miR-596 Modulates Melanoma Growth by Regulating Cell Survival and Death

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Tumors grow because cancer cells lack the ability to balance cell survival and death signaling pathways. miR-596, a microRNA located at the 8p23.3 locus, has been shown by the TCGA-Assembler to be deleted in a significant number of melanoma samples. Here, we also validated the low levels of miR-596 in melanoma compared to tissue nevi, and Kaplan-Meier curve analysis revealed that low miR-596 expression was associated with worse overall survival. Moreover, we showed that miR-596 overexpression effectively inhibited MAPK/ERK signaling, cell proliferation, migration, and invasion and increased the cell apoptosis of melanoma cells. In addition, we found that miR-596 directly targets MEK1 and two apoptotic proteins, MCL1, and BCL2L1, in melanoma cells. Our findings indicated that miR-596 is an important miRNA that both negatively regulates the MAPK/ERK signaling pathway by targeting MEK1 and modulates the apoptosis pathway by targeting MCL1 and BCL2L1, suggesting that miR-596 could be a therapeutic candidate for treating melanoma, and a prognostic factor for melanoma patients.


INTRODUCTION

Aberrant levels of microRNAs (miRNAs) have been noted in numerous diseases, including many types of cancer (Bader et al., 2010; Jansson and Lund, 2012; Macfarlane and Murphy, 2010). Moreover, miRNAs can influence or coordinate signaling pathways by regulating components of multiple oncogenic pathways in cancer (Chen et al., 2009; Kefas et al., 2008). The RAS-RAF-MEK-ERK (MAPK/ERK) pathway is highly dysregulated in cancer, especially melanoma (Inamdar et al., 2010). This pathway primarily controls a wide range of mammalian cellular responses such as cell proliferation, differentiation, survival, inflammation, motility, and apoptosis (Dhillon et al., 2007; Ikeda et al., 2012; Kim and Choi, 2010). Approximately 50–70% and 15% of melanomas contain mutations of BRAF or NRAS mutations, respectively, and approximately 90% of the BRAF mutations are the V600E substitution—this mutant markedly increases MAPK signaling pathway activity in melanoma (Fecher et al., 2009). Tumorigenesis is largely attributed to an imbalance between cell growth and death (Ding et al., 2006, Inamdar et al., 2010). The MAPK/ERK pathway has been implicated in regulating MCL1 and BCL2L1 transcription via Elk-1 or Stat and AP1 or c-Jun, respectively (Becker et al., 2014; Booy et al., 2011; Goncharenko-Khaider, 2012; Jung-Jung Changchien, 2015; Sevilla et al., 2001; Townsend et al., 1999).

Inhibition of ERK1/2 activity causes arrest at the G1 phase of the cell cycle and downregulation of the expression levels of Mcl-1 and Bcl-XL, the anti-apoptotic homologs of Bcl-2 (Booy et al., 2011; Boucher et al., 2000). In particular, the anti-apoptotic proteins MCL1 and BCL2L1 are present at higher levels in many tumor types, including in 80% of melanomas (Ding et al., 2006; Wong et al., 2008; Zhuang et al., 2007).

Copy number variations are thought to compose least 10% of the human genome and are commonly found in cancer (Shlien and Malkin, 2009). The 8p23.3 deletion is a frequent event in cancer that results in the loss or abnormal function of several tumor suppressor genes (Eguchi et al., 2010; Han et al., 2010; Muscheck et al., 2000). Although the coding genes within the 8p23.3 deletion have been well studied, the potential roles of miRNAs located at the 8p23.3 locus in melanoma remain unclear. miR-596 is an intergenic miRNA located at 8p23.3; however, the potential tumor-suppressive activities of miR-596 and the associated clinicopathologic characteristics in melanoma have not been well characterized, particularly the role of miR-596 is involved in oncogenic signaling.

In this study, we observed that miR-596 expression is lower in melanoma than in tissue nevi. In addition, miR-596 overexpression effectively inhibited MAPK/ERK signaling and melanoma cell proliferation, migration, and invasion as well as increased cell apoptosis in vitro. Our findings suggested that miR-596 not only negatively regulates the MAPK/ERK signaling pathway via the MEK1 3′ untranslated region (UTR), but also increases apoptosis by targeting the MCL1 and BCL2L1 3′ UTRs. These data are, to our knowledge, previously unreported to illustrate that miR-596 is an important

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Abbreviations: miRNA, microRNA; NC, negative control; UTR, untranslated region

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tumor-suppressor miRNA that can regulate both the survival and death of melanoma; thus, miR-596 could be a potent therapeutic candidate for melanoma.

RESULTS

miR-596 levels are repressed in melanoma cells with elevated MAPK/ERK activity

Data from a microRNA profiling analysis showed that miR-596 was downregulated in oral squamous cell carcinoma cells (Endo et al., 2012). However, the potential tumor-suppressive activity of miR-596 in melanoma is poorly understood. To assess the significant correlations of miR-596 levels with clinical characteristics in melanoma patients, we processed TCGA data from the TCGA-Assembler; miR-596, which is located at the center of the 8p23.3 locus, was deleted in 367 melanoma samples (Zhu et al., 2014). Our previous miRNA profile analysis showed that miR-596 was the one of most downregulated miRNAs in the melanoma cell line Malme-3M compared to the parental Malme-3 cell line (Liu et al., 2014). We hypothesized that miR-596 participates in melanoma tumorigenesis.

We investigated miR-596 expression in different cell lines expressing the BRAFV600E mutant (A2058, A375, Malme-3M, and SK-Mel-19, the NRAS mutant (SK-Mel-2, SK-Mel-30), wild-type NRAS and BRAF (Malme-3) (Figure 1a). The expression of miR-596 was at least eight-fold lower in melanoma cells with increased MAPK/ERK activity compared to normal cells. We further analyzed the public patient microarray data from the Gene Expression Omnibus database (22 benign nevus samples and 35 melanoma cancer samples, Gene Expression Omnibus accession no. GSE24996) and found that the miR-596 level was significantly lower in melanoma tissues than in nevus tissues (Figure 1b).

To gain a better understanding of endogenous miRNA levels in primary tissues, we analyzed miR-596 levels in nevus and melanoma tissues from patients in Taiwan and in a commercial tissue array using in situ hybridization (Figure 1c, 1d). We followed the Allred scoring system, which comprises the intensities index and the proportion index; each index was scored using four levels based on the individual staining intensity and the percentage of area stained (Supplementary Figure S1a, S1b online). S100 proteins, which are normally present in cells derived from the neural crest such as melanocytes, were used as a control. Elevated miR-596 expression levels were significantly correlated with the nevus samples, suggesting that miR-596 downregulation was associated with melanoma cells (Figure 1d). Receiver operating characteristic curve analysis was performed to evaluate the diagnostic value of the miRNAs. An area under the curve value close to 1 indicates more substantial differences between the melanoma and nevus samples. Receiver operating characteristic curve analysis revealed that miR-596 had significantly higher area under the curve values of 0.8857 and 0.8337 in the Taiwanese samples and the tissue array, respectively (Figure 1e). Therefore, we found that miR-596, which is within the 8p23.3 locus, was deleted in melanomas and that reduced miR-596 expression was common among human melanoma cell lines and primary patient tissues.

miR-596 decreases melanoma cell proliferation and growth and increases apoptosis

To examine the cellular function of miR-596, we overexpressed miR-596 in different melanoma cell lines (SK-Mel-19, A375, and Malme-3M) and evaluated cellular proliferation. PLX4032, a BRAFV600E inhibitor, was used as a positive control. Cells overexpressing miR-596 exhibited reduced proliferation colony formation and cell number as shown in Figure 2a (Figure S2a–2c online). We also examined the tumorigenic effects of miR-596 on melanoma cells using the soft agar assay. The size and number of colonies were significantly decreased (Figure 2b). In addition, overexpression of anti-miR-596 in SK-Mel-19 cells increased the proliferation rate by approximately 20% and enhanced the colony-forming ability (Supplementary Figure S2d online). Moreover, we overexpressed miR-596 in melanoma cells to determine the effects on apoptosis. After a 72-hour transfection with miR-596, the percentage of apoptotic cells was measured using Annexin V staining. The results indicated that miR-596 increased the number of apoptotic cells by approximately 10% compared to the negative control (NC) in both the SK-Mel-19 and A375 cell lines (Figure 2c). Consistent with the results of the Annexin V assay, caspase 3/7 activity increased by 6-to 8-fold in miR-596–transfected melanoma cells (Figure 2d). These results suggested that miR-596 repressed proliferation and enhanced apoptosis in melanoma cells.

miR-596 inhibits melanoma cell migration and invasion

To assess the migratory and invasive activities of miR-596, we monitored cell migration and invasion for 96 hours using xCELLigence technology (ACEA Biosciences, San Diego, CA), which utilizes gold microelectrodes to detect the mobility of cultured cells. SK-Mel-19 cells transfected with miR-596 exhibited a significant reduction in migration and invasion compared to NC cells (Figure 3a, 3b). Cell migration and invasion were inhibited by approximately 30% and 70%, respectively, over a 40- to 50-hour period (Figure 3a, 3b, right panel). Similar results were observed in SK-Mel-19 and A375 cells using the Transwell method, a more traditional measurement of cell motility (Figure 3c). To confirm the suppressive effects of miR-596 on melanoma cell migration, cell motility was measured using a wound-healing assay. A nontoxic concentration of actinomycin D was co-administered with miR-596 to cells to prevent cell proliferation while monitoring wound closure. At 48 hours in SK-Mel19 cells and at 24 hours in A375 cells, the wound closure rate was decreased in cells that overexpressed miR-596, but not those expressing NC miRs (Figure 3d). Taken together, these results indicated that miR-596 suppressed cancer cell migration and invasion.

miR-596 attenuates the activity of the MAPK/ERK and anti-apoptosis pathways

Many reports have suggested that dysregulating the MAPK/ERK pathway enhances cell proliferation and invasion and promotes metastasis in melanoma (Inamdar et al., 2010). Therefore, we examined whether overexpressing miR-596 decreases the proliferation, migration, and invasion of melanoma cells by regulating MAPK pathway activity. miR-596 overexpression reduced the levels of phosphorylated
Figure 1. miR-596 expression is reduced in melanoma cells and primary melanoma tissues. (a) Quantitative reverse transcription PCR analysis of miR-596 expression in seven cell lines. miR-596 expression was normalized to RNU48 expression in Malme-3 cells. The quantification data are reported as the mean ± standard deviation of three independent experiments (Student t test: ***P < 0.001). (b) Box-whisker plots of miR-596 expression in melanoma samples. The microRNA expression profiles were obtained from Gene Expression Omnibus (GEO) accession number GSE24996. (c) In situ hybridization and immunohistochemistry analyses using DIG*-labeled miRCURY LNA probes complementary to miR-596 and S100, respectively. (d) Left: The percentage of miR-596 expression was calculated based on the total scores in 36 melanoma tissues versus those in 22 normal nevus tissues procured from Taiwanese hospitals. Right: The percentage of miR-596 expression was calculated based on the total scores in 82 melanoma tissues versus those in 18 nevus tissues in a commercially available tissue array. (e) The receiver operating characteristic curve of miR-596 in primary Taiwanese samples or the tissue array showed accurate discrimination between the diagnostically confirmed melanoma and nevus groups. Bar = 5 μm.
ERK1/2, MEK, and RSK in the A375, SK-Mel-19, A2058, and Malme-3M cell lines (Figure 4a and Supplementary Figure S3 online). Additionally, the protein expression of the cyclin-dependent kinase inhibitor p27 and the apoptotic marker cleaved poly (ADP-ribose) polymerase was increased. In addition, overexpression of anti-miR-596 enhanced the lower endogenous MAPK/ERK activity in A375 and HEK293 cells (Figure 4b). These results indicated that miR-596...
expression attenuated MAPK/ERK activity and the expression of downstream targets, such as p27 and cleaved poly (ADP-ribose) polymerase, and pro-apoptotic proteins.

**miR-596 mediates cell survival by directly inhibiting MEK1, MCL1, and BCL2L1 expression through their 3' UTRs**

To investigate the mechanism of miR-596 activity in melanoma, we utilized in silico prediction programs and several known components associated with melanoma progression to predict potential targets of miR-596. We found that the 3' UTRs of MEK1, MCL1, and BCL2L1 mRNA were complementary to the miR-596 seed region (Figure 5a–5c, left panel). To verify whether MEK1, MCL1, and BCL2L1 are direct targets of miR-596, we cloned each 3' UTR downstream of the luciferase gene. In addition, we mutated the predicted miR-596 seed regions in the 3' UTR to validate target specificity (Figure 5a–5c, left panel). MEK1 contains a miR-596 binding site within 260–265 nucleotides of the MEK1 3' UTR, MCL1 contains a miR-596 binding site within 1,316–1,322 nucleotides of the MCL1 3' UTR, and BCL2L1 contains two miR-596 binding sites within 516–536 and 1,054–1,074 nucleotides of the BCL2L1 3' UTR. The wild-type luciferase reporters had the native miR-596 binding site, whereas the mutant reporters included mutations that deleted the miR-596 seed sequence. When miR-596 was co-transfected with the luciferase plasmids containing the wild-type 3' UTRs from MEK1, MCL1, or BCL2L1 into SK-MEL-19 cells, we observed a consistent reduction of luciferase activity. Conversely, co-transfection of miR-596 with the luciferase plasmids containing the mutant 3' UTRs from MEK1, MCL1, or BCL2L1 into SK-MEL-19 cells resulted in no significant change in luciferase activity (Figure 5a–5c, left panel).
right panel). Interestingly, when we transfected miR-596 into SK-Mel-19, A375 or A2058 cells, the endogenous protein levels of MEK1, MCL1, and BCL2L1 were reduced (Figure 5d and Supplementary Figure S3a online).

To confirm whether the growth inhibitory effect of miR-596 was due to its regulation of MEK1, MCL1, or BCL2L1 expression, we co-transfected cells with miR-596 and plasmids harboring MEK1, MCL1, or BCL2L1 without their 3′ UTR. Overexpressing the MEK1 coding region rescued the miR-596-induced inhibition of proliferation, and over-expressing either the MCL1 or BCL2L1 coding region attenuated the miR-596-mediated increase in caspase 3/7 activity in SK-Mel-19 and A375 cells (Figure 5e, 5f, and Supplementary Figure S4a–S4c). Taken together, these results indicated that miR-596 exerts inhibitory effects on MEK1, MCL1, and BCL2L1 by interacting with their respective 3′ UTRs, therefore, miR-596 regulates cell survival.

Low miR-596 expression is associated with a worse prognosis

Kaplan–Meier analysis was used to test for an association between microRNAs and overall survival. We utilized a public dataset of a miRNA microarray analysis of the clinical melanoma specimens (GSE59334). For miR-596, patients were separated into two groups based on the average value of miR-596. As shown in Figure 6a, patients with low miR-596 expression had significantly shorter overall survival than those in the high expression group.

DISCUSSION

We utilized the TCGA-Assembler to process the TCGA data on 37 cancer cell types. miR-596 was located at 8p23.3 locus, which is significantly deleted in eight cancer cell types, but especially in melanoma (Supplementary Table S1 online). We found that the 8p23.3 locus contains not only miR-596, but also miR-4674 and miR-7160. However, the effects of miR-4674 and miR-7160 on melanoma progression remain unclear. To the best of our knowledge, this is a previously unreported study to examine the mechanisms of action of miR-596 in melanoma. We observed lower levels of endogenous miR-596 in most melanoma samples compared to melanocytes and other tumor samples from patients (Figure 1a, 1b). Previous miRNA profiling studies showed that miR-596 is downregulated in oral cancer, bladder cancer, and craniopharyngioma (Olivieri et al., 2016; Samis et al., 2016).

We investigated whether the low levels of miR-596 expression are associated with activation of the MAPK/ERK signaling pathway. Interestingly, overexpression of BRAFV600E activates MAPK/ERK signaling in HEK293 cells, which decreased miR-596 expression by approximately
Figure 5. miR-596 directly targets the 3' UTRs of MEK1, MCL1, and BCL2L1. (a–c) Left panel: Schematic diagrams of the MEK1, BCL2L1, and MCL1 reporter constructs. Right panel: Co-transfection of 2, 10, or 20 nM miR-596 mimic with either the wild-type or mutant pGLO target reporter into SK-Mel-19 cells led to the expression of firefly and Renilla luciferase. The transfection efficiency was normalized to Renilla luciferase expression. The relative quantification values of firefly luciferase versus Renilla luciferase expression are shown in the lower panel and were measured by NIH ImageJ software. Ctr indicates that cells were transfected with plasmids without miRNAs. (d) SK-Mel-19 and A375 cells were transfected with either negative control (NC) or miR-596 mimic and cell lysates were collected at 48 hours after transfection for immunoblotting. (e) The proliferative activity of SK-Mel-19 and A375 cells transfected with miR-596 in the presence or absence of MEK1 was measured. (f) Caspase3/7 activity was assessed in SK-Mel-19 and A375 cells transfected with miR-596 in the presence of anti-apoptotic expression clones (BCL2L1 and MCL1). The quantification data are reported as the mean ± standard deviation of three independent experiments. Student t test: *P < 0.05, **P < 0.01, ***P < 0.001. The significant value of miR-596 was normalized to the NC. The significant value of miR-596 co-transfection with an expression clone was normalized to miR-596 with pcDNA3.1.
Thus, we addressed whether miR-596 expression is affected by inhibiting the hyperactivation of the MAPK/ERK signaling pathway. We treated SK-Mel-19 cells with the specific BRAFV600E inhibitor PLX4032 to block the overactivation of MAPK/ERK signaling and observed an approximately 7-fold increase in miR-596 expression (Supplementary Figure S5b). These results indicated that miR-596 expression was associated with MAPK/ERK pathway activity.

A recent study by Endo et al. (2012) reported that LGALS3BP is directly targeted by miR-596. LGALS3BP was shown to regulate the expression of IL-6 through the phosphorylation of ERK1/2, suggesting that LGALS3BP overexpression might play a pivotal role in oral squamous cell carcinoma progression by activating the MAPK/ERK signaling pathway. In our proposed model, increased MAPK/ERK signaling in melanoma cells results in enhanced cell proliferation, migration, and survival. Transcription factors downstream of the MAPK signaling pathway regulate the expression of the anti-apoptotic genes MCL1 and BCL2L1, which inhibit apoptosis pathways (Booy et al., 2011; Goncharenko-Khaider, 2012; Townsend et al., 1999). Overexpression of miR-596 in melanoma cells significantly suppressed MAPK/ERK activity via MEK1, MCL1, and BCL2L1, leading to enhanced apoptosis and decreased cell proliferation.
hallmark of melanoma (Dhillon et al., 2007; Inamdar et al., 2010; Kim and Choi, 2010). As part of the MAPK pathway, MEK1 is involved in many cellular processes, including cell proliferation, differentiation, and transcriptional regulation (Inamdar et al., 2010). MEK1 mutations have been identified in melanoma, non-small cell lung carcinoma, and pancreatic cancer (Inamdar et al., 2010; Kim and Choi, 2010; Sasaki et al., 2010, Zhou et al., 2010). Moreover, MEK1−/− mice are embryonic lethal due to placental defects, whereas MEK2−/− mice can survive with a normal lifespan and fertility, suggesting that MEK1 also has a critical function during embryonic development (Aoidi et al., 2016). Inhibiting or depleting MEK1 significantly decreases the self-renewal capability and tumor growth of liver cancer stem cells both in vitro and in vivo (Kasobhatla and Tseng, 2003). Several MEK inhibitors have been tested in clinical trials in patients with metastatic melanoma, but these inhibitors are ineffective in the treatment of melanoma. Thus, miR-596 mimics could be a potential tool for repressing high MEK1 levels by directly targeting its 3′ UTR and preventing its transcription.

Although many novel and current small-molecule inhibitors are specifically designed to target components of signaling pathways, patients often rapidly acquire drug resistance. Many miRNAs have been reported to repress several targets in a specific signaling pathway involved in cancer cell survival and death (Chen et al., 2011; Kent et al., 2010, Luo et al., 2012; Palma et al., 2014; Wang et al., 2015). Our data provide the unreported insight into the mechanism of action of miR-596 in melanoma. Additionally, miR-596 may be a potential therapeutic candidate for melanoma treatment.

**MATERIALS AND METHODS**

**Tissue specimens**

A total of 58 formalin-fixed paraffin-embedded samples (16 and 20 tissues collected from patients with melanoma at Landseed Hospital and at Saint Paul’s Hospital, respectively; 22 tissue nevi collected from patients at Landseed Hospital). The study was approved by Landseed Hospital and Saint Paul’s Hospital’s the ethics committees at both institutions (IRB-14-005-B1 and IRB-SPH-10502-01). Written informed consent was obtained before the study. The melanoma tissue array contains a total of 100 formalin-fixed paraffin-embedded tissues, including 82 melanoma samples and 18 nevus tissues (US Biomax, Rockville, MD).

**In situ hybridization**

The hsa-miR-596 probes were labeled using double DIG*-labeled miRCURY LNA miRNA (Exiqon, Vedbaek, Denmark). We performed in situ hybridization using an In Situ Hybridization Kit (BioChain Institute, Newark, CA) following the manufacturer’s protocol. The slides were prehybridized with a prehybridization solution at 65°C for 4 hours. The hybridization mixture was prepared by adding 0.2 μl DIG-labeled probe to 20 μl hybridization solution; this mixture was added to the slides and hybridized at 55°C for at least 12 hours. The staining scores were established according to the staining intensity index and the proportion score index. The total score was calculated by adding the staining intensity and proportion score indexes. After histologic sections were stained, they were evaluated by two pathologists. All formalin-fixed paraffin-embedded sections were evaluated using the Allred scoring system.

**Western blot analysis**

Proteins were extracted in NP-40 buffer, and a standard Western protocol was followed as described previously (Liu et al., 2014). Primary antibodies against p42/44 MAPK, phospho-p42/44 MAPK, phospho-ERK, phospho-ERK, cleaved PARP, MCL1, BCL2L1, and GAPDH were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against BRAF, caspase 3, and firefly luciferase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and Renilla luciferase and MEK1/2 antibodies were obtained from GeneTex (Irvine, CA).

**Luciferase reporter gene assay**

A total of 3 × 105 SK-Mel-19 cells were seeded into six-well plates. Two micrograms of either pGLO Reporter-WT 3′ UTR or pGLO Reporter-MT 3′ UTR was transfected into SK-Mel-19 cells; 4 hours later, the cells were co-transfected with 10 nM miR-596 or NC. The mimic miRNAs and plasmids were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) and X-tremeGENE HP (Roche, Branchburg, NJ), respectively. The cells were lysed and assayed for protein expression 48 hours after transfection.

**Cell proliferation assay**

Cells were plated into six-well plates and transfected with either miR-596 mimic or NC. After 48 hours, cell proliferation was determined using AlamarBlue assays (Invitrogen, Carlsbad, CA). The fluorescence values were measured at excitation wavelengths of 530–560 nm and an emission wavelength of 590 nm. All measured values were obtained using a Synergy HT (BioTek, Winooski, VT).

**Soft agar assay**

A soft agar assay was performed as described previously (Liu et al., 2014). At 48 hours post transfection, viable transfected cells were plated into 12-well plates containing a suitable percentage of soft agar (0.6% bottom layer, 0.3% top layer) and maintained in complete medium for 3–4 weeks. The colonies were stained with 0.005% crystal violet for 5 minutes and then photographed.

**Annexin V assay**

Annexin V staining was used to detect apoptotic cells. Cells were transfected with either miR-596 mimic or NC for 72 hours, after which they were stained using a Muse Annexin V and Dead Cell Assay Kit (Millipore, Billerica, MA) following the manufacturer’s instructions. The stained cells were analyzed using a Muse Cell Analyzer (Millipore, Billerica, MA).

**xCELLigence real-time cell analysis: migration and invasion**

Cell migration and invasion experiments were performed using 16-well plates (CIM-16; Roche Diagnostics GmbH, Mannheim, Germany). Each well consists of an upper and lower chamber separated by a microporous membrane containing randomly distributed 8-μm pores. Prior to each experiment, cells were maintained in serum-free medium for 24 hours. All the procedures were conducted according to the manufacturer’s guidelines. Each condition was performed in duplicate with a programmed signal detection schedule that records every 15 minutes for 96 hours. All the data were recorded and stored using the supplied real-time cell analysis software (version 1.2.1). The cell index data from the experiments were normalized to the last data point prior to treatment initiation using the real-time cell analysis software. All the other results (migration and invasion) were based on raw data without cell index.
normalization and were processed as described here to compare the real-time cell analysis results with those obtained from conventional methods.

Survival curve analysis
A public dataset of a miRNA microarray analysis of the clinical melanoma specimens (GSE59334) was downloaded from the Gene Expression Omnibus database at https://www.ncbi.nlm.nih.gov/geo/. Then, miR-596 expression values from clinical melanoma specimens (ID: 2365) were used to perform Kaplan–Meier survival curve analysis according to the clinical parameter provided in the same dataset. High and low miR-596 expression groups were created by using the average value of miR-596 as a cutoff. Finally, the survival curve was generated by using a bioinformatics tool from R2 platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). Two-tailed P-values were calculated, and P < 0.05 indicated statistical significance.

Statistical analysis
Differences between two groups were analyzed using Student t test (two-tailed). Differences were considered statistically significant at P < 0.05. All the statistical analyses were performed using SigmaPlot software 10.0 (SYSTAT, San Jose, CA).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
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REFERENCES


