNA assays kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions in an Applied Biosystems 7900HT Fast Real-Time PCR apparatus. Amplification of RNU19 was used for normalization. The data analysis was done using the SDS (Sequence Detection Systems) 2.2.2 program (Applied Biosystems, Foster City, CA).

DNA Methylation Analysis

The following human cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ): JURKAT, MOLT-4, KARPAS-45, and PEER (T cell acute lymphoblastic leukemia; T cell ALL); K562, KCL22, and EM-2 (CMLs). Primary human T cell ALLs (S0171, S020201, and 220T), CMLs (51226, 60514, 60515, 07CG1139, 07CG1187, 07CG1212, 07CG1257, and 08BG0061), B cell ALLs (06CG0664, 06CG0630, 60344, 07CG0144, 07CG0178, 07CG0223, 07CG0267, 07CG0331, 07CG0354, 07CG0356, 07CG0407, 07CG0674, 07CG0689, 07CG0750/0806, 07CG0751, 07CG0758, 07CG0912, 07CG0956, 07CG0995, 07CG1001, 07CG1035, 07CG1092, 07CG1214, 08CG0100, and 08CG0115), acute myelogenous leukemias (AMLs: 07BL1509, 07CG1192, and 07CG1198), or chronic myeloproliferative diseases (CMPDs: 07CG1165, 07CG1155, and 07CG1197) were obtained from our repository or were kindly provided by J. Benitez, R. Villuendas, or M.A. Piris (CNIO, Madrid). Leukemic bone marrow samples were obtained by the Cytogenetics Laboratory at our Center under our approved protocol that complies with the recommendations included in the Spanish Law for Clinical Diagnostic Laboratories. All used samples had previously informed consent, corresponded to the remaining exceeding material after diagnosis, and were anonymously surveyed. The Supplemental Data include Supplemental Experimental Procedures, and Isidro Sánchez-García, CIC-Salamanca) and with a pBabe-puro-mycin vector expressing miR-203 under a nucleotideffect. Cells were maintained in puromycin selection during 5 days, and then EGFP-positive cells isolated by FACS were used to determine the cell growth rate. For RNA interference, four different vectors that express small-hairpin RNAs against the human ABL1 were used (see Supplemental Experimental Procedures and Table S2). Apoptotic cell death was monitored using an Annexin V staining kit (PharMingen BD Biosciences) following the manufacturer’s recommendations.

Protein Analysis and Luciferase Assays

Protein lysates were obtained from tissues or cells using RIPA Cell Lysis buffer (150 mM NaCl, 50 mM [pH 8] Tris HCl, 0.5% deoxycholate sodium, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA) and the recommended amounts of Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail 2 (Sigma, St. Louis, MO), and Benzonase (Novagen, Darmstadt, Germany). Samples were spun at 20,000 × g at 4°C for 15 min, and the supernatant was stored at −80°C or immediately quantified using a protein assay (Bio-Rad). Seventy micrograms of protein lysates were loaded onto 8% polyacrylamide-SDS gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with ABL1 antibodies (Calbiochem, Darmstadt, Germany). In addition, anti-α-tubulin antibody (Sigma, St. Louis, MO) was used as a loading control. After washing, blots were incubated with the appropriate secondary antibodies coupled to Alexa Fluor 680 or 800 (Invitrogen). Subsequently, the membrane was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Luciferase constructs were made after amplification of the ABL1 3′UTR and subcloning in a modified pGL3-Control vector (Promega). 293 or HeLa cells were transfected with 0.3 μg of firefly luciferase reporter vector containing the ABL1 3′UTR and 0.3 μg of the control vector containing Renilla luciferase pRL-CMV (Promega), using Effectene (Qiagen). Three micrograms of pmCMV-203 or the empty vector were used to analyze the effect of microRNA expression on luciferase signal. Luciferase assays were performed 48 hr after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase was normalized to Renilla luciferase activity.

ACCESSION NUMBERS

Array data have been deposited in the Gene Expression Omnibus ( GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE10858. MicroRNA array data have been deposited in the GEO database under accession number GSE10891. Microarray data have been deposited in the GEO database under accession number GSE10859, and the entire set of T cell lymphoma arrays can be accessed under GSE10861.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, seven supplemental figures, and two supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/6/496/DC1/.
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REFERENCES


