Epigenetic Changes of EGFR Have an Important Role in BRAF Inhibitor–Resistant Cutaneous Melanomas

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BRAF mutations are frequent in cutaneous melanomas, and BRAF inhibitors (BRAFi) have shown remarkable clinical efficacy in BRAF mutant melanoma patients. However, acquired drug resistance can occur rapidly and tumor(s) often progresses thereafter. Various mechanisms of BRAFi resistance have recently been described; however, the mechanism of resistance remains controversial. In this study, we developed BRAFi-resistant melanoma cell lines and found that metastasis-related epithelial to mesenchymal transition properties of BRAFi-resistant cells were enhanced significantly. Upregulation of EGFR was observed in BRAFi-resistant cell lines and patient tumors because of demethylation of EGFR regulatory DNA elements. EGFR induced PI3K/AKT pathway activation in BRAFi-resistant cells through epigenetic regulation. Treatment of EGFR inhibitor was effective in BRAFi-resistant melanoma cell lines. The study demonstrates that EGFR epigenetic activation has important implications in BRAFi resistance in melanoma.

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INTRODUCTION

Despite recent major advances in therapies, the prognosis for melanoma patients with advanced stage disease remains very poor (Gopal *et al.*, 2010; Siegel *et al.*, 2012). Approximately 50% of melanomas harbor an activating BRAF V600 mutation (BRAFmt; Davies *et al.*, 2002; Long *et al.*, 2011; Jakob *et al.*, 2012), an oncogene known to have an important role in the proliferation and progression of melanoma cells through activation of the rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)/extracellular signal–regulated kinase (ERK) cell signaling pathway (Gopal *et al.*, 2010). FDA approved BRAF inhibitors (BRAFi) such as vemurafenib (PLX4032, Roche, San Francisco, CA) and recently dabrafenib (GSK2118436, GlaxoSmithKline, London, UK) were used to inhibit the RAF/MEK/ERK signaling pathway by targeting BRAFmt (Flaherty *et al.*, 2012; Lito *et al.*, 2012). Clinical studies have shown that stage IV BRAFmt melanoma patients respond rapidly to BRAFi therapies with high response rates (Flaherty *et al.*, 2010; Chapman *et al.*, 2011; Falchook *et al.*, 2012; Hauschild *et al.*, 2012; Long *et al.*, 2012). However, a majority of patients eventually develop resistance.

Recently, studies have attempted to identify mechanisms and changes associated with BRAFi resistance. Studies have shown that other signal transduction pathways, such as the IGF1R/PI3K/AKT pathway (Villanueva *et al.*, 2010) and the MAPK pathway (Johannessen *et al.*, 2010), were hyper-activated when the RAS/RAF/MAPK/ERK pathway was blocked by BRAFi. Studies have also demonstrated that overexpression of proteins such as CRAF, N-RAS, cyclin D1, FGF Receptor 3, EGFR, and platelet-derived growth factor receptor β contribute to melanoma BRAFi resistance (Montagut *et al.*, 2008; Smalley *et al.*, 2008; Nazarian *et al.*, 2010; Shi *et al.*, 2011; Yadav *et al.*, 2012; Girotti *et al.*, 2013). Mutation of PTEN (Paraiso *et al.*, 2011) and gatekeeper residue in BRAF (Whittaker *et al.*, 2010) have also been associated with acquired BRAFi resistance in melanoma. An increase in RTK-ligand levels, through autocrine tumor-cell production, paracrine contribution from tumor stroma, or systemic sources, could confer resistance to BRAFi (Wilson *et al.*, 2012). BRAFi resistance have been reported to be developed through NRAS mutations (Nazarian *et al.*, 2010), $BRAF^{V600E}$ mutation amplification (Shi *et al.*, 2012), and $BRAF^{V600E}$ alternative splice variants (Poulikakos *et al.*, 2011). However, the acquired mechanisms of BRAFi resistance remains elusive in melanoma patients.

In this study, we found that EGFR expression was significantly upregulated in BRAFi-resistant melanoma cells and tumor tissues and that the EGFR/PI3K/AKT pathway is hyper-activated in BRAFi-resistant melanoma cells. Our results

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Abbreviations: BRAFi, BRAF inhibitor; BRAFmt, BRAF V600 mutation; EGFRi, EGFR inhibitor; EMT, epithelial to mesenchymal transition; ERK, extracellular signal–regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; TMA, tissue microarray

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suggested that, in melanoma, BRAFi resistance is caused by enhanced EGFR expression through epigenetic regulation and that activation of the EGFR/PI3K/AKT pathway contributes to melanoma progression.

RESULTS

Epithelial to mesenchymal transition characterizes melanoma resistance to BRAFi

Melanoma, a tumor of neuroectodermal origin, can display epithelial to mesenchymal transition (EMT), which is a complex process associated with invasion and metastasis of various types of solid tumors (Kudo-Saito *et al.*, 2009; Weiss *et al.*, 2012; Craene and Berx, 2013). We developed several BRAFi melanoma cells (see Materials and Methods) and compared their phenotype with respective parental cells. BRAFi-resistant melanoma cells were more elongated and fibroblast-like in shape compared with respective parental cells and resembled mesenchymal cells (Figure 1a; Supplementary Figure 1 online). To determine whether expression of known mesenchymal protein markers was changed, we performed immunoblotting on paired cells. The expression of vimentin, fibronectin, α-smooth muscle actin, and N-cadherin was upregulated in BRAFi-resistant cells (Figure 1b). This confirmed that development of BRAFi resistance is associated with an EMT phenotype.

Resistant cells have aggressive growth

We characterized BRAFi-resistant melanoma cells and showed that migration and invasion were higher in BRAFi-resistant cells than in respective parental cells (Figures 1c and d; Supplementary Figures 2 and 3 online). The soft agar colony formation assay was performed to investigate the cell's ability to grow unattached to a surface suspended in agar. The 3D matrigel assay was used to monitor invasive potential of cells. To compare growth and invasive potential of parental and resistant cells, we performed the soft agar colony formation assay and 3D matrigel gel culture. Our results showed that BRAFi-resistant cells grew faster compared with respective parental cells in soft agar and 3D matrigel culture (Figures 1e and f). Furthermore, quantification of colony formation was shown in Supplementary Figure 4A online, and morphology of M14 to M14R and M219 to M219R in 3D matrigel growth medium (days 1 and 12) was shown for comparison in Supplementary Figure 4B online and Figure 1f, respectively. Analysis of cell growth also confirmed faster growth of BRAFi-resistant cells compared with respective parental cells in 2D adherent cell culture (Supplementary Figures 5A and B online). Combining together, this suggested that resistant cells have more aggressive growth compared with parental cells.

Cyclin D1 activity is related to cell cycle regulation and cell proliferation (Ray *et al.*, 2010; Choi *et al.*, 2012). Immunoblot analysis demonstrated that expression of cyclin D1 was approximately twofold higher in BRAFi-resistant cells than in respective parental cells (Supplementary Figures 5C and D online). These results supported the aggressive growth properties of BRAFi-resistant cells. Upregulated expression of cyclin D1 accelerates G(1)-phase progression in the cell cycle through the RAS/RAF/MEK/ERK pathway (Cai *et al.*, 2013).

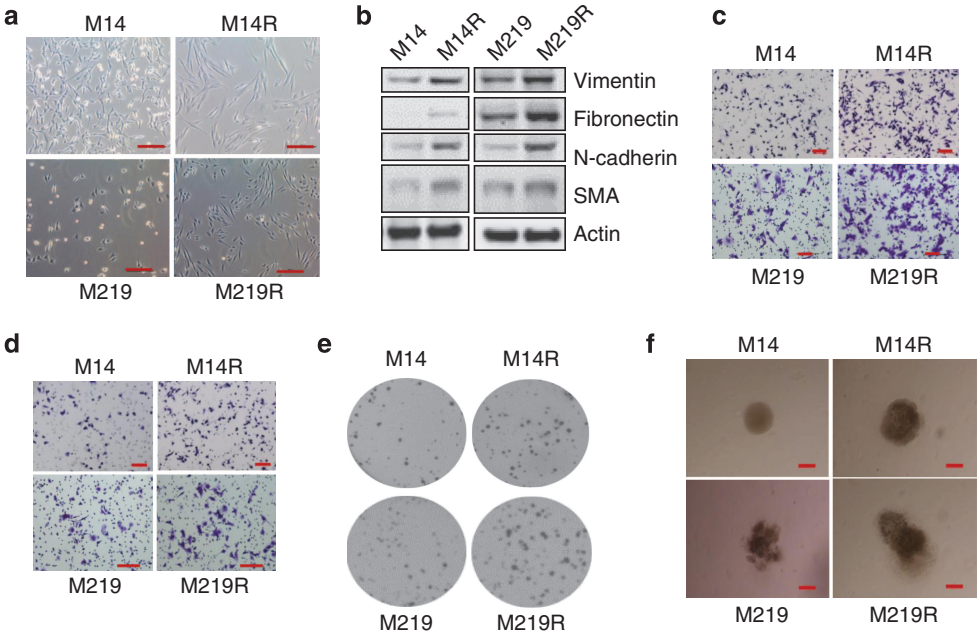

Figure 1. Epithelial to mesenchymal transition (EMT) of BRAF inhibitor (BRAFi)-resistant cells. Proliferation, migration, and invasion of BRAFi-resistant cells. (**a**) BRAFi-resistant melanoma cells (M14R and M219R) were more elongated and fibroblast-like in shape compared with respective parental cells (M14 and M219). Bar = 100 μm. (**b**) Immunoblots of EMT protein markers (vimentin, fibronectin, N-cadherin, and alpha-smooth actin (SMA)). (**c**) Migration of melanoma parental cells (M14 and M219) and BRAFi-resistant cells (M14R and M219R). Bar = 100 μm. (**d**) Invasion of parental cells and BRAFi-resistant cells. Bar = 100 μm. (**e**) Cell growth in soft agar. (**f**) Cell growth in 3D matrigel. Migration and invasion were higher in BRAFi-resistant cells than in respective parental cells, and BRAF-resistant cells grew faster compared with parental cells in soft agar and 3D matrigel. Bar = 100 μm.

Tumorigenicity of parental versus BRAFi-resistant melanoma xenografts

To assess the tumorigenic properties of parental versus BRAFi-resistant cells *in vivo*, 1×10^6 cells from M219, and M219R cell lines were inoculated subdermally into the thigh of male nude-BALB/c mice (6 mice per cell line). BRAFi-resistant cells had significantly higher tumorigenicity and faster growth compared with respective parental cells (Supplementary Figure 6 online). The xenograft study demonstrated the aggressive growth properties of BRAFi-resistant cells (M219R) *in vivo*, supporting the respective analysis of the *in vitro* cell line studies (Supplementary Figure 5A online). This *in vivo* xenograft analysis is one paired parental and resistant cell line.

EGFR expression is enhanced in BRAFi-resistant melanoma cells

Gene expression profiling by gene expression microarray (Affymetrix array) analysis was performed on M14, M14R, M219, and M219R cell lines to identify major significant changes in BRAFi cells compared with respective parental cells. EGFR mRNA expression level was identified 5.51-fold higher in M14R than in M14 cells and 2.76-fold higher in M219R than in M219 cells (Figure 2a, left). RNA-sequence analysis of total RNA from M14 and M14R cells verified higher(6.23-fold) RNA expression for EGFR in M14R cells than in M14 cells (Supplementary Figure 7 online). Quantitative real-time reverse-transcriptase–PCR analysis further confirmed higher expression of mRNA EGFR in M14R(3.85-fold higher) and M219R(5.62-fold higher) cells than in respective parental cells (Figure 2a, right).

To determine whether protein expression of EGFR was enhanced in BRAFi-resistant cell lines, we assessed EGFR expression by immunoblotting and flow cytometry. As shown in Figures 2b and c, EGFR expression was significantly higher in BRAFi-resistant cells than in respective parental cells.

EGFR expression is enhanced in BRAFi-resistant melanoma metastases

We assessed the EGFR expression level by immunohistochemistry in American Joint Committee on Cancer stage III and IV metastatic melanomas using a melanoma tissue microarray

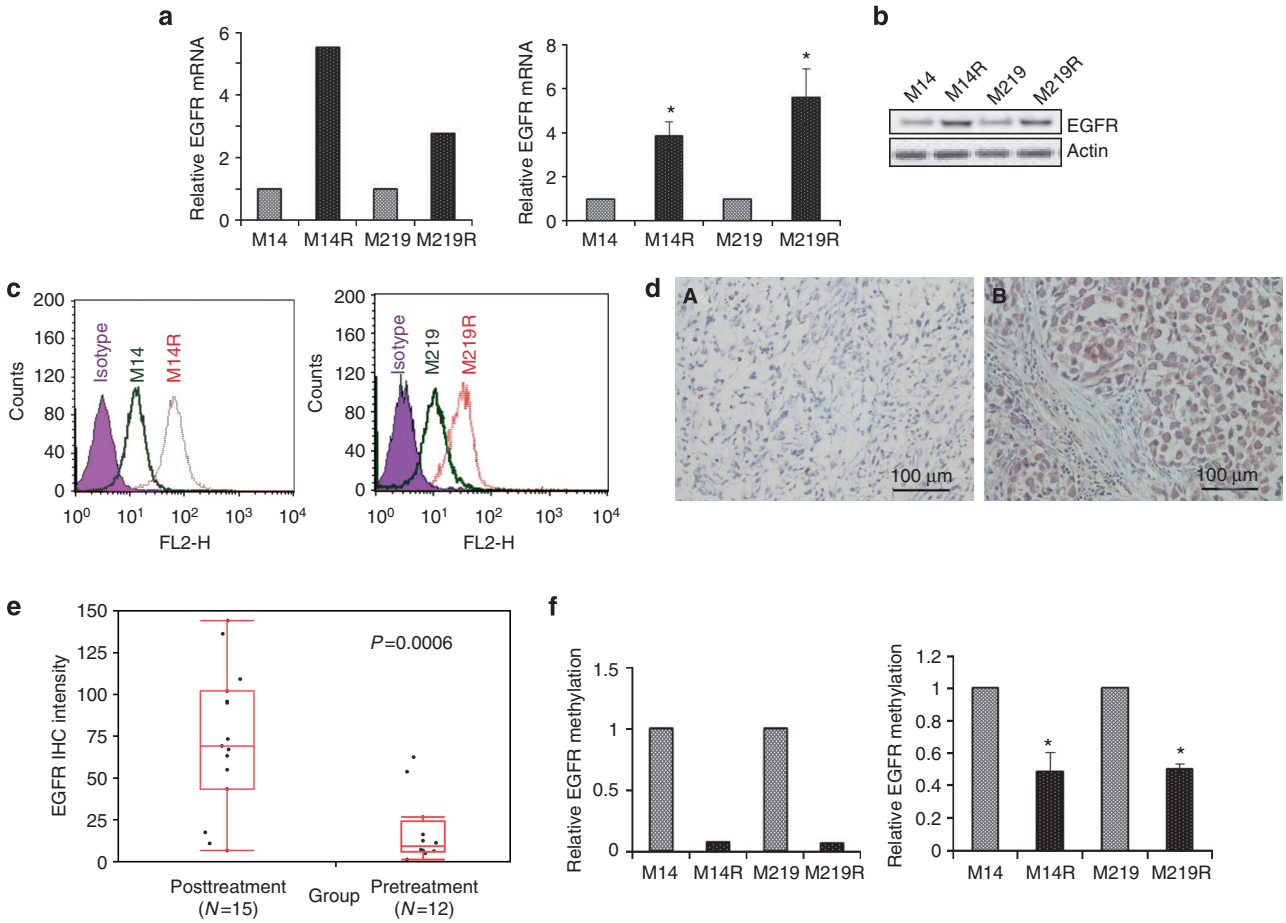

Figure 2. EGFR expression in metastatic melanoma cells (panels a–c) and metastatic melanoma tissues (panels d–f). (**a**) EGFR mRNA expression in parental (M14 and M219) and BRAF inhibitor (BRAFi)-resistant (M14R and M219R) cells assessed by gene expression microarray (left) and quantitative real-time reverse-transcriptase–PCR (right) analyses. (**b**) Immunoblot of EGFR expression in cells. (**c**) Flow cytometric analysis of EGFR expression in cells. (**d**) Representative immunohistochemistry (IHC) stain of EGFR in a metastatic tumor specimen obtained before (**A**) and after (**B**) treatment with BRAFi. In picture **b**, melanoma has higher EGFR expression compared with normal cells in tissue. Bar = 100 µm. (**e**) Intensity of EGFR IHC staining in melanoma tumors obtained from patients before and after treatment with BRAFi (three pre-treatment patients were not available). (**f**) EGFR methylation array (left) and EGFR methylation-specific PCR (right). Error bars = SD (*P <0.05).

(TMA) that was annotated with long-term clinical follow-up data (non BRAFi treatment; Nguyen *et al.*, 2010). American Joint Committee on Cancer stage III and IV melanomas generally had low levels of EGFR expression independent of BRAFmt presence (Supplementary Figure 8 online).

To determine whether EGFR expression was enhanced in BRAFi-resistant melanomas, we immunostained melanoma tumor specimens obtained from 15 patients before and after BRAFi treatment with dabrafenib (13 patients) or vemurafenib (2 patients; Figure 2d and Supplementary Figure 9 online). Analysis of staining intensity showed that EGFR expression was higher in BRAFi-resistant tumors (Figure 2e). To confirm these results, we compared EGFR expression in autologous pairs of BRAFmt melanoma specimens obtained from 12 different patients before and after treatment with BRAFi (Supplementary Figure 10 online). EGFR expression post treatment was significantly higher in BRAFi-resistant recurrent tumor.

EGFR gene hypomethylation in BRAF-resistant cells

As EGFR was demonstrated to be significantly activated in BRAFi-resistant cells, we investigated its expression regulation. Methylation profiling with a HumanMethylation450 BeadChip (Illumina, San Diego, CA) analysis was carried out to determine whether EGFR was epigenetically regulated by CpG site methylation status. The methylation level of the EGFR promoter region was 14.9-fold lower in M14R cells than in M14 cells and 16.4-fold lower in M219R cells than in M219 cells (Figure 2f, left). Methylation-specific PCR confirmed lower EGFR methylation in M14R and M219R cells than in respective parental cells (Figure 2f, right). To verify whether hypermethylation of EGFR affects its expression, M14 and M219 cells were treated with a 5-azacytidine demethylation agent and assessed for EGFR protein expression by immunoblotting. EGFR expression was enhanced in cells treated by 5-azacytidine (Figure 3d; Supplementary Figure 11 online). These results supported that EGFR expression is regulated by the CpG methylation status in the promoter region.

EGFR expression is regulated by the methylation status of EGFR enhancers

Genome-wide DNA methylation and transcriptomic integrative analyses were carried out to further determine whether enhanced EGFR expression in BRAFi-resistant cells was regulated by changes in CpG site methylation. The data from bead chip methylation array were assessed to establish a linear regression and were used to estimate Pearson's correlation coefficient (r) between the methylation level and the EGFR expression level. The analysis included a 65 kb upstream region and a 180 kb downstream region, both measured from the transcription start site of the *EGFR* gene (Figure 3a). The targeted genomic region analysis included the promoter, 5′UTR, 3′UTR, and gene coding regions. The promoter region of *EGFR* gene presents two CpG islands with respective shores and shelves that were also targeted by this analysis. One enhancer region was located upstream of the transcription start site, represented by probe number 4, and the other was located downstream of the transcription start site in the first intron of the gene, represented by probe number 51 (Figure 3b). The regions that showed significant correlation between DNA methylation and the EGFR expression level were located in enhancer elements (r; $P<0.05$; Figure 3c). The comparative methylation analysis of parental and BRAFi-resistant cells demonstrated a consistent hypomethylation in BRAFi-resistant cells in CpG sites located in the upstream and downstream enhancers. EGFR downstream enhancer methylation status was verified by methylation-specific PCR. Supplementary Figure 12 online demonstrated that methylation of EGFR downstream enhancers was much lower in M14R and M219R cells than in respective parental M14 and M219 cells. This strongly suggested that EGFR expression is regulated by methylation status of EGFR enhancers in the BRAFi-resistant melanoma cells.

EGFR expression is associated with cell resistance to BRAFi

The effect of EGFR inhibitor (EGFRi) on BRAFi-resistant melanoma cells was evaluated by treating M14 and M14R cells by BRAFi with or without EGFRi. M14 cells were sensitive to BRAFi but not EGFRi, whereas M14R were not sensitive to BRAFi but sensitive to EGFRi (Figure 4a and Supplementary Figures 13 and 14 online). Furthermore, M14R cells were more sensitive to the combination of EGFRi and BRAFi than to EGFRi alone.

To determine whether EGFR expression confers BRAFi resistance to melanoma cells, we transfected M219 cells with an EGFR expression plasmid and selected stable clones with high EGFR expression (Figure 4b, left). We also transfected M14R with EGFR small interfering RNA to reduce expression of EGFR (Figure 4b, right). M219 cells were more sensitive to BRAFi than were M219-EGFR cells, and M14R-small interfering RNA cells were more sensitive to BRAFi than were M14R (Figures 4c–e). Taken together, these results suggested that EGFR activation was associated with BRAFi resistance in melanoma cells.

EGFR/PI3K/AKT pathway is activated in BRAFi-resistant melanoma cells

To determine whether growth of BRAFi-resistant cells depends on EGFR activation, parental and resistant cells were cultured in RPMI-1640 medium with or without EGF (20 ng ml^{-1}). There was no significant difference in cell growth of parental and resistant cells cultured in medium without EGF (Figure 5a). However, BRAFi-resistant cells grew faster compared with parental cells when cultured in medium with EGF. This suggested that growth of resistant cells was more dependent on EGF compared with respective parent cells.

To further investigate EGFR activation in resistant melanoma cells, we employed Human Phospho-RTK array to assess phosphorylation of receptor tyrosine kinase. EGFR was more phosphorylated in resistant cells than in parental cells (Figure 5b). This suggested that overexpression of EGFR in resistant cells leads to enhanced tyrosine phosphorylation of EGFR. To further confirm that EGFR activation in cells was involved in acquired resistance to BRAFi, we performed immunoblotting to evaluate phosphorylation of EGFR at Tyr1068, which indicated kinase activation. Results

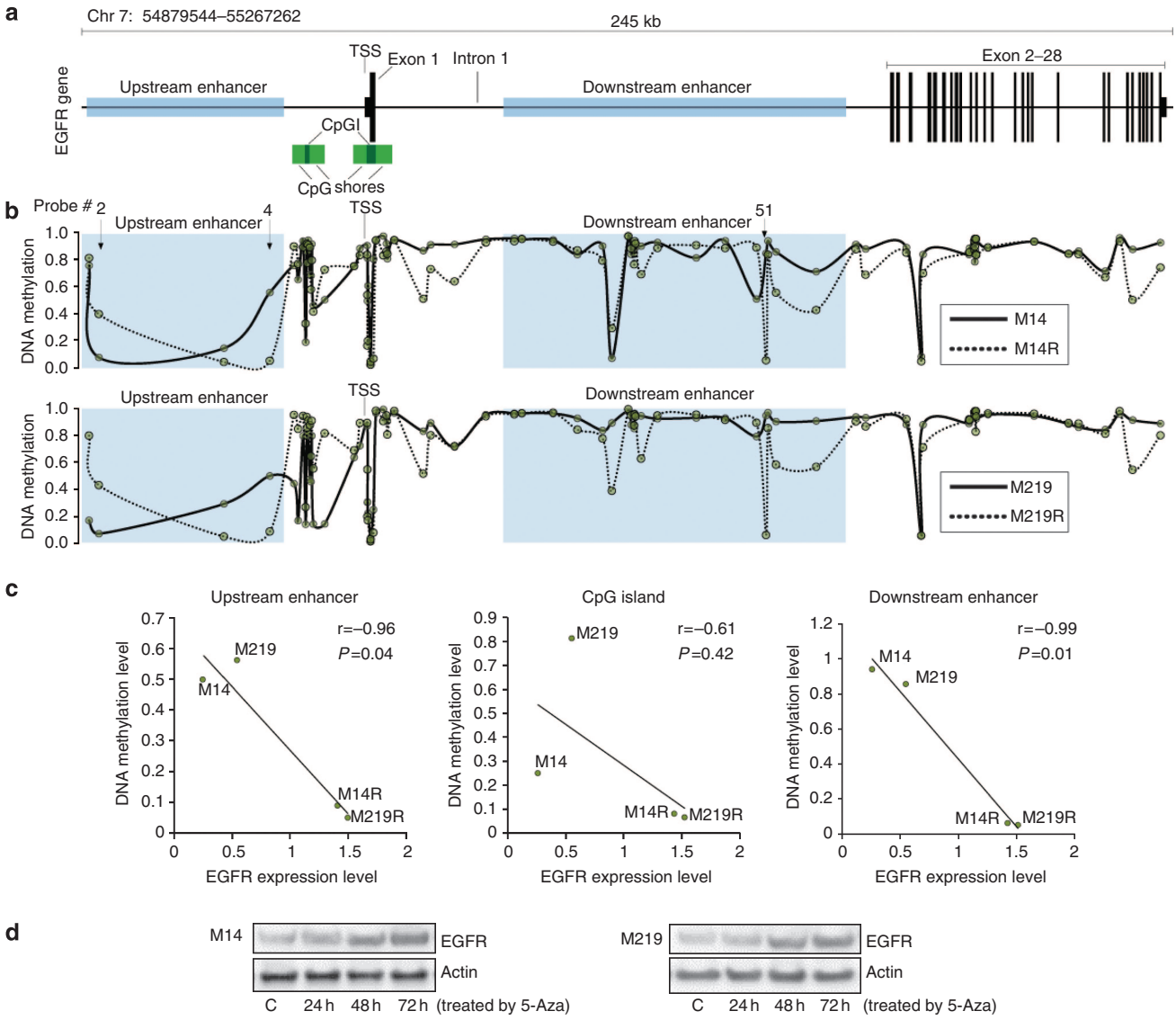

Figure 3. Hypomethylation of EGFR enhancers in BRAF inhibitor (BRAFi)-resistant melanoma cells. (**a**) The 245 kb region surrounding EGFR gene (65 kb upstream and 180 kb downstream to the transcription start site (TSS). (**b**) Profiles of DNA methylation at region surrounding EGFR gene. One enhancer region located upstream of the TSS is represented by the probe #4, and the other located downstream of the TSS, specifically in the first intron of the gene, represented by probe #51 defined in HumanMethylation450K BeadChip array (Illumina), respectively. (**c**) There was significant correlation between DNA methylation, and expression levels were located in enhancer elements. (**d**) Immunoblot of EGFR expression in M14 and M219 treated with 5-azacytidine (5-Aza). EGFR expression was increased in M14 and M219 treated with 5-Aza compared with treatment by DMSO control for 72 h.

demonstrated that phosphorylation of EGFR was significantly enhanced (Figure 5c).

To examine whether EGFR was activated when the RAS/RAf/MAPK/ERK pathway is blocked, M225 cells (high expression of EGFR, Figure 5d) were treated with the MEK inhibitor AZD6244 or with BRAFi. After 24 hours, phosphorylated-EGFR (Tyr1068) and p-AKT were significantly increased, EGFR was also significantly increased, and p-ERK1/2 (p42/p44) was significantly decreased in BRAFi-treated cells (Figure 5e and Supplementary Figure 15 online). Expression of EGFR was increased in BRAFi-treated cells. EGFR dimerization is formed as the EGFR level increases. EGFR dimerization stimulates its intrinsic intracellular protein tyrosine kinase activity (autophosphorylation of EGFR), thereby the expression of p-EGFR increases.

These results suggested that EGFR was activated when the RAS/RAF/MAPK/ERK pathway was blocked.

To determine whether activation of EGFR leads to signaling via the PI3K/AKT pathway, AKT phosphorylation was monitored after treatment of M14 and M14R cells with EGF, alone or in combination with the AKT inhibitor CCT128930(10 μM). AKT phosphorylation was significantly higher in M14R cells and was inhibited by CCT128930 (Figure 5f).

To further confirm activation of the EGFR/PI3K/AKT pathway, phosphorylation of p85, expression of PI3K components, and downstream targets of the EGFR/PI3K/AKT pathway

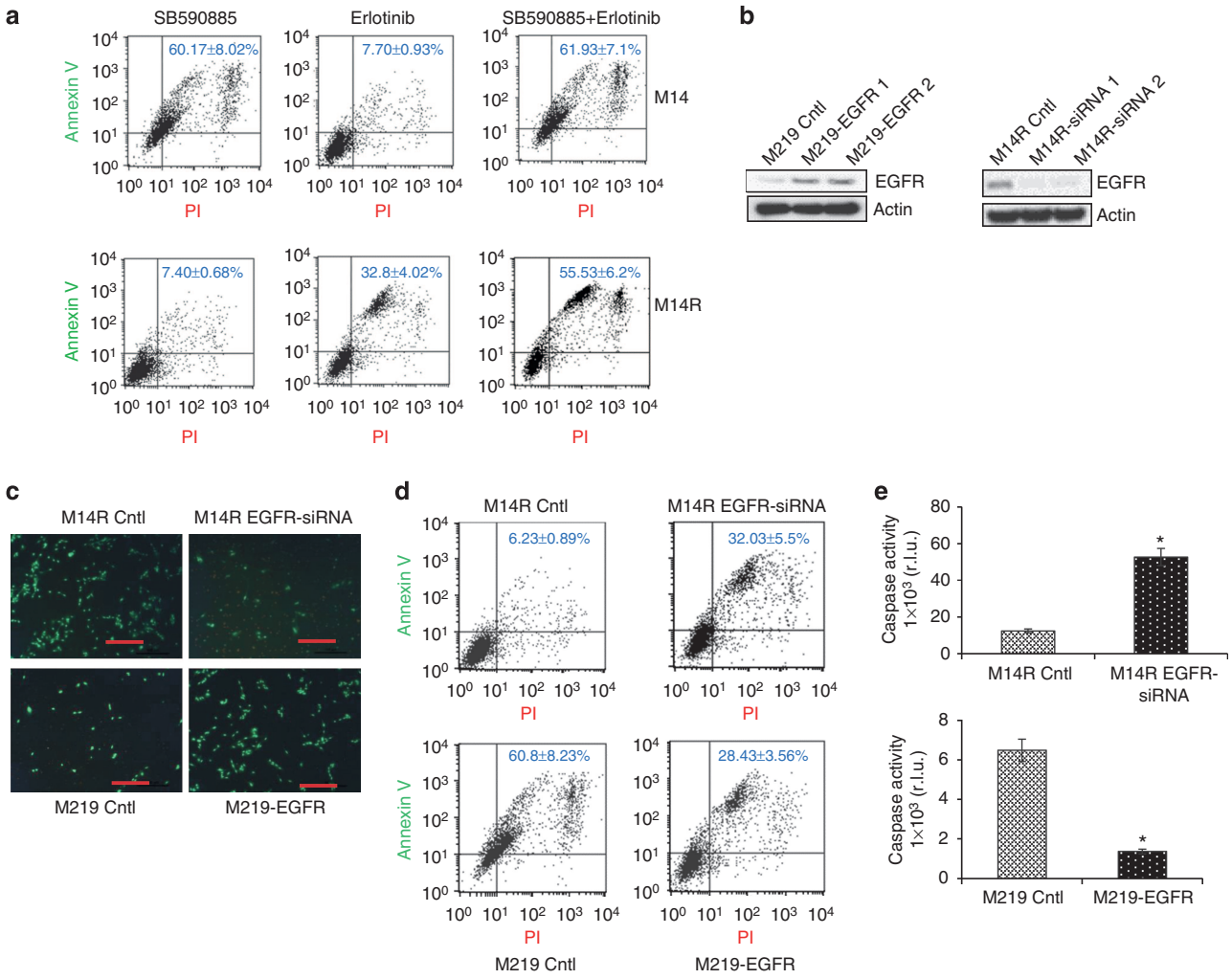

Figure 4. EGFR confers resistance to BRAF inhibitor (BRAFi) in melanoma lines. (**a**) Representative FACS scatter plots of treated cells stained with Annexin V (*y*-axis) versus propidium iodide (PI; *x*-axis). (**b**) Immunoblots of EGFR expression in M219 (left) and M14R (right) cells, before and after transfection with EGFR expression plasmid or EGFR small interfering RNA (siRNA), respectively. (**c**) Fluorescence microscopy of cells treated by SB590885 and stained with PI (5 μg ml^{-1}) and SYTO-13 (1 μmol ml^{-1}). Bar = 100 μm. (**d**) Representative FACS scatter plots of SB590885-treated cells that were stained with Annexin V (*y*-axis) versus PI (*x*-axis). (**e**) The Caspase3/7-Glo luminescent assay of cells treated with SB590885. Error bars, SD (*$P<0.05$).

were assessed. Expression levels of p110 alpha, phosphorylated-p85 (Tyr458), p-AKT (473), and phosphorylated 4E-BP1 (downstream targets of the EGFR/PI3K/AKT pathway) were higher in resistant cells than in parental cells (Figure 5g). Taken together, these results suggested that the EGFR/PI3K/AKT pathway is activated in BRAFi-resistant melanoma cells.

Combination of EGFRi and BRAFi overcomes acquired resistance to BRAFi

To further confirm the role of EGFR in acquired resistance to BRAFi, the effect of combining EGFRi and BRAFi on M14, M14R, M219, and M219R cells grown in a 3D matrix tumor-like microenvironment was assessed. M14R and M219R cells were more sensitive to combined inhibitors than to either single inhibitor. However, M14 and M219 cells (low EGFR expression) were sensitive to BRAFi but not to EGFRi. The photographs of the cells were taken at days 1 and 18 for comparison (Figure 6a and Supplementary Figures 16–18 online). M14R and M219R cells (high EGFR expression) were sensitive to the combined inhibitors, whereas M14 and M219 (low EGFR expression) were not sensitive to EGFRi. These results indicated that activated EGFR is responsive to EGFRi in BRAFi-resistant melanoma cells.

DISCUSSION

We present evidence that BRAFi-resistant melanoma cells develop an EMT phenotype, and EGFR-mediated activation of the PI3K-AKT pathway is induced as a consequence through BRAFi resistance. Enhanced expression of EGFR in BRAFi-resistant cells was due to epigenetic changes in EGFR activation. This epigenetic changes led to hyperactivity of the EGFR/PI3K/AKT pathway. BRAFi-resistant melanoma cells were sensitive to combined treatment with EGFRi and BRAFi. Our results suggested that epigenetic changes of EGFR led

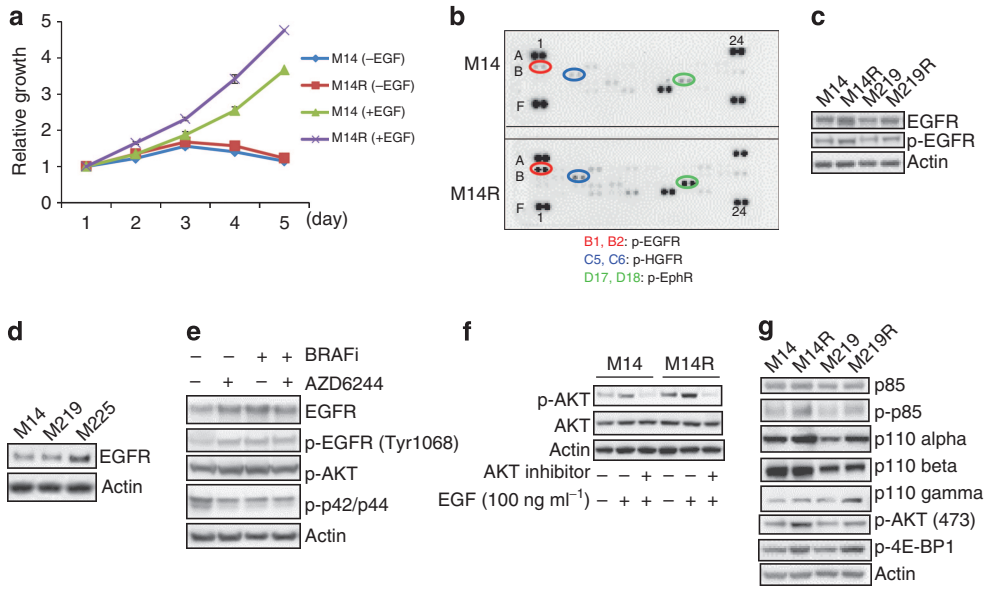

Figure 5. EGFR/PI3K/AKT pathway activation in BRAF inhibitor (BRAFi)-resistant melanoma cells. (**a**) Growth curve of M14 and M14R cells in RPMI-1640 medium with or without EGF. (**b**) The Human Phospho-RTK array assay of M14 cells (top figure) and M14R cells (bottom figure) (B1, B2 (red circle): p-EGFR; C5, C6 (blue circle):p-HGFR; D17, D18 (green circle): p-EphR). Both pictures were exposured for 10 seconds. (**c**) Immunoblots of p-EGFR (Tyr1068) expression in parental and resistant cells. (**d**) Expression of EGFR in three BRAF V600 mutation (BRAFmt)-bearing cell lines: M14, M219, and M225. (**e**) Immunoblots show that MAPK/ERK kinase (MEK) inhibitor AZD6244 blocked MEK-mediated regulation of EGFR in BRAFmt cells. (**f**) Immunoblots of AKT and phosphorylated-AKT expression in M14 and M14R cells exposed to EGF after a 1-hour treatment with AKT inhibitor. (**g**) Immunoblots show expression of components on the PI3K/AKT pathway. Expression levels of p110 alpha, phosphorylated-p85 (Tyr458), p-AKT (473), and phosphorylated 4E-BP1 were higher in resistant cells than in parental cells.

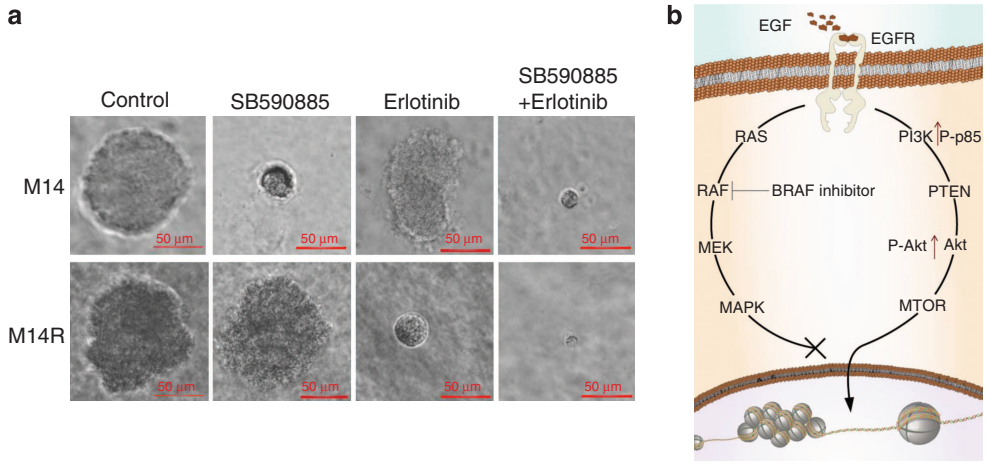

Figure 6. Response of cells exposed to EGFR inhibitor (EGFRi) and BRAF inhibitor (BRAFi) in a 3D-tumor-like microenvironment. (**a**) M14 parental cells and M14R resistant cells grown in a 3D-tumor-like microenvironment were treated with DMSO, BRAFi (SB590885), and/or EGFRi (erlotinib). Bar = 50 μm. (**b**) Flow diagram of EGFR/PI3K/AKT pathway activation in resistant cell lines treated by BRAFi. The EGFR/PI3K/AKT pathway was activated when the RAS/RAF/MEK/ERK pathway was blocked by BRAFi.

to enhanced expression and played an important role in BRAFi-resistant cells. The drug combination co-targeting BRAFmt and EGFR-positive melanomas may provide an effective alternative treatment strategy to improve durable clinical responses.

BRAFi-resistant cells displayed a distinct EMT-like phenotype with spindle-shaped morphology as well as upregulation of EMT markers. These morphological changes of BRAFi-resistant cells confirmed previous results (Nazarian *et al.*, 2010). EMT has been associated with metastasis of lung and pancreatic cancers that are resistant to treatment with EGFR and AKT inhibitors (Byers *et al.*, 2013; Marais *et al.*, 2013). Our results demonstrated that migration and invasion of resistant cells were enhanced compared with parental cells. The EMT phenotype and EGFR expression are known to be closely related to cell migration and invasion (Zhang *et al.*,

2012; Zhu *et al.*, 2012). This may partially explain why recurrent melanomas are more aggressive in BRAFi-resistant melanoma patients. We believe these resistant cells revert to an EMT-like phenotype with activation of the EGF signal pathways. Melanoma is of embryonic neuroectoderm origin, and during advanced stages may revert to its embryonic ectoderm phenotype.

The modulation of EGFR transcription is a complex mechanism that involves both genetic and epigenetic factors. Our results suggested that the hypomethylation of two different related promoters' DNA elements significantly regulates EGFR expression. Remarkably, these two regions are located in the genomic enhancer elements. DNA enhancer elements interact with protein complexes that regulate the transcriptional rate of a gene or a group of genes. In breast cancer, the mechanism involving the enhancers of *EGFR* gene was shown to be related to anti-EGFR therapy response (McInerney *et al.*, 2001; Brandt *et al.*, 2006).

In a study of colon cancer, investigators found that BRAFi effect on BRAFmt tumors causes a rapid feedback activation of EGFR, which supports cell growth (Prahallad *et al.*, 2012). EGFR-mediated reactivation of MAPK signaling allows BRAFmt colorectal cancers to resist vemurafenib treatment (Corcoran *et al.*, 2012). Our results demonstrated that expression of EGFR and phosphorylated-EGFR was increased in BRAFi-resistant melanoma cells. Expression levels of P110α and phosphorylated-p85 (Tyr458) in PI3K/AKT were enhanced, whereas no significant enhancement on other components of the MAPK/MEK/ERK pathway was observed. We believe that the EGFR/PI3K/AKT pathway is activated when the MAPK/MEK/ERK pathway is blocked by BRAFi. EGFR IHC further confirmed that there was higher EGFR expression in resistant melanoma tumors than in BRAFi pretreated melanoma patient tumors. EGFR-mediated reactivation of PI3k/AKT signaling provided BRAFi-resistant melanoma cells with an alternative mechanism to grow and progress.

Oncogene addiction is a phenomenon in which some cancers that contain multiple genetic, epigenetic, and chromosomal abnormalities remain dependent on (addicted to) one or a few genes for both maintenance of the malignant phenotype and cell survival, especially in drug-resistant cancer cells (Weinstein, 2002; Torti and Trusolino, 2011; Kuehl and Bergsagel, 2012). Our studies indicated that BRAFi-resistant melanoma cells became ''addicted to'' EGFR oncogenic activity as do EMT cells during tumor progression. Overexpression of EGFR rendered cells resistant to BRAFi, whereas knockdown of EGFR rendered cells sensitive to BRAFi. Expression of EGFR in BRAFi-resistant tumors was increased, which also supported the findings that EGFR rendered cells sensitive to BRAFi. These findings suggested that EGFR has an important role in BRAFi resistance. Development of resistance to tyrosine kinase inhibitors is a common problem often associated with chronic treatment with these receptor tyrosine kinase-targeted anticancer drugs (Solit and Rosen, 2011). Drug combinations have recently been employed widely to treat aggressive drug-resistant melanomas (Kim *et al.*, 2011; O'Day *et al.*, 2013). We demonstrated that growth of resistant cells was significantly inhibited by a combination of BRAFi and EGFRi, whereby growth was not affected by BRAFi alone. These findings coincided with studies described in BRAFi treatment of colon cancer (Prahallad *et al.*, 2012). The EGFR/PI3K/AKT pathway is activated when RAS/RAF/MEK/ERK is inhibited by BRAFi. BRAFi-resistant cells are epigenetically activated to overexpress EGFR and respond to EGF (Figure 6b).

This study is to demonstrate that EGFR conferred epigenetic activation upon resistant development to BRAFi *in vitro* and *in vivo*. Our MSP results showed that the methylation level of EGFR in resistant cells was much lower than that in parental cells. It is known that enhancer elements can have a major regulation of gene expression and not strictly the methylation status of the gene promoter region. Our results also showed that there was consistent hypomethylation of EGFR in BRAFi-resistant cells in CpG sites located in the enhancer region. Taken together, our results suggested that the increase in EGFR expression in resistant cells was due to epigenetic regulation including hypomethylation of the promoter and enhancer regions. We believe that epigenetic changes of EGFR have an important role in aggressive tumor growth in BRAFi-resistant cutaneous melanomas.

Our study sheds light on the molecular basis of resistance and suggestive mechanisms of growth when BRAFi resistance develops. The EGFR and EGF axis activation in melanomas may be underestimated. It is known that primary melanoma metastasizes to distant organ sites that are ectoderm in origin. When invaded and damaged these organ sites release EGF for repair and regrowth. As a consequence, the melanoma cells bearing EGFR can take advantage of this microenvironment damage repair to survive and continue to grow through this process.

MATERIALS AND METHODS

Cell lines and tissues

The established human melanoma cell lines (M14, M219, and M225) that contained $BRAF^{V600E}$mt were used in the studies. Tumor tissues from stage III/IV melanoma patients were obtained from Melanoma Institute Australia, Sydney, Australia and JWCI (John Wayne Cancer Institute, Santa Monica, CA) with written informed consent from each patient under approved Human Research Ethics committee protocols. Melanoma metastatic biopsies were taken from patients prior to commencing BRAFi and after surgical resection when resistance and progression occurred.

TMA

TMA were developed as previously described and were well clinically annotated with >5 years follow-up (Camp *et al.*, 2008; Nguyen *et al.*, 2010). AJCC Stage IV melanoma TMA included 268 distant organ metastases and 39 autologous stage III/LN metastases, as well as 29 normal tissues from each respective organ (cancer-free).

BRAFi-resistant BRAFmt melanoma lines

To obtain melanoma-resistant cells, BRAFmt melanoma cells from established cell lines M14, M219, and M225 of early passages were cultured with gradually increasing concentration (0.1 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1 μM) of BRAFi(SB590885 in DMSO). SB590885, a potent and highly selective inhibitor of BRAF kinase (Villanueva *et al.*,

2010), was purchased from SYMANSIS NZ (Timaru, New Zealand). Resistant melanoma cells were maintained in the continuous treatment of 1 μM of SB590885 for 3 months. These BRAFi-resistant melanoma cell lines were designated as M14R, M219R, and M225R. The resistant cells were maintained in the BRAFi drug treatment after 3 months. All the experiments were performed while cells were maintained in the presence of the BRAFi drug.

RNA extraction and array-based gene expression analysis

Total RNA was extracted from melanoma-related specimens using the RNeasy Mini Kit (QIAGEN, Valencia, CA). RNA samples were analyzed by GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) at the USC/CHLA Genome Core Laboratory. Data were analyzed in Expression Console 1.1 software (Affymetrix) using the core transcript set and employing the robust multi-array average algorithm for background correction and normalization of data.

RNA deep sequencing

Samples of high quality RNA (RIN $\geq$ 8.0) were used to create mRNA libraries using the Illumina TruSeq RNA Sample Preparation Kit v2 (Illumina). The mRNA libraries were then sequenced on the Illumina HiSeq 2500 high-throughput mode using the TruSeq SBS v3–HS 200 cycle kit (Illumina) according to standard procedures generating over 35 million 100-base pair paired-end reads per sample. Base calling and demultiplexing were processed using CASAVA v1.8 (Illumina), alignment was performed using TopHat2 (Baltimore, MD) (Kim *et al.*, 2013), and expression values were generated using Cufflinks (Roberts *et al.*, 2011).

Infinium HumanMethylation450K beadchip analysis

DNA was extracted from cell lines with the QIAamp DNA mini kit (QIAGEN, Valencia, CA). Extracted DNA (1 μg) was first treated with sodium bisulfite and then recovered using the Zymo EZ DNA methylation kit (Zymo Research, Irvine, CA), quantified and assessed for quality according to the manufacturer's specifications (Campan *et al.*, 2009). The Illumina HumanMethylation450K BeadChip (Illumina) was used to assay the methylation status of >450K CpG sites across the genome (Marzese *et al.*, 2014). Data were processed at the USC High Performance Computing Center (HPCC) using a dedicated, Linux-based, high performance computational cluster. The methylation level was reported as a beta-value (β = intensity of the methylated allele/intensity of the unmethylated allele + intensity of the methylated allele), which was calculated using the signal intensity value for each CpG site.

Biostatistical analysis

The results are presented as mean ± SD of samples measured in triplicate and repeated three times. Student's *t*-test was used to calculate differences between the various study groups. Significant difference was considered at $P<0.05$.

Additional materials and methods information is available in Supplementary Material online.

Data access

The study data are deposited in NCBI's Sequence Read Archive (SRA) data bank (http://www.ncbi.nlm.nih.gov/sra/) and accession file SRP022029.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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