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miR-200c/Bmi1 axis and epithelial–mesenchymal transition contribute to acquired resistance to BRAF inhibitor treatment

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Summary

Resistance to BRAF inhibitors (BRAFi) is one of the major challenges for targeted therapies for BRAF-mutant melanomas. However, little is known about the role of microRNAs in conferring BRAFi resistance. Herein, we demonstrate that miR-200c expression is significantly reduced whereas miR-200c target genes including Bmi1, Zeb2, Tubb3, ABCG5, and MDR1 are significantly increased in melanomas that acquired BRAFi resistance compared to pretreatment tumor biopsies. Similar changes were observed in BRAFi-resistant melanoma cell lines.

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Author contributions XX and SL designed the experiments; SL, MTT, TW, GZ, and XX wrote the manuscript; SL, RY, YY, and LX performed the experiments; CK, MX, and MB generated the PDX and RPDX mouse model; WX, GK, LS, LZ, and RKA collected and organized all the clinical data; GZ, WX, ZW, and MH performed the bioinformatics analysis. All authors commented on the study and revised manuscript.

Conflict of interest The authors declare no conflict of interest.

Overexpression of miR-200c or knock-down of Bmi1 in resistant melanoma cells restores their sensitivities to BRAFi, leading to deactivation of the PI3K/AKT and MAPK signaling cascades, and acquisition of epithelial–mesenchymal transition-like phenotypes, including upregulation of E-cadherin, downregulation of N-cadherin, and ABCG5 and MDR1 expression. Conversely, knock-down of miR-200c or overexpression of Bmi1 in BRAFi-sensitive melanoma cells activates the PI3K/AKT and MAPK pathways, upregulates N-cadherin, ABCG5, and MDR1 expression, and downregulates E-cadherin expression, leading to BRAFi resistance. Together, our data identify miR-200c as a critical signaling node in BRAFi-resistant melanomas impacting the MAPK and PI3K/AKT pathways, suggesting miR-200c as a potential therapeutic target for overcoming acquired BRAFi resistance.

Keywords

melanoma; BRAF inhibitor; miR-200c; Bmi1; epithelial–mesenchymal transition

Introduction

The identification of the oncogenic mutation in BRAF^{V600E} (Davies et al., 2002) is one of the most pivotal advances in our understanding of the biology of melanoma in recent years. The BRAF^{V600E} mutation results in constitutive activation of the mitogen-activated protein kinase (MAPK) signaling cascade. The prevalence of the BRAF^{V600E} mutation in cutaneous melanomas (approximately 50–70%) led to the design of targeted anti-BRAF^{V600E} therapies such as vemurafenib and dabrafenib. These anti-BRAF^{V600E} therapies have led to improvements in the survival of patients with melanomas harboring the BRAF^{V600E} mutation (Flaherty et al., 2010). However, most patients treated with BRAF inhibitors (BRAFi) develop resistance to these therapies after only several months (Flaherty et al., 2012; Franco et al., 2011; Sullivan and Flaherty, 2013). A number of BRAFi-resistant mechanisms have been identified, including reactivation of the MAPK pathway through secondarily acquired activating mutations in MEK1/MEK2 and N-RAS (Nazarian et al., 2010); alternative splice variants of BRAF^{V600E} itself (Poulikakos et al., 2011), or activation of the PI3K pathway through loss of PTEN or activation of receptor tyrosine kinases (Dankort et al., 2009; Villanueva et al., 2010). Melanomas also employ more general mechanisms of drug resistance common to many types of cancers, besides the BRAFi-specific mechanisms of resistance, including changes directed at reducing intra-cellular drug accumulation during the acquisition of chemotherapy resistance (Gottesman, 2002; Gottesman et al., 2002; La Porta, 2009), such as upregulation of members of the ATP-binding cassette (ABC) transporters – a superfamily of transmembrane proteins that transport a variety of substrates across biological membranes in an ATP-dependent fashion (Chen and Tiwari, 2011).

Despite a wealth of studies describing the critical roles of miRNAs in melanoma development, progression, and metastasis, relatively few studies have explored the roles of miRNAs in mediating resistance to antimelanoma therapy (Luo et al., 2014). There is emerging evidence that the dysregulation of microRNA expression contributes to the acquisition of drug resistance by cancer cells (Ma et al., 2010). In our previous study, we

have shown a progressive reduction in the expression of miR-200c in primary and metastatic melanomas and melanoma cells with lower miR-200c expression is more resistant to PLX4720 in vitro (Liu et al., 2012). However, the mechanism underlying miR-200c-induced BRAFi resistance is unknown.

Recent studies demonstrate an intricate relationship between chemotherapy resistance and changes associated with the epithelial to mesenchymal transition (EMT; Wang et al., 2010). Epithelial to mesenchymal transition encompasses a series of morphological and biochemical changes, in part related to the expression profile of cell adhesion molecules E-cadherin and N-cadherin. Tumor cells with an 'epithelial' phenotype express high E-cadherin and low N-cadherin, while loss of E-cadherin and increased N-cadherin expression heralds the 'mesenchymal' phenotype as the cell loses polarity, adapts a spindled morphology together, and acquires an enhanced migratory capacity to produce a more invasive tumor phenotype (Gregory et al., 2008; Hsu et al., 2004; Shimono et al., 2009; Tucci et al., 2007). Epithelial to mesenchymal transition is controlled by a complex signaling network involving numerous transcriptional repressors, including SNAIL, ZEB (Thiery, 2002; Thiery and Sleeman, 2006) and Bmi1 (Guo et al., 2011; Song et al., 2009), which repress E-cadherin expression. Many of the transcription repressors directing EMT have also been shown to regulate ABC transporter expression, establishing a mechanistic link between EMT and the development of a drug resistance phenotype (Saxena et al., 2011).

miR-200 family (including miR-200a, miR-200b, miR-200c, and miR-141) regulates epithelial to mesenchymal cell transition (EMT) and chemotherapy resistance (Wang et al., 2010). The miR-200c miRNA directly represses ZEB1/ZEB2 (Dykxhoorn et al., 2009; Gregory et al., 2008; Park et al., 2008) and Bmi1 (Shimono et al., 2009; Wellner et al., 2009), resulting in increased expression of E-cadherin. Epithelial to mesenchymal transition is controlled by a complex signaling network involving a number of transcription repressors, including SNAIL, ZEB (Thiery, 2002; Thiery and Sleeman, 2006), and Bmi1 (Guo et al., 2011; Song et al., 2009), which repress E-cadherin expression. Many of the transcriptional repressors involved in directing EMT have also been shown to regulate ABC transporter expression. These findings establish a link between EMT and development of drug resistance (Saxena et al., 2011). Nevertheless, the role of EMT in acquired melanoma BRAFi resistance has not been explored.

Here, we demonstrate the clinical significance of the miR-200c/Bmi1 axis in conferring acquired resistance to BRAFi therapy. We show that loss of miR-200c expression not only correlates with the development of resistance to BRAFi therapy in clinical samples of melanomas and BRAFi-resistant cell lines, but also is sufficient to generate a BRAFi-resistant phenotype in melanoma cells. These results highlight a new signaling network that includes miR-200c, Bmi1, and EMT that plays a critical role in mediating sensitivity to BRAFi.

Results

Reduction of miR-200c expression in BRAFi-resistant patient-derived xenografts and clinical melanoma tissues

To investigate the correlation between miR-200c expression and melanoma BRAFi resistance, we first examined miR-200c expression in patient-derived xenografts (PDXs) (tumor biopsies derived from BRAFi-naïve melanoma patients) and resistant PDXs (RPDXs) (tumor biopsies derived from progressive melanomas after BRAFi treatment) mouse models. We found that there was a significantly lower miR-200c expression in six RPDX mouse models compared to that in four PDX mouse models (Figure 1A).

Next, we determined the expression of miR-200c using paired pretreatment and post-treatment tumor biopsies from fourteen patients who were treated with vemurafenib and experienced tumor progression. Realtime PCR analysis demonstrated a striking reduction in miR-200c expression in post-treatment tumor biopsies compared with pretreatment tumor biopsies (Figure 1B).

We further interrogated the mRNA expression pattern of miR-200c target genes using gene expression microarrays of paired pretreatment and post-treatment tumor biopsies (GEO accession number GSE50509). The bioinformatics analysis showed that expression of miR-200c target genes, *Bmi1* (Figure 1C), *Zeb2*, and *Tubb3* (Figure 1D,E) is significantly increased in post-treatment tumor biopsies compared to that in paired pretreatment tumor biopsies.

Bmi1 has been shown to be a direct target of miR-200c (Shimono et al., 2009; Wellner et al., 2009). *Bmi1* expression inversely correlated with miR-200c expression in BRAFi post-treatment tumor biopsies (Figure 1C,F). Because miR-200 family members are also well-known regulators of EMT, we examined the relative expression of *E-cadherin*, *N-cadherin*, and *SNAIL* using melanoma tumor biopsies. The results showed a reduction in *E-cadherin* expression and an increase in both *N-cadherin* and *SNAIL* expression in post-treatment tumor biopsies (Figure 1G).

Resistance to BRAFi is associated with reduction in miR-200c expression

We utilized two induced BRAFi-resistant melanoma cell lines, Mel1617BR and 451LuBR, to investigate the potential roles of decreased miR-200c in acquired drug resistance to BRAFi. In comparison with their parental cell lines (Mel1617 and 451Lu), Mel1617BR and 451LuBR exhibited resistance to increasing concentrations of PLX4720, an analog of vemurafenib (Figure 2A,B) and there was a 17- and 40-fold reduction of miR-200c expression in Mel1617BR and 451LuBR cells, respectively (Figure 2C). In both resistant cell lines, there was a significant decrease in the expression of *E-cadherin* and an increase in both *N-cadherin* and *SNAIL* at mRNA and protein levels (Figure 2D,E), compared to their parental cell lines.

Bmi1 is known to activate the phosphatidylinositol 3- kinase/protein kinase B (PI3K/AKT) signaling pathway (She et al., 2008; Wu et al., 2011). Indeed, we found increased levels of p-AKT in both Mel1617BR and 451LuBR cell lines compared to their parental cell lines

(Figure 2F) without alterations in the overall levels of AKT protein. We have previously shown that ABC transporter genes are miR-200c targets. We determined whether the expression of ABC transporter genes is correlated with acquired drug resistance to BRAFi. Indeed, both Mel1617BR and 451LuBR cell lines exhibited increased ABCG5 and MDR1 at mRNA and protein levels compared to their parental cell lines (Figure 2D,E). Together, our data demonstrate that resistant cells exhibit perturbations in a signaling network that is regulated by miR-200c.

Overexpression of miR-200c overcomes BRAFi-resistant phenotypes

We examined whether acquired BRAFi resistance depends on miR-200c because miR-200c appears to be a nexus point that governs the activity of multiple signaling pathways in melanomas. We infected 451LuBR cells with a lentivirus-overexpressing miR-200c and overexpression of miR-200c was confirmed by qRT-PCR (Figure 3A, left panel). Overexpression of miR-200c restored the sensitivity of 451LuBR cells to BRAFi to the level that is comparable of 451Lu parental cell line (Figure 3A, right panel). Furthermore, overexpression of miR-200c in 451LuBR cells resulted in a marked reduction in *Bmi1* mRNA (Figure 3C, top panel) and protein expression compared to that of control 451LuBR cells (Figure 3C). Restoration of sensitivity to BRAFi in these cells was accompanied with diminished phospho-AKT and phospho-ERK (Figure 3C). In addition, overexpression of miR-200c in 451LuBR cells resulted in a significant increase in the expression of E-cadherin at both mRNA (Figure 3B) and protein levels (Figure 3D) and a decrease in the expression of N-cadherin and SNAIL (Figure 3B,D). Overexpression of miR-200c also significantly reduced the expression of ABCG5 and MDR1 (Figure 3B,D).

Knock-down of miR-200c confers BRAFi resistance

To further establish a casual role of reduced miR-200c expression in BRAFi resistance, we knocked down miR-200c in parental 451Lu cells. Decreased miR-200c expression was confirmed by qRT-PCR (Figure 3E, left panel). Downregulation of miR-200c in 451Lu cells decreased melanoma sensitivity to BRAFi (Figure 3E, right panel), and this effect is correlated with the increase in the expression of N-cadherin, SNAIL, ABCG5, and MDR1 and the decrease in E-cadherin at both mRNA and protein expression (Figure 3F,G). Moreover, we also observed an increase in the expression of *Bmi1* and phosphorylation of AKT and ERK (Figure 3H).

To investigate whether similar results can be observed in other melanoma cell lines, we introduced anti-miR-200c shRNA constructs into two BRAF^{V600E} melanoma cell lines, WM35 and WM793 cells (Paraiso et al., 2011). Reduction of miR-200c expression was confirmed by qRT-PCR (Figures S1A and S2A). Antagonizing miR-200c reduced susceptibility of WM35 cells (Figure S1B) and WM739 cells (Figure S2B) to BRAFi. Anti-miR-200c resulted in a significant increase in the expression of N-cadherin, SNAIL, ABCG5, and MDR1 (Figures S1C,D and S2C,D) and diminished the expression of E-cadherin at both mRNA (Figures S1C and S2C) and protein levels (Figures S1D and S2D). Finally, antagonizing miR-200c led to an increase in the expression of *Bmi1*, as well as the phosphorylation of AKT and ERK levels in these cells (Figures S1E and S2E).

Bmi1 is required for miR-200c-mediated BRAFi resistance

We next asked whether the effects of miR-200c overexpression in 451LuBR were dependent on Bmi1. *Bmi1* was introduced into miR-200c-overexpressing 451LuBR cells using a lentiviral expression vector. Increased Bmi1 mRNA and protein levels were confirmed by quantitative RT-PCR and Western blot analysis, respectively (Figure 4A,E). Bmi1 overexpression counteracted the effect of miR-200c overexpression in melanoma cells (Figure 4B). Bmi1 overexpression similarly reversed miR-200c-induced reduction of N-cadherin, SNAIL, ABCG5, and MDR1 mRNA (Figure 4C) and protein expression (Figure 4D) and a reversal of miR-200c-induced upregulation of E-cadherin mRNA (Figure 4C) and protein expression (Figure 4D). Finally, overexpression of *Bmi1* in miR-200c-overexpressing 451LuBR cells restored AKT and ERK phosphorylation (Figure 4E).

We then asked whether reduction of Bmi1 would exert effects similar to those seen after miR-200c overexpression in BRAFi resistance. We knocked down Bmi1 in 451LuBR cells using a shRNA construct (Onder et al., 2012). Bmi1 knock-down led to both decreased Bmi1 mRNA and protein expression (Figure 5A,E). Bmi1 knock-down in 451LuBR cells recapitulated the effects of miR-200c overexpression. Bmi1 knock-down led to increased sensitivity to varying concentrations of PLX4720 (Figure 5B), downregulation of N-cadherin, SNAIL, ABCG5, and MDR1 mRNA (Figure 5C) and protein expression (Figure 5D), upregulation of E-cadherin mRNA (Figure 5C) and protein expression (Figure 5D), as well as diminished phosphorylation of AKT and ERK (Figure 5E).

Bmi1 mediates the effects of miR-200c on BRAFi resistance

To investigate whether the miR-200c is mediated through Bmi1, we introduced *Bmi1* into 451Lu parental cells. Increased expression of Bmi1 mRNA and protein was confirmed by qRT-PCR and Western blot analysis, respectively (Figure 6A,E). The forced expression of Bmi1 reduced the sensitivity of 451Lu cells to BRAFi (Figure 6B). The decrease in sensitivity of 451Lu cells was correlated with a reversal of expression of N-cadherin, SNAIL, ABCG5, and MDR1 at both mRNA (Figure 6C) and protein levels (Figure 6D). Furthermore, Bmi1 overexpression led to the decrease in E-cadherin at both mRNA (Figure 6C) and protein expression (Figure 6D). In addition, overexpression of Bmi1 in 451Lu parental cells resulted in a significant increase in the phosphorylation of AKT and ERK (Figure 6E). We then overexpressed Bmi1 in WM35 and WM793 melanoma cell lines. Increased expression of Bmi1 mRNA and protein was confirmed by qRT-PCR and Western blot analysis, respectively (Figures S1I and S2I). Overexpression of Bmi1 increased WM35 and WM793 resistance to BRAFi (Figures S1F and S2F). Overexpression of Bmi1 resulted in an increased expression of N-cadherin, SNAIL, ABCG5, and MDR1 mRNA (Figures S1G and S2G) and protein expression in WM35 and WM793 cells (Figures S1H and S2H). This increase in BRAFi resistance correlated with diminished expression of E-cadherin mRNA (Figures S1G and S2G) and protein (Figures S1H and S2H). Finally, overexpression of Bmi1 led to a significant increase in both AKT and ERK1 phosphorylation in WM35 cells (Figure S1I) and WM793 cells (Figure S2I).

Discussion

Here, we show that expression of miR-200c is diminished in acquired BRAFi-resistant melanoma patient tissues. The reduction of miR-200c induces expression of Bmi1, which in turn leads to the acquisition of features seen in EMT, upregulation of ABC transporter expression, and activation of the PI3K and MAPK signaling cascades. Restoration of miR-200c expression in BRAFi-resistant melanoma cells is sufficient to overcome the resistant phenotype through a Bmi1-dependent mechanism. Furthermore, knock-down of miR-200c or overexpression of Bmi1 in parental melanoma cell lines resulted in the induction of a BRAFi-resistant phenotype. These results identify miR-200c as a crucial regulator for the acquisition of BRAFi resistance in melanoma and a pivotal relationship between miR-200c, Bmi1, EMT, and ABC transporters – the balance of which is likely essential to the preservation of sensitivity to antineoplastic agents.

Bmi1 is a member of the Polycomb group of proteins, which regulate a wide spectrum of signaling pathways in many types of cancers. Bmi1 overexpression has been demonstrated in numerous human cancers (Jacobs and Van Lohuizen, 2002; Piunti et al., 2014), including melanoma (Mihic-Probst et al., 2007), consistent with an important role for Bmi1 during oncogenesis. Furthermore, overexpression of Bmi1 correlates with a more aggressive tumor phenotype and a chemoresistance phenotype in a wide range of other human tumors (Cui et al., 2007; Glinsky et al., 2005; Hayry et al., 2008; Huber et al., 2011). In this study, we demonstrated increased levels of Bmi1 protein expression in BRAFi-resistant melanoma cell lines and clinical specimens. Abrogation of Bmi1 expression in BRAFi-resistant melanoma cell lines significantly reversed their drug resistance phenotype, while overexpression of Bmi1 in BRAFi-naïve melanoma cells was sufficient to confer a BRAFi resistance phenotype. These findings indicate a pivotal role for Bmi1 in directing BRAFi resistance in melanoma.

In breast cancer cells, miR-200c binds directly to the 3'UTR of *Bmi1* and reduces Bmi1 protein expression (Shimono et al., 2009). In pancreatic cancer cells, transfection of miR-200c reduces the activity of a luciferase–*Bmi1* 3'UTR-reporter construct, and loss of miR-200c correlates with the acquisition of a chemotherapy resistance phenotype (Wellner et al., 2009). Here, we demonstrate a striking inverse relationship between miR-200c and Bmi1 in clinical specimens of melanoma as well as melanoma cell lines exhibiting a BRAFi resistance phenotype. Overexpression of miR-200c in BRAFi-resistant cell lines restored their sensitivity to BRAFi, whereas antagonizing miR-200c in BRAFi-naïve melanoma cell lines is sufficient to induce a BRAFi resistance phenotype. Together, these findings suggest a model in which acquisition of resistance to BRAFi is intrinsically linked to loss of miR-200c, which in turn relieves repression of Bmi1.

Bmi1 is a known mediator of EMT and induction of EMT is associated with an increased expression of ABC transporter expression (Paranjape et al., 2014; Saxena et al., 2011). In BRAFi-resistant melanoma tissues and cell lines, we observe a consistent activation of EMT pathways and upregulation of ABC transporter genes, suggesting that miR-200c/Bmi1-mediated BRAFi resistance is dependent on the activation of EMT and ABC transporter.

miR-200c is a master regulator of EMT. Induction of EMT is associated with an increased expression of ABC transporter expression (Saxena et al., 2011). In BRAFi-resistant melanoma tissues and cell lines, we observed a consistent activation of EMT pathways coincident with upregulation of ABC transporter genes, suggesting that the miR-200c/Bmi1 axis-mediated BRAFi resistance is related to the activation of EMT and ABC transporters. Several studies have implicated that a relationship between alterations in miR-200c/Bmi1 and the activation of the PI3K/AKT pathway might be central to the acquired resistance to chemotherapy (Kopp et al., 2012; Segal, 2003). PTEN loss and the resultant activation of PI3K signaling pathways are key mediators of resistance to BRAFi (Paraiso et al., 2011). Melanoma with BRAF resistance exhibited increased ERK activation that was associated with activation of the PI3K/AKT pathway (Jiang et al., 2011). Our study showed that miR-200c/ Bmi1-mediated BRAFi resistance is associated with increased phosphorylation of AKT and ERK in melanoma cells, suggesting that the miR-200c/Bmi1 axis-mediated BRAFi resistance is at least in part through activation of the MAPK and PI3K signaling cascades.

Together, our study defines a new pivotal signal network involved in the regulation of the molecular pathogenesis of resistance to BRAFi therapy, and our findings highlight important new opportunities for the rational design of future targeted therapy combinations. Targeting miR-200c/Bmi1 provides a potential therapeutic strategy for overcoming BRAFi resistance in melanomas.

Methods

Cell culture and reagents

Human melanoma cell lines (WM35, WM793, Mel1617, Mel1617BR, 451Lu, and 451LuBR) were kind gifts of Meenhard Herlyn (The Wistar Institute, Philadelphia). Human melanoma cell lines WM35 and WM793 were maintained in MCDB medium supplemented with 2% fetal bovine serum (FBS) as previously described (Liu et al., 2011). Mel1617 and 451Lu were maintained in DMEM medium supplemented with 10% FBS; Mel1617BR and 451LuBR were maintained in 1 μ M PLX4720 as described (Villanueva et al., 2010). The human 293T cell line was a gift from Frank Lee at the University of Pennsylvania. 293T cells were maintained in high-glucose Dulbecco's modified Eagle's medium with 10% FBS and penicillin/ streptomycin (100 U/ml and 100 mg/ml).

The following antibodies were used: goat anti-Bmi1 (c1510; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilution), rabbit monoclonal anti-human phospho-AKT, AKT, phospho-ERK, ERK (Cell Signaling Technology, Danvers, MA, USA; 1:1000 dilution), monoclonal mouse anti-human E-cadherin antibody (Dako, Carpinteria, CA, USA; 1:500 dilution), mouse monoclonal anti-human N-cadherin antibody (3B9, 1:1000 dilution; Life Technologies™, Grand Island, NY, USA), rabbit polyclonal anti-human Snail1 antibody (sc-28199; Santa Cruz Biotechnology; 1:500 dilution), rabbit polyclonal to ABCG5 antibody (ab69713; Abcam Inc., Cambridge, MA, USA; 1:500 dilution), rabbit polyclonal MDR1 antibody (LS-B1448; LifeSpan Biosciences Inc., Seattle, WA, USA; 1:200 dilution), and mouse anti- β -actin monoclonal antibody (010M4816; Sigma-Aldrich, St. Louis, MO, USA; 1:2500 dilution).

pEIZ-HIV-ZsGreen-miR-200c, pEIZ-HIV-ZsGreen-vector, c-cherry vector, and c-cherry-Bmi1 vector were gifts from Dr. Michael F. Clarke at Stanford University (Stanford, CA; Shimono et al., 2009). Anti-miR-200c and anti-microRNA controls were purchased from Ambion (Austin, TX, USA). sh-Bmi1 and sh-vector were kindly provided by Dr. George Q. Daley from the Dana Farber Cancer Institute (Onder et al., 2012).

FuGENE6 transfection reagent was purchased from Roche Diagnostics GmbH (12132000) and was used according to the manufacturer's instructions.

Generation of mice PDX and RPD model

Patient-derived xenografts were established by grafting melanoma tumor biopsies from patients subcutaneously into immunodeficient NSG mice in 1:1 complete media and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Animals are monitored weekly for tumor growth and live tissue is banked or serially transplanted when tumors reach the maximal volume. Four mouse tumor tissues with passage <6 were used in this study. If PDXs are established from BRAF inhibitor therapy relapsed patients (RPDX), tumors are grown in mice fed with PLX4720 200 mg/kg additive diet. Six mouse tumor tissues with passage <7 were used in this study. All studies are conducted in accordance with the guidelines set by the Wistar Institutional Animal Care and Use Committee.

MicroRNA from fresh tissues was extracted followed the manufacturer's instructions (mirVana™ miRNA Isolation Kit, AM1561; Ambion), real-time PCR was conducted as described previously (Liu et al., 2012).

Bioinformatics

Illumina data (GSE50509) were normalized, background corrected, and summarized using the R package 'lumi' (Du et al., 2008). We then selected patients whose 30% of the genes in the miR-200c targeting gene set under the prog condition is positive. We then conducted one-way clustering of the miR-200c targeting genes over the selected samples. The normalized RNA-seq data (GSE50535) were analyzed directly by conducting one-way clustering of Tubb3, FLT1, Zeb2, Zeb1, and Bmi1 over the samples.

Isolation of microRNA from formaldehyde fixed paraffin embedded melanoma patient tissues

The human tissue samples were obtained under a human subject research protocol; informed consent form was approved by the Institutional Review Boards (IRBs) of the University of Pennsylvania and Abramson Cancer Center's Clinical Trials Scientific Review and Monitoring Committee. The study was conducted in compliance with regulations of the Health Insurance Portability and Accountability Act and the Declaration of Helsinki. We collected 14 specimens from patients prior to treatment with a substantial dermal component and 14 matched specimens from the same patients following treatment with PLX4720 with a substantial dermal component (Clark level IV, Breslow thickness 41.0 mm). Two of 5-μm sections per sample were obtained from the selected block, and the area of interest was macrodissected from a glass slide for the extraction of total microRNA using the

RecoverALL™ Total Nucleic Acid Isolation (Cat#1975; Ambion Inc.) according to the manufacturer's instructions.

Isolation of miRNA from cell lines

Mel1617, Mel1617BR, 451Lu, and 451LuBR cells were harvested, and microRNA was isolated using the mirVana™ miRNA Isolation Kit (Cat# AM1561; Ambion Inc.) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) of miRNAs

Real-time quantitative RT-PCR was carried out on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) machine using the TaqMan MicroRNA Assay for miR-200c (Applied Biosystems, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, total RNA template was prepared, and the concentration and integrity were assessed as previously described (Liu et al., 2011). Ten nanogram of total RNA was used for reverse transcription using the specific miRNA primers for hsa-miR-200c (Applied Biosystems), and the U6B snRNA (Applied Biosystems) was used as a control. The mean C_T was determined from duplicate or triplicate reactions. Delta C_T was calculated as $C_{TmiRNA} - C_{TU6B}$. Relative gene expression was calculated as $2^{-(\Delta C_T)}$ and multiplied by 10^5 to ease data presentation as previously described (Liu et al., 2011, 2012).

Isolation of total RNA and quantitative PCR in cell lines

Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA) followed by cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the iQ SYBR green supermix (Bio-Rad Laboratories) with specific primers (listed below). cDNA corresponding to 1 µg of RNA was added to the iQSYBER green supermix and analyzed with iCYCLER (Bio-Rad Laboratories) according to the manufacturer's instructions. The cycling condition was as follows: 40 cycles of 95°C for 30 s and 56°C for 30 s. Melting curve analysis was carried out for each PCR to confirm the specificity of amplification. At the end of each phase, fluorescence was used to qualify PCR product. Bmi1 RT-PCR was performed as previously described (Liu et al., 2012; Park et al., 2003).

The following primers were used: E-cadherin forward (Fw) 5'-TTC CCT GCG TAT ACC CTG GT-3', E-cadherin reverse (Rev) 5'-GCC ATC TCT TGC TCG AAG TCC-3', N-cadherin Fw 5'-CAC TGC TCA GGA CCC AGA T-3', N-cadherin Rev 5'-TAA GCC GAG TGA TGG TCC-3', Snail1 Fw 5'-GAC TAG AGT CTG AGA TGC CC-3', Snail1 Rev 5'-CAG ACA TTG TTA AAT TGG CCG-3', ABCG5 Fw 5'-TGG GAC ATC ACA TCT TGC CG-3', ABCG5 Rev 5'-CCG TTC ACA TAC ACC TCC CC-3', MDR1 Fw 5'-AGG AAG CCA ATG CCT ATG ACT TTA-3', MDR1 Rev 5'-CAA CTG GGC CCC TCT CTC TC-3', β -actin Fw 5'-TGA CTG ACT ACC TCA TGA AGA TCC-3', and β -actin Rev 5'-GCC ATC TCT TGC TCG AAG TCC-3'.

Western blot analysis

Western blot analysis was performed as previously described (Liu et al., 2008).

Infection of pEIZ–HIV–ZsGreen–miR-200c, c-Cherry–Bmi1, and sh-Bmi1

pEIZ-HIV-ZsGreen-vector control, pEIZ-HIV-ZsGreen-vector containing miR-200c, c-cherry–vector control, and c-cherry–vector containing Bmi1 (gifts from M. Clarke), sh-control, and sh-Bmi1 were transfected into 293T cells with packing vector (pCMV-dR8.2-dupr and pCMV VSV). Viral supernatants were collected 72 h after transfection. Infection of human melanoma cells was performed (Bmi1 for 451Lu, WM35, and WM793. sh-Bmi1 or miR-200c for 451LuBR) as previously described (Liu et al., 2012). Forty-eight hours after infection, miR-200c-infected green fluorescent protein-positive (green) cells were sorted by fluorescence-assisted cell sorting. For the rescue assay, we co-infected the Bmi1 virus into the miR-200c-overexpressing cells. Expression of miR-200c and Bmi1 was confirmed in the sorted cells by PCR and/or Western blot analysis.

Transfection of anti-miR-200c

Anti-miR-200c miRNA and the scrambled anti-miR miRNA (negative control) were both purchased from Ambion and were used according to the manufacturer's instructions. Briefly, 5×10^4 451Lu, WM35, and WM793 cells were seeded per well in 2 ml of 2% FBS MCDB tumor media and were incubated with siPORT™ NeoFX™ Transfection Agent (10 μ l in 200 μ l of OPTI-MEM–I medium without serum) for 5 min. Ten micromolar anti-miR-200c or miR control was then added, and cells were incubated for 10 min at room temperature to allow the formation of transfection complexes. After 24 h, the medium was replaced with 2% FBS MCDB tumor media. After additional 48 h of incubation, cells were harvested and analyzed.

Cell growth/viability

A total of 5×10^4 cells per well were seeded into a 24-well plate. After 48 h of treatment, either the MTT assay or cell counting was used for quantifying cell growth/viability as previously described (Liu et al., 2012).

Statistical analysis

Unpaired Student's *t*-test and Wilcoxon test were used to assess for a statistically significant difference between the relative miRNA expression levels in melanoma tissues compared with control ($P < 0.05$). The student's *t*-test or one-way ANOVA was used to analyze gene expression and cell viability using GRAPHPAD PRISM software (La Jolla, CA, USA). Statistical significance was reached if ($P < 0.05$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance

Despite the successful development of BRAF inhibitors (BRAFi) for BRAF-mutant melanomas, resistance inevitably develops. However, the role of the microRNA on BRAFi resistance remains largely unexplored. We found that loss of miR-200c occurs in BRAFi-resistant melanomas, which leads to increased activation of the PI3K/AKT and MAPK pathways via a transcriptional factor, Bmi1-dependent mechanism. Our study identifies miR-200c as a central signaling node that modulates many signaling mechanisms related to anticancer therapies resistance. Our data provide new insights into how microRNA affects the efficacy of BRAFi and targeting microRNA might be a potential novel strategy for overcoming BRAFi resistance.

Figure 1.

Reduction of miR-200c expression and increase of miR-200c target genes in BRAFi-resistant patient melanoma tissues. (A) Quantitative RT-PCR analysis was used to analyze the relative expression of miR-200c in xenograph from patient-derived xenograft (PDX) (n = 4) and resistant PDX (RPDX) (n = 6) mouse model. (B) Quantitative RT-PCR analysis was used to analyze the relative expression of miR-200c in tissues from 14 paired pretreatment and 14 post-treatment patients (n = 3). Right panel is summary of the results of left panel. (C) One-way clustering was performed analyzing the expression of *Bmi1* gene in twenty-one paired pre- and post-treatment frozen melanoma tumor tissues (Left panel). Right panel is the quantitative analysis of left panel. Bioinformatics analysis of the expression of miRNA200c target genes. (D, E) One-way clustering was performed analyzing the expression of *Zeb2* (D) and *Tubb3* (E) genes in 21 paired pre- and post-treatment frozen melanoma tumor tissues (Left panel). Right panel is a summary of left panel. (F) RT-PCR analysis was used to analyze *Bmi1* expression in tissues from (B). Left panel: Result of one representative experiment with a tissue from (B) is presented. Right panel is summary of left panel (n = 3). (G) Quantitative RT-PCR analysis of *E-cadherin*, *N-cadherin*, and *Snail1* relative mRNA expression in tissue from (B) (n = 3). Data are given as mean $\pm$ SD. *P < 0.05 compared with pretreatment group. Results are representative of three independent experiments.

Figure 2.

Reduction of miR-200c expression in BRAFi-resistant melanoma cell lines. (A, B) Mel1617 and Mel1617BR (A), and 451Lu and 451LuBR (B) cells were treated with increasing concentrations of PLX4720 for 48 h. Cell survival was quantified with MTT assay ($n = 3$), $*P < 0.05$ compared with parental cell lines. (C) Quantitative RT-PCR analysis was used to analyze miR-200c relative expression in cell lines from (A) and (B) ($n = 3$). Data are given as mean $\pm$ SD. $*P < 0.05$ compared with parental cells group. (D) Quantitative RT-PCR analysis was used to analyze relative mRNA expression of *E-cadherin*, *N-cadherin*, *Snail1*, *ABCG5*, and *MDR1* in cell lines from (A) and (B) ($n = 3$). Data are given as mean $\pm$ SD. $*P < 0.05$ compared with parental cells group. (E) Western blot analysis of E-cadherin, N-cadherin, Snail1, ABCG5, and MDR1 protein expression in cell lines from (A) and (B). (F) RT-PCR analysis of *Bmi1* (top panel) and Western blot analysis (lower panel) of Bmi1, phospho- AKT expression in cell lines from (A) and (B). β -actin was used as a loading control. Results are representative of three independent experiments.

Figure 3.

Overexpression of miR-200c reverses drug resistance in 451LuBR cells. (A) miR-200c-overexpressing 451LuBR cells and 451LuBR control cells were treated with increasing concentrations of PLX4720 for 48 h. Left panel, qRT-PCR confirmation of miR-200c overexpressed in miR-200c-overexpressing 451LuBR cells, Right panel: Cell survival was quantified with MTT assay (n = 3). *P < 0.05 compared with control group. (B) Quantitative RT-PCR analysis of *E-cadherin*, *N-cadherin*, *Snail1*, *ABCG5*, and *MDR1* mRNA expression in cell lines from (A) (n = 3). Data are given as mean $\pm$ SD. *P < 0.05 compared with 451LuBR control group. (C) RT-PCR analysis of *Bmi1* (top panel) and Western blot analysis (bottom panel) of *Bmi1*, phospho-AKT, and phospho-ERK expression in cell lines from (A). (D) Western blot analysis of E-cadherin, N-cadherin, *Snail1*, *ABCG5*, and *MDR1* protein expression in 451LuBR cells with overexpression of miR-200c compared to 451LuBR control cells. β -actin was used as a loading control. Results are representative of three independent experiments. (E) Left panel: Quantitative RT-PCR analysis was used to analyze miR-200c relative expression in 451Lu cells transfected with anti-miR-200c and control 451Lu cells (n = 3). Data are given as mean $\pm$ SD. *P < 0.05 compared with 451Lu control group. Right pane: Cells from (E) were treated with increasing concentrations of PLX4720 for 48 h. Cell survival was quantified with MTT assay (n = 3). *P < 0.05 compared with 451Lu control group. (F) Quantitative RT-PCR analysis of *E-cadherin*, *N-cadherin*, *Snail1*, *ABCG5*, and *MDR1* relative mRNA expression in cells from (E) (n = 3). Data are given as mean $\pm$ SD. *P < 0.05 compared with 451Lu control group. (G) Western blot analysis of E-cadherin, N-cadherin, *Snail1*, *ABCG5*, and *MDR1* protein expression in cells from (E). (H) RT-PCR analysis of *Bmi1* (top panel) and Western blot analysis (bottom panel) of *Bmi1*, phospho-AKT, and phospho-ERK expression in cells from (E).

Figure 4.

Requirement of Bmi1 expression for miR-200c-mediated BRAFi resistance in 451LuBR cells. (A) RT-PCR analysis was used to analyze *Bmi1* expression in 451LuBR cells overexpressing miR-200c alone or in 451LuBR cells overexpressing both miR-200c and Bmi1 ($n = 3$). (B) Cells from (A) were treated with increasing concentrations of PLX4720 for 48 h. Cell survival was quantified with MTT assay ($n = 3$). * $P < 0.05$ compared with 451LuBR cells with overexpression of miR-200c alone. (C) Quantitative RT-PCR analysis of *E-cadherin*, *N-cadherin*, *Snail1*, *ABCG5*, and *MDR1* relative mRNA expression in cells ($n = 3$). Data are given as mean $\pm$ SD. * $P < 0.05$ compared with 451LuBR cells with overexpression of miR-200c alone. (D) Western blot analysis of E-cadherin, N-cadherin, Snail1, ABCG5, and MDR1 protein expression in cells from (A). (E) Western blot analysis of Bmi1, phospho-AKT, and phospho-ERK expression in cells from (A). β -actin was used as a loading control. Results are representative of three independent experiments.

Figure 5.

Knock-down of Bmi1 restores sensitivity of 451LuBR cells to PLX4720. (A) RT-PCR analysis was used to analyze *Bmi1* expression in 451LuBR cells where Bmi1 expression was knocked down compared to 451LuBR parent control cells. (B) Cells from (A) were treated with increasing concentrations of PLX4720 for 48 h. Cell survival was quantified with MTT assay ($n = 3$). * $P < 0.05$ compared with 451LuBR control cells. (C) Quantitative RT-PCR analysis of *E-cadherin*, *N-cadherin*, *Snail1*, *ABCG5*, and *MDR1* relative mRNA expression in cells from (A). Data are given as mean $\pm$ SD. * $P < 0.05$ compared with 451LuBR control group. (D) Western blot analysis of E-cadherin, N-cadherin, Snail1, ABCG5, and MDR1 protein expression in cells from (A). (E) Western blot analysis of Bmi1, phospho-AKT and phospho-ERK expression in cells from (A). β -actin was used as a loading control. Results are representative of three independent experiments.

Figure 6.

Knock-down miR-200c or overexpression of Bmi1 or antimiR- 200C confers BRAFi resistance in 451Lu cells. (A) Bmi1 overexpression was confirmed by RT-PCR. (B) Cells from (A) were treated with increasing concentrations of PLX4720 for 48 h. Cell survival was quantified with MTT assay (n = 3). *P < 0.05 compared with 451LuBR control group. (C) Quantitative RT-PCR analysis was used to analyze *E-cadherin*, *N-cadherin*, *Snail1*, *ABCG5*, and *MDR1* relative mRNA expression in cells from (A). Data are given as mean $\pm$ SD. *P < 0.05 compared with the 451Lu control group. (D) Western blot analysis of E-cadherin, N-cadherin, Snail1, ABCG5, and MDR1 protein expression in cells from (A). (E) Western blot analysis of Bmi1, phospho-AKT and phospho-ERK expression in cells from (A) β -actin was used as a loading control. Results are representative of three independent experiments.