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BRAF Inhibitor Therapy for Melanoma, Thyroid and Colorectal Cancers: Development of Resistance and Future Prospects.

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ABSTRACT

BRAF is a major oncoprotein and oncogenic mutations in *BRAF* are found in a significant number of cancers, including melanoma, thyroid cancer, colorectal cancer and others. Consequently, BRAF inhibitors have been developed as treatment options for cancers with *BRAF* mutations which have shown some success in improving patient outcomes in clinical trials. Development of resistance to BRAF kinase inhibitors is common, however, and overcoming this resistance is an area of significant concern for clinicians, patients and researchers alike. In this review, we identify the mechanisms of BRAF kinase inhibitor resistance and discuss the implications for strategies to overcome this resistance in the context of new approaches such as multi-kinase targeted therapies and emerging RNA interference based technologies.

INTRODUCTION

BRAF (v-RAF murine sarcoma viral homolog B1) is a proto-oncogene and a member of the RAF (Rapidly accelerated fibrosarcoma) family of proteins which are serine/threonine kinases. *RAF* genes were originally identified as retroviral oncogenes in 1983 [1-4]. Three RAF kinase proteins (ARAF, BRAF and CRAF) have been identified so far. CRAF (also known as RAF-1) was first discovered in 1985, ARAF in 1986 and BRAF in 1988 [4-8].

BRAF mutation is common and is present in around 8% of all cancers. Mutations most frequently occur in melanoma (40-70%), followed by thyroid (36-53%) and colorectal (5-22%) cancers [3-4, 9-12]. In cutaneous melanoma, BRAF mutation was found in 64% of trunk, 50% of arm/leg, 46% of head/neck and 20% of hand/foot cancers [13]. In thyroid cancer, *BRAF* mutations are only found in papillary thyroid carcinoma (45%) and papillary thyroid carcinoma-derived undifferentiated thyroid carcinoma (25%). Moreover, in different subtypes of papillary thyroid carcinoma the highest frequency of *BRAF* mutations are seen in tall-cell papillary thyroid carcinoma (77%) and the lowest frequency of *BRAF* mutations are seen in the follicular variant of papillary thyroid carcinoma (12%), whereas the frequency in conventional papillary thyroid carcinoma is 60% [4, 12, 14]. In addition, *BRAF* mutations are also found in ovarian serous carcinoma, gliomas, non-small cell lung carcinoma, hepatobiliary carcinoma and hairy cell leukaemia [4, 11, 15].

BRAF mutation has been associated with poor outcomes in a few cancers. The median survival for *BRAF* mutated metastatic melanoma patients was found to be only 5.7 months without any BRAF inhibitor treatment [16]. In papillary thyroid carcinoma, the *BRAF* V600E mutation was found to be associated with high risk clinicopathological factors as well as poor outcomes in a study performed by Kim *et al*. The result in the study also suggested that presence of *BRAF* V600E mutation should be considered as poor prognostic marker [17]. In addition, *BRAF* V600E mutation was found to be associated with poor outcome in patients with colorectal cancer in independent studies [18-19].

Most of the *BRAF* mutations appear in the glycine rich loop and activation segment of the BRAF kinase protein [4, 9-10, 20]. Although more than 65 *BRAF* mutations have been discovered so far, the V600E mutation is the most frequent, comprising more than 90% of mutations [8-9, 11]. This *BRAF* V600E mutation occurs near an Asp-Phe-Gly (DFG) motif in the activation segment of the BRAF protein where valine (V) is substituted with glutamic acid (E) [4, 9, 20]. This mutation destabilizes the hydrophobic interaction between the glycine rich loop and activation segment of BRAF kinase protein which results in the flipping of the DFG motif to its active orientation (DFG-in). As a result, BRAF returns to its active conformation, achieving a ~500 fold

increased kinase activity as compared to wild-type BRAF as well as self-sufficiency in sending proliferation and survival signals without any upstream or external stimuli. The result triggers uncontrolled cellular proliferation and survival [4, 9-10, 20]. Consequently, BRAF is an important target for interventions to control the growth of cancer cells.

Currently, different kinds of BRAF inhibitors are being used for the treatment of patients with *BRAF* mutated cancer. All these BRAF inhibitors are small molecule kinase inhibitors which are divided into two types: Type-I BRAF inhibitors (like Vemurafenib, Dabrafenib *etc.*) and Type-II BRAF inhibitors (like Sorafenib, Regorafenib, *etc.*) [21-24]. As all the BRAF inhibitors are ATP competitive kinase inhibitors, they are to some extent multi-kinase inhibitors and are able to inhibit other kinases beyond the BRAF V600E kinase, though the strength of their capacity to do so varies. Type-I BRAF inhibitors bind with the protein kinase in its active (DFG-in) conformation, whereas type-II BRAF inhibitors bind with the protein kinase in its inactive (DFG-out) conformation [21-22, 25-29]. By binding with the mutant BRAF protein kinase, a BRAF inhibitor inhibits the activities of mutant *BRAF*, which results in the inhibition of uncontrolled cellular proliferation driven by these mutations.

RESISTANCE

The response rates (complete or partial response) of different BRAF inhibitors in Phase-III clinical trials were found to be 1-50% [30-35]. The highest partial or complete response (50%) in Phase-III clinical trials was found with Dabrafenib which was studied in patients with metastatic melanoma [32]. Vemurafenib showed 48.4 % partial or complete response in its Phase-III clinical trial in patients with metastatic melanoma [30-31]. In addition, BRAF inhibitors Sorafenib and Regorafenib showed only partial response in Phase-III clinical trials where Sorafenib showed 2% partial response which was utilised in patients with advanced hepatocellular carcinoma and Regorafenib showed only 1% partial response tested in patients with metastatic colorectal cancer [33-35].

The median progression free survivals of patients treated with different BRAF inhibitors in Phase-III clinical trials were seen to vary from 1.9-6.9 months [30-35]. The highest median progression free survival in Phase-III clinical trials was observed with Vemurafenib which was 6.9 months in patients with metastatic melanoma and the lowest median progression free survival (1.9 months) was found with Regorafenib in its Phase-III clinical trials on patients with metastatic colorectal cancer [30-31, 34-35]. Dabrafenib showed 5.1 months median progression free survival in its Phase-III clinical trials on patients with metastatic melanoma

whereas the median time to radiological progression with Sorafenib was 5.5 months which was studied in patients with advanced hepatocellular carcinoma [32-33, 35].

The data to date demonstrate that at least half of the patients treated with BRAF inhibitors did not respond to the therapy. Also, patients who initially responded to BRAF inhibitor therapy eventually developed progressive disease after only a few months. Therefore, it is clear that some patients are resistant to BRAF inhibitor therapy from the beginning, whereas others develop resistance to the therapy a few months later. Based on the presence of immediate and developing resistance in patients, it has been hypothesised that resistance mechanisms for BRAF inhibitor therapy can be divided into two broad categories: intrinsic resistant and acquired resistance.

Intrinsic Resistance

There are some factors in tumour microenvironment whose pre-existing dysregulation or mutations have been found to contribute to intrinsic resistance of BRAF inhibitor therapy in melanoma (Fig. 1). These factors are found to include: cell cycle regulators (cyclin D1), regulators of alternative proliferation signalling pathways (PTEN) and hepatocyte growth factor (HGF) [36-39].

Cyclin D1 is the regulatory subunit of holoenzymes that inhibits the activity of the retinoblastoma protein which acts a gatekeeper of G_1 phase in cell cycle [40-42]. Smalley *et al* found in an experiment that cyclin D1 was amplified in 17% of *BRAF* V600E mutated metastatic melanoma. They also discovered that overexpression of cyclin D1 contributes to the resistance of BRAF inhibitor therapy in melanoma, which further increases with the overexpression of cyclin-dependent kinase-4 (CDK4) [37]. Cyclin D1 (*CCND1*) also acts as a collaborative oncogene by increasing oncogenic transformation of other oncogenes like *RAS*, *SRC* and *E1A* [43-45].

Phosphatase and tensin homolog (PTEN) is a lipid phosphatase and regulator of PI3K, which is a member of an alternative pathway (PI3K-AKT/PKB-mTOR pathway) for cellular proliferation and survival. It has been found in melanoma that alteration in PTEN is associated with the lowest response rates in BRAF inhibitor therapy. In addition, it has been also discovered that PTEN loss contributes to the resistance of BRAF inhibitor therapy through suppression of BIM (Bcl-2 interacting mediator of cell death)-mediated apoptosis [36, 46-47].

Hepatocyte growth factor is secreted from stromal cells and is able to activate receptor c-MET (MNNG HOS transforming gene) which is a receptor tyrosine kinase. Activated receptor c-MET is able to activate both

RAS-RAF-MEK-ERK pathway as well as PI3K-AKT/PKB-mTOR pathways which are important for cellular proliferation and survival. It has been found that stromal cell secretion of hepatocyte growth factor contributes to the resistance of BRAF inhibitor therapy in melanoma, which may be due to activation of both of the proliferation and survival pathways [38-39]. Deregulation of these proliferation and survival pathways also plays an important role in the development and progression of carcinogenesis [38-39, 48].

Acquired Resistance

Acquired resistance mechanisms develop during BRAF inhibitor therapy. They are subdivided into different categories: ERK (extracellular signal-regulated kinase)-dependent acquired resistance, ERK-independent acquired resistance and ABC (ATP-binding cassette) transporter mediated acquired resistance.

ERK-dependent Acquired Resistance

Reactivation of ERK signalling in spite of continuous presence of BRAF inhibitor is responsible for the development of ERK-dependent acquired resistance. Most of the ERK-dependent resistant mechanisms are due to overexpression, feedback activation, transactivation, truncated protein isoforms or mutation of the drug targets, upstream or downstream signalling molecules (Fig. 2, 3 & 4). However, no further mutation has been found in V600E mutated BRAF proteins either in pre-clinical BRAF inhibitor resistant models or from biopsies taken from BRAF inhibitor resistant patients [49-56].

Elevated CRAF Expression

Montagut and colleagues showed in a preclinical experiment that elevated levels of CRAF protein were responsible for the reactivation of ERK signalling in melanoma despite the continuous presence of selective BRAF inhibitor AZ628 [49]. They revealed that the resistance was associated with switching dependency of the pathway from BRAF to CRAF. Overexpressed CRAF activated MEK (Mitogen activated protein kinase [MAP] or ERK kinase), which resulted in reactivation of ERK signalling through ERK kinase, which drove further cellular proliferation and ultimately resulting in resistance against BRAF inhibitor therapy. They also showed that elevated levels of CRAF protein were not associated with gene amplification or increased gene transcription. Rather, it was associated with a post-transcriptional regulatory mechanism. In addition, expression levels of ARAF and BRAF proteins were found to be unchanged in this study [49].

Splice Variants of Mutant BRAF

A 61kD variant of V600E mutated BRAF protein was found to cause resistance to Vemurafenib in a subset of melanoma cells. This 61 kD variant lacks exons 4-8 which encode the RAS-binding domain (RBD) [50]. In addition, this variant exhibited an elevated tendency for dimerization as compared to conventional V600E mutated BRAF protein. In the 61kD variant, dimerization occurs independent of RAS. However, this type of dimerization also happens at low levels of RAS activation [50]. The proposed mechanism for this type of resistance is that these dimers are transactivated by ATP-competitive BRAF inhibitors which then activate MEK kinase. Activated MEK kinase phosphorylates ERK kinase which reactivates ERK signalling and caused the development of BRAF inhibitor resistance [50, 55].

Amplification of Mutant BRAF

V600E mutant *BRAF* amplification has been found in 20% of melanoma patients resistant to BRAF inhibitors [51]. Overexpressed V600E mutant *BRAF* was found to hyperactivate MEK kinase which was 5 to 6 times higher than the basal level in melanoma. Hyperactivated MEK then reactivated ERK signalling through phosphorylation of ERK kinase which resulted in BRAF inhibitor resistance. This type of resistance does not depend on CRAF protein [51]. However, amplification of V600E mutant *BRAF* had been found to be largely mutually exclusive with receptor tyrosine kinase overexpression, NRAS mutation or alternative splicing of V600E mutated *BRAF* [51, 57].

NRAS Mutation

Q61K mutated NRAS had been found in both pre-clinical BRAF inhibitor resistant models of melanoma as well as biopsies taken from patients with BRAF inhibitor (PLX4032) resistant melanoma. While different alleles of *RAS* (*HRAS*, *KRAS* and *NRAS*) had been sequenced in such patients, only *NRAS* mutations were found to date [52, 58]. The Q61K mutation resulted in a marked increase in activated NRAS levels. Activated NRAS used CRAF to activate the MEK kinase. Activated MEK then reactivated ERK signalling through phosphorylation of ERK kinase despite continuous presence of BRAF inhibitors. However, *NRAS* mutation was found to be mutually exclusive with platelet-derived growth factor receptor-β (PDGFRβ) overexpression in melanoma [52, 58-59]. PDGFRβ is a kind of receptor tyrosine kinase.

CRAF Mutation

Multiple CRAF point mutations (S257P, P261T and G361A) were identified by Antony *et al* in a preclinical BRAF inhibitor resistance model in melanoma which were capable of inducing biochemical and pharmacological resistance to BRAF inhibitors (specifically vemurafenib and PLX4720) [60]. These CRAF mutations significantly upregulated CRAF kinase activity in a dimerization dependent manner (homodimerization or heterodimerization with BRAF) and kinase activated CRAF was able to phosphorylate MEK kinase. Activated MEK kinase then reactivated the ERK signalling through phosphorylation of ERK kinase which eventually resulted in the development of resistance to BRAF inhibitor therapy [60].

MEK Mutation

Different MEK1 mutations (Q56P, C121S, P124L and E203K) have been found in pre-clinical BRAF inhibitor resistance melanoma models. These mutations were also identified in biopsies taken from melanoma patients resistant to BRAF inhibitors (PLX4032 and PLX4720). All these mutations markedly increased kinase activity of MEK1. Kinase activated MEK1 then phosphorylated ERK kinase which contributed to the reactivation of ERK signalling and helped in the development of resistance against BRAF inhibitors [53, 56, 58].

COT (Cancer Osaka Thyroid) Overexpression

Cancer Osaka thyroid (COT) is an enzyme that in humans is encoded by the *MAP3K8* (mitogen-activated protein kinase kinase kinase 8) gene. Johannessen and co-authors discovered overexpression of COT (Tp12) in pre-clinical BRAF inhibitor resistance melanoma models. They also identified COT overexpression in biopsies taken from melanoma patients resistant to the BRAF inhibitor PLX4720 [54]. Elevated levels of COT were found to reactivate ERK signalling through phosphorylation of ERK kinase in a RAF independent manner, which resulted in the development of resistance to BRAF inhibitor therapy. In addition, COT was also found to be able to activate ERK signalling both in MEK-dependent and in certain contexts MEK-independent manners [54].

Feedback Activation of Epidermal Growth Factor Receptor (EGFR)

Feedback activation of EGFR (epidermal growth factor receptor), a kind of receptor tyrosine kinase, was found to be responsible for development of resistance to BRAF inhibitor (vemurafenib) therapy in

melanoma and colorectal cancers [61-64]. Overexpression of activated EGFR (p-EGFR) was also found in biopsies taken from BRAF inhibitor (vemurafenib) resistant colorectal cancer patients [64]. ERK signalling was strong in BRAF V600E mutated cancer which produced strong feedback inhibition of EGFR through expression of SPRY2 (Sprouty homolog 2) and CDC25C (cell division cycle 25 homolog C**,** a phosphatase of EGFR). SPRY2 and CDC25C suppressed activation of EGFR, preventing related growth signalling in *BRAF* V600E mutated cancer [61-63]. When BRAF V600E mutated cancer was treated with BRAF inhibitors, ERK reduced was suppressed which also resulted in the reduced expression of SPRY2 and CDC25C. Therefore, the feedback inhibition mediated through the SPRY2 and CDC25C, was diminished which resulted in the ligand-dependent activation of EGFR. Activated EGFR is able to phosphorylate RAS and through it reactivate ERK signalling via activation of the CRAF-MEK-ERK pathway [61-64]. In addition, activated EGFR was also found to be responsible for developing ERK-independent resistance through activation of the PI3K-AKT-mTOR pathway in BRAF mutated cancers [62-63].

Feedback Activation of HER3 (V-erb-b2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 3)

BRAF inhibitor (vemurafenib) resistance was found to occur in thyroid cancer through feedback activation of HER3 which is a kind of receptor tyrosine kinase [62, 65]. In BRAF mutated cancer, strong ERK signalling increased binding of transcriptional repressors CTBP1 (C-terminal-binding protein 1) and CTBP2 (C-terminal-binding protein 2) to the *HER3* gene promoter. As a result, expression of HER3 was suppressed [62, 65]. But, CTBP1 and CTBP2 were released from the *HER3* gene promoter when *BRAF* mutated cancer was treated with BRAF inhibitors. In a manner similar to EGFR based resistance, BRAF inhibitors initially suppressed ERK signalling, which resulting in reduced binding of CTBP1 and CTBP2 to the *HER3* gene promoter [65]. This phenomenon triggered the expression of *HER3* gene and its subsequent translation. HER3 then bound with its ligand NRG1 (Neuregulin-1) and activated RAS through heterodimerization with HER2 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2), another receptor tyrosine kinase as HER3 is a kinase inactive receptor. HER2 was also found to be overexpressed in the same research by Montero-Conde *et al* [65]. Levels of basal HER2 and activated HER2 (p-HER2) were also found to be high in another experiment done by Corcoran *et al* on colorectal cancer [64]. HER2/HER3 activated RAS was then able to reactivate ERK signalling through activating the CRAF-MEK-ERK pathway. Moreover, like EFGR based resistance, HER3/HER2 heterodimers were also found to activate the PI3K-AKT-mTOR pathway in *BRAF* mutated cancer, which resulted in the ERK-independent resistance [65].

ERK-independent Acquired Resistance

ERK-independent acquired resistance mechanisms do not depend on ERK signalling for cellular proliferation and survival. This type of resistance is due to activation of parallel or alternative pathways (other than the RAS-RAF-MEK-ERK pathway) for cellular proliferation and survival. Mechanisms for activation of these pathways are summarised in figures 3, 4, 5 & 6.

Overexpression of PDGFRβ

Overexpression of platelet-derived growth factor receptor-β (PDGFRβ) had been found in preclinical BRAF inhibitor resistance melanoma and thyroid cancer models [52, 65]. Up-regulated expression of PDGFRβ (another receptor tyrosine kinase) was also seen in biopsies taken from melanoma patients resistant to the BRAF inhibitor PLX4032 [52]. It had been also found that melanoma cells that overexpressed PDGFRβ had low levels of activated RAS. However, significant reactivation of ERK signalling was not found while the patients were being treated with the BRAF inhibitor (PLX4032). Therefore, the proposed mechanism for development of resistance in this case was the activation of a PDGFRβ dependent alternative proliferation and survival pathway, specifically the PI3K-AKT-mTOR pathway. Stable knockdown of PDGFRβ in BRAF inhibitor resistant cells showed growth inhibition in the continuous presence of BRAF inhibitor (PLX4032), though it failed to induce apoptosis [52, 57].

Elevated Activation of IGF-1R

Increased levels of activated insulin-like growth factor 1 receptor (IGF-1R), a member of the receptor tyrosine kinase group, had been discovered in preclinical BRAF inhibitor resistant melanoma and colorectal cancer models [64, 66]. Overexpression of IGF-1R was also identified in biopsies taken from melanoma patients resistant to the BRAF inhibitor PLX4032 [66]. Activated IGF-1R is usually able to activate both the RAS-RAF-MEK-ERK pathway as well as the PI3K-AKT/PKB-mTOR pathway. Despite this, overexpressed IGF-1R showed increased phosphorylation of AKT and no significant phosphorylation of ERK in the experiment done by Villanueva *et al* in melanoma. Therefore, the resistance in this case was hypothesised to be due to activation of the PI3K-AKT/PKB-mTOR pathway which agrees with a similar study done by Corcoran *et al* in colorectal cancer [64, 66].

Activation of EGFR-SFK-STAT3 Signalling

Activation of the EGFR-SFK-STAT3 signalling pathway was found to be responsible for BRAF inhibitor (PLX4720) resistance in a preclinical experiment done by Girotti *et al* on melanoma. Elevated activation of epidermal growth factor receptor and SFK (SRC family kinase) were also found in biopsies taken from melanoma patients resistant to the BRAF inhibitor vemurafenib [67]. In this resistance mechanism, EGF (epidermal growth factor) binds with the epidermal growth factor receptor and activates it, resulting in the phosphorylation of STAT3 (signal transducer and activator of transcription 3) with the help of the SFK protein. After activation, STAT3 translocates to the nucleus and induces transcription of genes responsible for proliferation and survival which finally result in functional resistance to BRAF inhibitor therapy [67-68]. Moreover, this pathway activation was found to induce not only proliferation and survival activities, but it also activated invasion and metastatic processes, making it potentially even more clinically significant [67].

ABC Transporters Mediated Acquired Resistance

ATP-binding cassette (ABC) transporter, ABCG2 was found to be responsible for developing BRAF inhibitor resistance in a preclinical study done by Wu *et al* [69]. The experiment was done on *BRAF* V600E mutated A375 melanoma cell lines treated with the BRAF inhibitor vemurafenib. They showed that overexpression of active ABCG2 transporters was able to efflux vemurafenib from the A375 melanoma cell line (Fig. 7). Therefore, vemurafenib concentration inside the A375 melanoma cells was reduced which resulted in decreased efficacy of vemurafenib in inhibiting mutated BRAF kinase. Insufficient inhibition of mutant BRAF kinase triggered reactivation of the BRAF pathway and contributed to the development of BRAF inhibitor resistance. It was also found in the study that vemurafenib had no effects in modulating the protein expression of ATP-binding cassette (ABC) transporters like ABCG2, ABCB1 and ABCC1 [69]. In addition, vemurafenib was shown to have a strong binding affinity with the ABCG2 transporter and a low binding affinity with another ATP-binding cassette (ABC) transporter ABCB1, which further contributes to the development of resistance. This may open up ways to block or evade this pathway [69].

FUTURE THERAPY

Multi-kinase Targeted Therapy

Because of emergence of resistance due to activation of alternative proliferation and survival mechanisms, a single kinase targeted therapy is ineffective in *BRAF* mutated cancer. Therefore, it is wise to

target multiple kinases to inhibit cancer cell proliferation and induce apoptosis. The target kinases should be selected from both of the major proliferation and survival pathways to stop cancer cells from switching to alternative proliferation and survival mechanisms. Moreover, we need to find out the most effective kinases whose inhibition not only will arrest proliferation but also will induce apoptosis.

In Vitro Multi-kinase Targeted Study

A number of attempts to apply combination therapy on BRAF mutated cancers have already been put in place. One of the combination studies was performed in melanoma using MEK and PI3K inhibitors [70]. In that study, U0126 was used as the MEK inhibitor and LY294002 was used as the PI3K inhibitor. They found that when BRAF mutated melanoma cell lines were treated with both U0126 and LY294002, cell growth was inhibited by 60% due to their combined action. However, this growth inhibition was actually reversible, which means further incubation of the treated cell line without the drugs showed cell growth again. Cell cycle and apoptosis analysis showed that growth inhibition caused by the combined drug treatment was associated with a G_1 phase cell cycle arrest, rather than induction of apoptosis in that study.

A range of combination studies were done in experiment on *BRAF* mutated melanoma cell lines where they checked the inhibitory effect of combined BRAF and MEK inhibitor, BRAF and PI3K/mTOR inhibitor as well as MEK and PI3K/mTOR inhibitor on BRAF inhibitor resistant melanoma cell line clones [71]. In the study, the authors used GSK2118436 (Dabrafenib) as the BRAF inhibitor, GSK1120212 (Trametinib) as the MEK inhibitor and GSK2126458 as the PI3K/mTOR inhibitor. It was noted that all the three combinations gave enhanced growth inhibition when compared to individual drugs. However, the anti-proliferative effect of BRAF and PI3K/mTOR inhibitor combination was less potent than either of the other two combination studies. Furthermore, BRAF and MEK inhibitor combination failed to increase apoptosis activity when compared to individual drugs. However, slightly increased apoptotic activities were seen in the other two combination treatment groups when compared to individual drugs.

In a combination study on thyroid cancer, a MEK inhibitor (AZD6244) was combined with an mTOR inhibitor (Rapamycin) [72]. This treatment showed at least 60% growth arrest in BRAF mutated thyroid carcinoma cell lines, which was found to be a consequence of cytostatic effects rather than a cytotoxic effect. They also did the same dual inhibition study on a *RET-PTC* (rearranged during transfection- papillary thyroid carcinoma) mutated thyroid cancer cell line (TPC1) and xenograft model of that cell line in nude mice. It was found that dual pathway inhibition by MEK inhibitor and mTOR inhibitor caused an intense G_1 phase cell cycle

arrest in the cell culture and a cytostatic inhibition in the xenograft model, which was actually reversible. They did not observe any significant apoptotic activity both in cell line and xenograft models.

A further combination study on thyroid cancer was done by combining the AKT inhibitor MK2206 with BRAF inhibitor PLX4032 or MEK inhibitor AZD6244 [73]. In the experiment, synergistic proliferation inhibition was observed compared to individual drugs when *BRAF* mutated thyroid cancer cell lines were treated with both of the combination drugs. Cell cycle and apoptosis analysis revealed that synergistic proliferation inhibition was mainly due to increased cell cycle arrest at G_1 phase rather that significant cell apoptosis, although MK2206 and AZD6244 combination therapy showed a modest apoptosis activity in that study.

In a combination drug therapy study on *BRAF* mutated colorectal cancer, the BRAF inhibitor vemurafenib was combined with the EGFR inhibitor, grfitinib [64]. When *BRAF* mutated colorectal cancer cell lines were treated with the combined drugs, a higher inhibition of proliferation was observed as compared to the individual drugs. Effect of combination therapy was also evaluated in a xenograft mouse model of *BRAF* mutated colorectal cancer cell lines, where the BRAF inhibitor vemurafenib was combined with the epidermal growth factor receptor inhibitor, erlotinib. A significant level of tumour inhibition as well as regression was observed in that xenograft experiment.

Another combination therapy was assessed by Prahallad *et al.* They studied the effect on *BRAF* mutated colorectal carcinoma in which the BRAF inhibitor PLX4032 was combined with EGFR inhibitor cetuximab or grfitinib [63]. As compared to individual drugs, strong synergistic proliferation inhibition was seen when *BRAF* mutated colorectal cancer cell lines were treated with both of the combination drugs. Although individual drug treatment did not show any apoptosis activity, the combined therapies did induce apoptosis in that study. Prahallad *et al* also tested the combination therapy in a xenograft mice model of *BRAF* mutated colorectal cancer cell lines. In this xenograft experiment, they combined the BRAF inhibitor PLX4032 with the epidermal growth factor receptor inhibitors (cetuximab, erlotinib). Also, potent tumour growth inhibition was observed for combination therapy as compared to either drug alone.

Clinical Trial of Multi-kinase Targeted Therapy

A Phase 1 and 2 clinical trial of combination therapy with BRAF inhibitor (Dabrafenib) and MEK inhibitor (Trametinib) was performed on 247 patients with metastatic melanoma having *BRAF V600* mutations (V600E, V600K, V600R) [74]. A total of 85 patients were included in the safety & pharmacokinetic study, whereas 162 patients were randomly selected for combination therapy with Dabrafenib (150 mg) and

Trametinib (1 or 2 mg) or Dabrafenib monotherapy. In this trial, complete or partial response increased to 76% in patients who received 150 mg Dabrafenib and 2 mg Trametinib (combination 150/2) treatment, where as it was only 54% in patients who received monotherapy treatment. The median progression free survival also increased to 9.4 months in patients who received combination 150/2 therapy, whereas it was only 5.8 months in patients who received monotherapy. In addition, 41% patients were alive and progression free in the combination 150/2 group, whereas it was only 9% in monotherapy group at study completion. BRAF inhibitor treatment associated skin lesions were also seen less frequently in patients with combination 150/2 therapy group as compared to the monotherapy group, although MEK inhibitor treatment associated toxicities and other toxicities were more frequent in combination 150/2 group as compared to monotherapy group in that clinical trial.

Multi-kinase Targeted Treatment Efficacy

The lack of increased apoptosis in combination therapies has also been an area of research interest. Gray-Schopfer *et al* showed that tumour necrosis factor-α (TNF-α) inhibited induction of apoptosis when BRAF signalling was inhibited in melanoma cell lines [75]. They also showed that the survival mechanism was based on the activation of nuclear factor-κB (NF-κB) signalling which was activated by TNF-α. Although elevated basal NF-κB signalling activities were seen in melanoma cells, it was not enough to inhibit apoptosis induced by BRAF signalling inhibition. However, inhibition of apoptosis occurred when TNF-α further increased NF-κB signalling activities beyond basal levels [75]. It had also been shown in several studies that immune cells (like macrophages and mast cells) infiltrate into melanoma and these immune cells are capable of secreting TNF-α [76-78]. Elevated basal activities of NF-κB signalling as well as high level of invasive macrophages (secreting TNF-α) found in melanoma contributes to constitutive activation of NF-κB signalling [75, 79]. In addition, expression of NF-κB was also found to be high in melanomas as compared to melanocytes of normal skin that could also contribute to the constitutive activation of NF-κB signalling [80-82]. Elevated basal as well as constitutive activity of NF-κB was also found in thyroid and other carcinomas [83-86]. This constitutive activity of NF-κB in cancer cells gives them capability of escape from apoptosis.

As most of the combination drug treatments failed to induce significant amount of apoptosis activity in BRAF mutated cancer cells despite the successful inhibition of proliferation, it seems that induction of apoptosis in growth arrested *BRAF* mutated cancer cells is a real challenge. To overcome this, we need to find out the target kinases whose inhibition will stop proliferation, induce apoptosis and arrest NF-κB signalling [75, 79]. In

the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR proliferation and survival pathways, CRAF and AKT (protein kinase B) are involved in the activation of NF-κB [4, 87-98]. Therefore, we can consider CRAF and AKT as important target kinases along with mutant *BRAF* for inhibiting the proliferation and survival mechanisms of *BRAF* mutated cancer cells. We can also consider the use of a separate inhibitor of NF-κB in combination with other drugs to help close off additional activation paths.

To prevent the efflux of BRAF inhibitors or other kinase inhibitors from cancer cells as well as to increase the concentration of BRAF inhibitors or other kinase inhibitors inside cancer cells, the delivery method of BRAF inhibitors or other kinase inhibitors should be improved so that ATP-binding cassette (ABC) transporters would not able to recognise and efflux the BRAF or other kinase inhibitors out of the cytoplasm into interstitial fluid. This would result in higher concentrations of all those inhibitors inside the cancer cells and would effectively inhibit all those target kinases. In addition, a suitable suppressor of ATP-binding cassette (ABC) transporters could also be combined with multi-kinase targeted therapy for better outcomes.

Gene Therapy

BRAF inhibitors are actually not highly specific to mutant *BRAF*, and are known to have several off target effects. As a result, BRAF inhibitor therapy shows a lot of toxicities. To get rid of these toxicities or reduce the toxicities at least to a tolerable range, we should ideally design a drug that would be highly specific to its target, inhibiting the target and nothing else. We can explore different available as well as potentially more effective approaches to achieve that objective. For example, we can use targeted siRNA, shRNA, bi-shRNA and miRNA therapy in the treatment of patients with *BRAF* mutated cancers.

Therapy with siRNA, shRNA and bi-shRNA

siRNA is a small interfering RNA that silences a gene through the RNA interference (RNAi) mechanism which was first discovered in 1998 [99]. RNAi is an evolutionarily conserved process which is used to control developmental processes, to create defence against parasitic nucleic acids as well as heterochromatic silencing in nature [99-101]. Dicer is an endoribonuclease from the RNase III family that cleaves double-stranded RNA and pre-microRNA into short double-stranded RNA (dsRNA) fragments about 20-25 base pairs long, with a two-base overhang on the 3' end. In RNAi mechanisms, the Dicer protein first recognises and cleaves a long dsRNA to an siRNA duplex. The siRNA duplex is then taken up into the RNA-induced silencing complex (RISC) where the antisense strand of the siRNA duplex is incorporated into RISC, to guide RISC to a

homologous target mRNA for specific cleavage. Dicer is also able to recognise and cleave double stranded RNA (dsRNA) derived from vector based shRNA to siRNA [100, 102-105]. Silencing of a gene by siRNA is highly specific and efficient [102, 106]. Because of this high specificity and efficiency, siRNA is being used to silence different target oncogenes and other genes responsible for tumour cell growth, angiogenesis, metastasis and chemo-resistance. Some siRNA based cancer therapies are in clinical trials where all the siRNA based drugs are found to be well tolerated and no dose limiting toxicities have been seen so far [101, 107-111].

siRNA based therapy can be used in the treatment of *BRAF* mutated cancer patients. Specially, mutant *BRAF* genes can be silenced through siRNA to minimise its effect on the proliferation pathways. Subsequently, genes that are overexpressed, mutated or otherwise responsible for reactivation of the RAS-RAF-MEK-ERK pathway or activation of the alternative PI3K-AKT-mTOR pathway could also be silenced through siRNA to inhibit further growth of resistance. In addition, siRNA therapy can also be used to silence the genes responsible for inhibiting apoptosis, or those promoting angiogenesis and metastasis which will result induction of apoptosis as well as preventing further growth and spread of cancer. Again, a combined siRNA therapy consisting of a couple of siRNA drugs which will inhibit cancer cell proliferation, inducing apoptosis or inhibiting angiogenesis as well as metastasis, could be designed for the treatment of patients with *BRAF* mutated cancers.

For increasing the efficacy, durability as well as rapid onset of gene silencing, bi-functional shRNA (bi-shRNA) can be designed instead of either siRNA or shRNA. A bi-shRNA has two stem-loop shRNAs structure where one strand is perfectly matched to the target sequence and other strand is imperfectly matched to the target sequence [101, 112-113]. The perfectly matched strand will cause complete cleavage of the target mRNA and the imperfectly matched strand will activate alternative gene suppression mechanisms most often seen in response to natural micro-RNAs. As a result, more accuracy, efficiency and rapid gene silencing could be achieved [101, 112-115]. Therapies based in bi-shRNA are currently in clinical trial in other cancers [116].

Therapy with miRNA

Micro-RNAs (miRNAs) are a class of highly conserved 18~25 nucleotides long, endogenous, non-coding RNA which were first discovered in 1993 [117-118]. Approximately 1 to 4% genes in human genome actually produce miRNAs [119-120]. miRNAs function similarly to siRNAs through RNAi mechanisms, though they are typically imperfectly matched to their target genes. This imperfect matching allows them to silence multiple genes simultaneously, and as a result, miRNAs are involved in control of a range of cellular

processes like proliferation, differentiation, apoptosis, development, cell cycle progression, immunity, metabolism, stem cell maintenance, aging etc. [100, 121-133]. In the cell, miRNA is first transcribed as a primary miRNA (pri-miRNA) which is then cleaved by Drosha, an RNase III endonuclease, to pre-miRNA in nucleus. Pre-miRNA is then transported to the cytoplasm where Dicer, the RNase III enzyme involved in processing siRNA, cleaves pre-miRNA to a miRNA duplex which consists of a mature miRNA and its complimentary sequence. Afterwards, matured miRNA incorporates into RISC which is capable of silencing target genes [134-139]. miRNA usually binds with the complete or partially complimentary 3'-UTR region of target mRNA and inhibits its protein synthesis. As complete match is not mandatory to inhibit protein synthesis, a single miRNA is capable of silencing hundreds of different genes [119, 136, 140-142]. In addition, miRNA is able to bind with the 5'-UTR region, coding region or a combination of sites of target mRNA to inhibit its protein synthesis although with less potency as compared to 3'-UTR region binding [101, 143-144]. As deregulation like overexpression or down-regulation of a range of miRNAs can contribute to carcinogenesis, miRNA or anti-miRNA based therapy has the potential to minimize the effects of these kinds of deregulation [101, 120].

Control of cancer cell proliferation, survival, angiogenesis and metastasis by using miRNAs or anti-miRNA have already proved to be effective in a number of experiments with different types of cancers [145-164]. In the case of *BRAF* mutated cancer patients, different miRNAs that control the proliferation and survival pathways can be used for treatment. Specially, miRNAs that control the RAS-RAS-MEK-ERK pathway can be used for treatment as mutant *BRAF* deregulates this pathway. In addition, miRNAs that control the PI3K-AKT-mTOR pathway can be combined with previous type of miRNAs as activation of the PI3K-AKT-mTOR pathway is found to be responsible for proliferation and survival of *BRAF* mutated cancer when the RAS-RAS-MEK-ERK pathway is inhibited. Furthermore, miRNAs that control the NF-κB signalling can be combined with the previous type of miRNA therapy to induce significant apoptosis in *BRAF* mutated cancer cells. Also, some miRNAs control angiogenic and metastatic process through controlling the expression of angiogenic factors like vascular endothelial growth factors or other factors responsible for angiogenesis and metastasis [165]. These miRNAs could be used to stop further growth and spread of BRAF mutated cancer.

CONCLUSION

BRAF inhibitor therapy is a single kinase targeted drug therapy which can be a pathway to successful treatment against *BRAF* mutated cancer. The main obstacle to be overcome in making BRAF inhibitor

treatment successful is the development of resistance, due to switching of the cancer to alternative proliferation and survival mechanisms. As cancer is a consequence of multiple genetic disorders, multiple primary kinase targeted drug therapies need to be put in place to combat the wide range of potential growth mechanisms at work. Therefore, future trials and research should concentrate on applying reasonable multiple drug therapy protocols to make more successful treatments against *BRAF* mutated cancers. Also, due to the potential for excessive toxicity from such combination approaches, the drugs should be highly specific to the target kinases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Figure Legends

Figure 1: Mechanisms of intrinsic resistance. High plasma level of hepatocyte growth factor (HGF) is able to activate both of the proliferation and survival pathways through activation of c-MET. In addition, PTEN is a regulator of PI3K-AKT/PKB-mTOR pathway. If PTEN is mutated, the PI3K-AKT/PKB-mTOR pathway becomes uncontrolled, which triggers spontaneous cellular proliferation and survival activities. Moreover, the overexpression of cyclin D1 is also able to send spontaneous cellular proliferation and survival signals to other proteins. All these events help *BRAF* mutated cancer cells escaping from BRAF inhibitor therapy. Pathways blocked by BRAF inhibitor therapies are shown with dotted arrows, resistance pathways are indicated with solid arrows and mutated protein is indicated with solid star.

Figure 2: Mechanisms of ERK-dependent acquired resistance. Any one of multiple events like CRAF overexpression or mutation, COT overexpression, splice variant or amplification of mutant *BRAF*, new RAS mutations, new MEK mutations can trigger spontaneous ERK signalling which results uncontrolled proliferation and survival activities. As a result, *BRAF* mutated cancer cells are able to show resistance against BRAF inhibitor therapy. Pathways blocked by BRAF inhibitor therapies are shown with dotted arrows, resistance pathways are indicated with solid arrows and potentially mutated proteins are indicated with solid star.

Figure 3: Mechanism of ERK-dependent/ERK-independent acquired resistance through feedback activation of epidermal Growth Factor Receptor (EGFR). Feedback changes result in overexpression of EGFR, which binds with its ligand (EGF), and becomes activated. Activated EGFR can result in uncontrolled proliferation and survival through activating both of the downstream proliferation and survival pathways. Activation of these pathways result in *BRAF* mutated cancer cells developing BRAF inhibitor resistance. Pathways blocked by BRAF inhibitor therapies are shown with dotted arrows, resistance pathways are indicated with solid arrows and mutated protein is indicated with a solid star.

Figure 4: Mechanism of ERK-dependent/ERK-independent acquired resistance through feedback activation of HER3. Feedback changes due to BRAF inhibition result in overexpressed HER3. HER3 then binds with its ligand NRG1 and then activates both the downstream proliferation and survival pathways through dimerization with HER2. Activation of these two pathways contributes to developing resistance against BRAF inhibitor therapy. Pathways blocked by BRAF inhibitor therapies are shown with dotted arrows, resistance pathways are indicated with solid arrows and mutated protein is indicated with solid star.

Figure 5: Mechanisms of ERK-independent acquired resistance. By binding with growth factors, overexpressed receptor tyrosine kinases (RTKs) like platelet-derived growth factor receptor-β (PDGFRβ) and insulin-like growth factor 1 receptor (IGF-1R) are able to activate the PI3K-AKT/PKB-mTOR pathway, which is an alternative proliferation and survival pathway. These events assist *BRAF* mutated cancer cells to escape from BRAF inhibitor therapy. Pathways blocked by BRAF inhibitor therapies are shown with dotted arrows, resistance pathways are indicated with solid arrows and mutated protein is indicated with solid star.

Figure 6: Mechanisms of ERK-independent acquired resistance through activation of EGFR-SFK-STAT3 signalling. Overexpressed EGFR binds with its ligand EGF and becomes activated. With the help of SFK, activated EGFR then stimulates STAT3 signalling which induces uncontrolled proliferation, survival, invasion and metastatic activities. All these activities contribute to develop BRAF inhibitor resistance. Pathways blocked by BRAF inhibitor therapies are shown with dotted arrows, resistance pathways are indicated with solid arrows and mutated protein is indicated with solid star.

Figure 7: Mechanism of ABC transporter mediated acquired resistance. Overexpressed and active ATP-binding cassette (ABC) transporters like ABCG2 and ABCB1 pump BRAF inhibitors out of the cytoplasm into interstitial fluid. As a result, BRAF inhibitor concentration in cytoplasm decreases. Low concentration of BRAF inhibitor in cytoplasm produces no or insufficient inhibition of mutant *BRAF* which results in reactivation of the mutant BRAF pathway and contributes in the development of BRAF inhibitor resistance.

Figure 1:

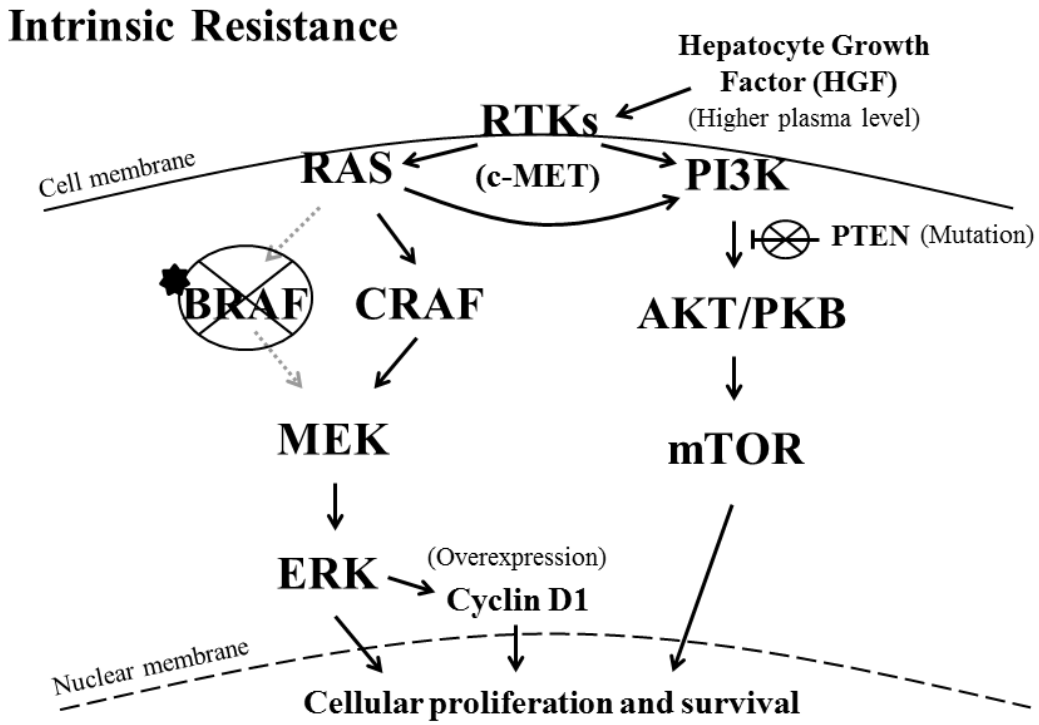

Intrinsic Resistance
Hepatocyte Growth Factor (HGF)
(Higher plasma level)
RTKs
Cell membrane
RAS
(c-MET)
PI3K
PTEN (Mutation)
BRAF
CRAF
AKT/PKB
MEK
mTOR
ERK
(Overexpression)
Cyclin D1
Nuclear membrane
Cellular proliferation and survival

Figure 2:

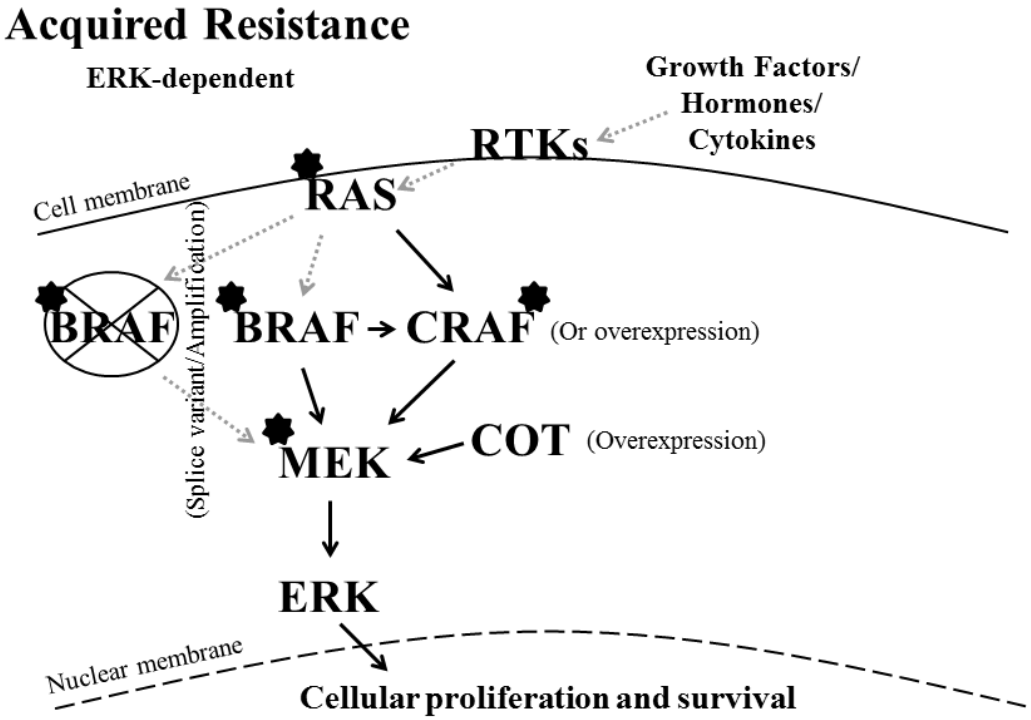

Acquired Resistance
ERK-dependent
Growth Factors/
Hormones/
Cytokines
RTKs
Cell membrane
RAS
BRAF
BRAF → CRAF
(Or overexpression)
(Splice variant/Amplification)
MEK
COT
(Overexpression)
ERK
Nuclear membrane
Cellular proliferation and survival

Figure 3:

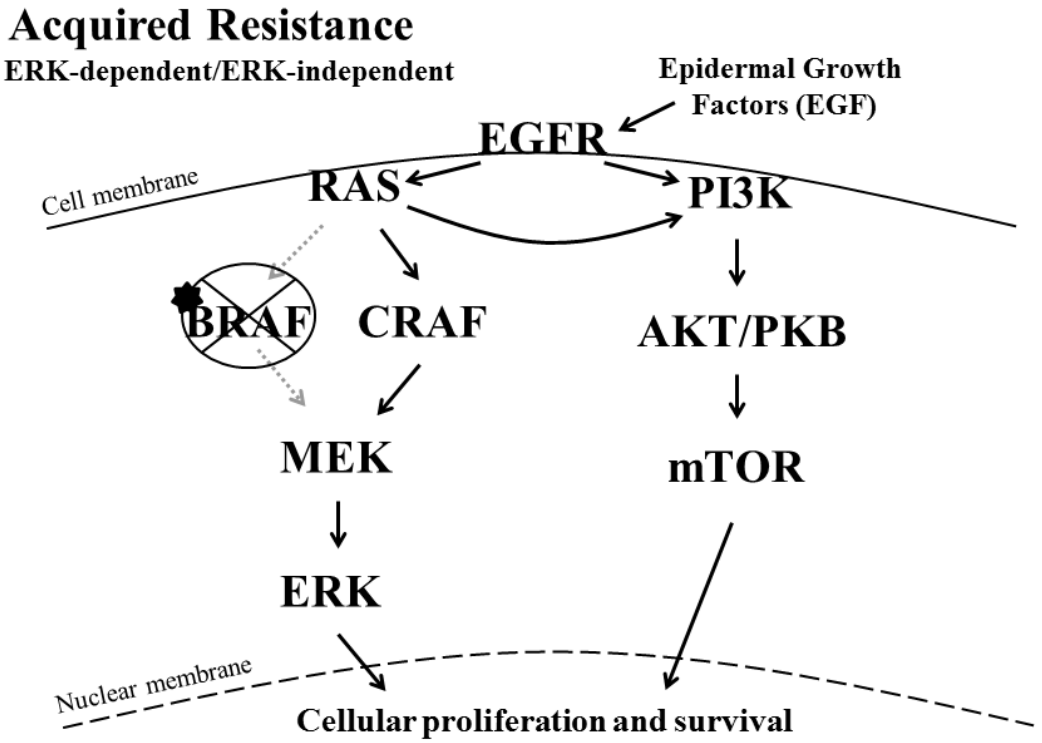

Acquired Resistance
ERK-dependent/ERK-independent
Epidermal Growth Factors (EGF)
EGFR
Cell membrane
RAS
PI3K
BRAF
CRAF
AKT/PKB
MEK
mTOR
ERK
Nuclear membrane
Cellular proliferation and survival

Figure 4:

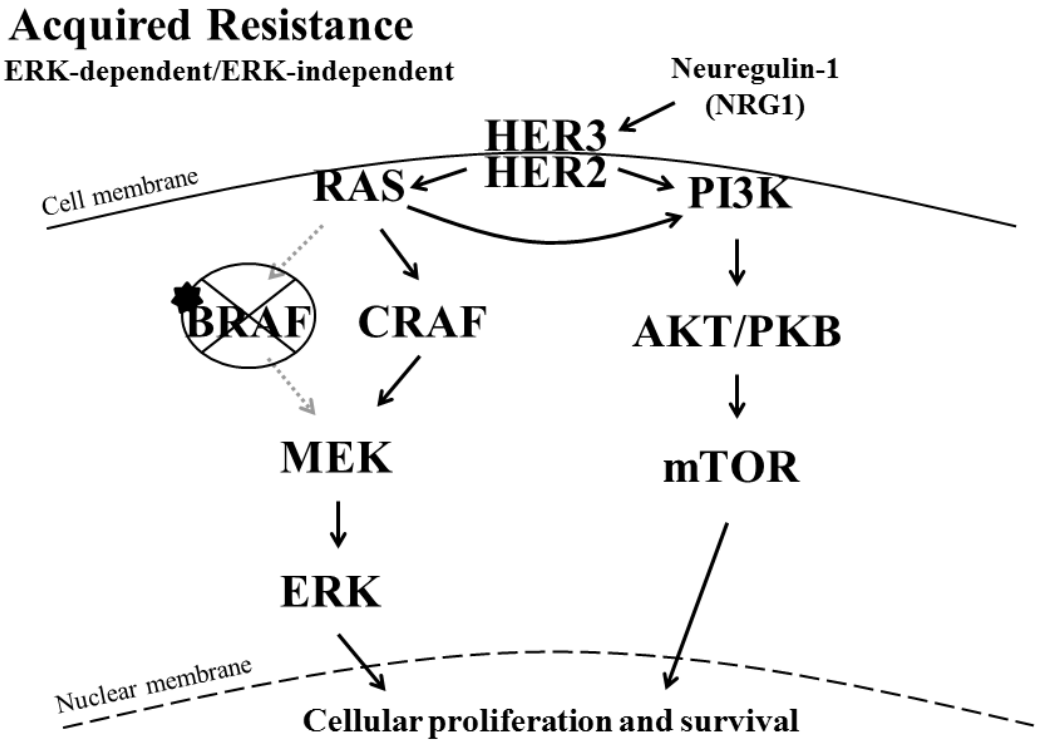

Acquired Resistance
ERK-dependent/ERK-independent
Neuregulin-1
(NRG1)
HER3
HER2
Cell membrane
RAS
PI3K
BRAF
CRAF
AKT/PKB
MEK
mTOR
ERK
Nuclear membrane
Cellular proliferation and survival

Figure 5:

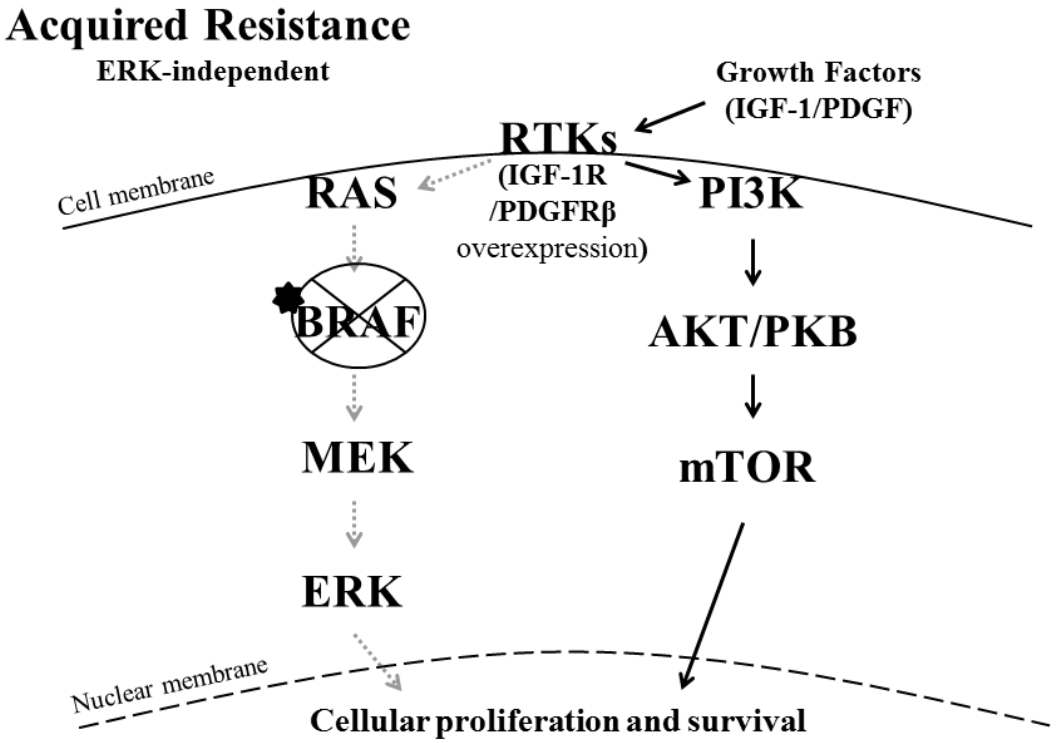

Acquired Resistance
ERK-independent
Growth Factors
(IGF-1/PDGF)
RTKs
(IGF-1R
/PDGFRβ
overexpression)
Cell membrane
RAS
PI3K
BRAF
AKT/PKB
MEK
mTOR
ERK
Nuclear membrane
Cellular proliferation and survival

Figure 6:

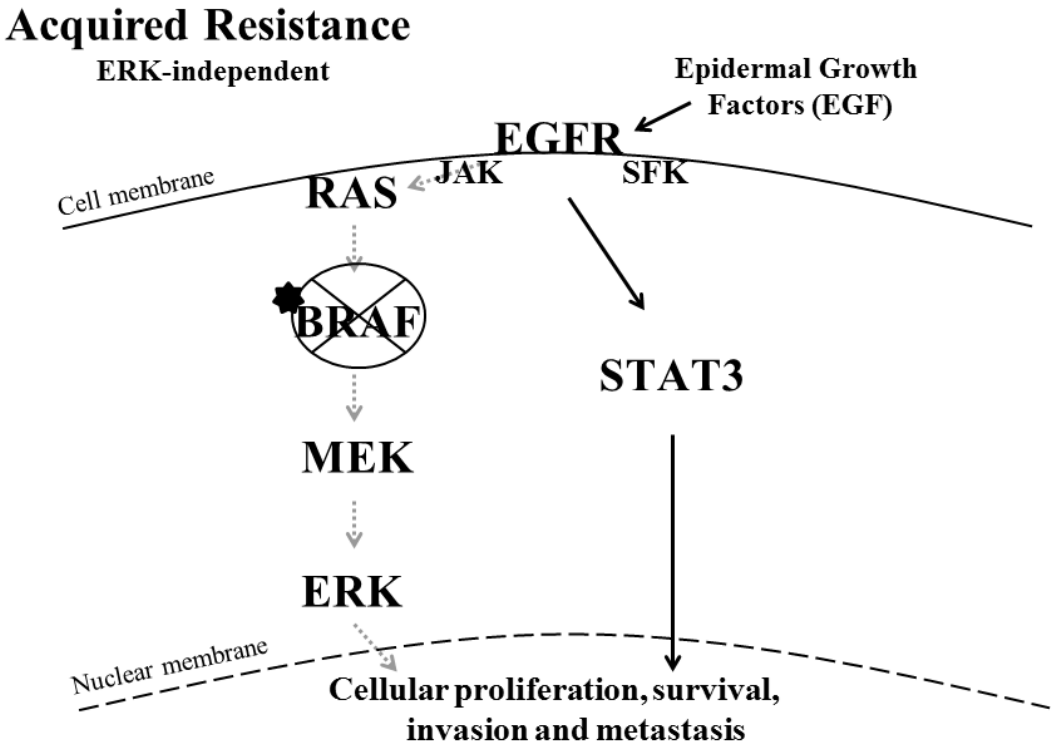
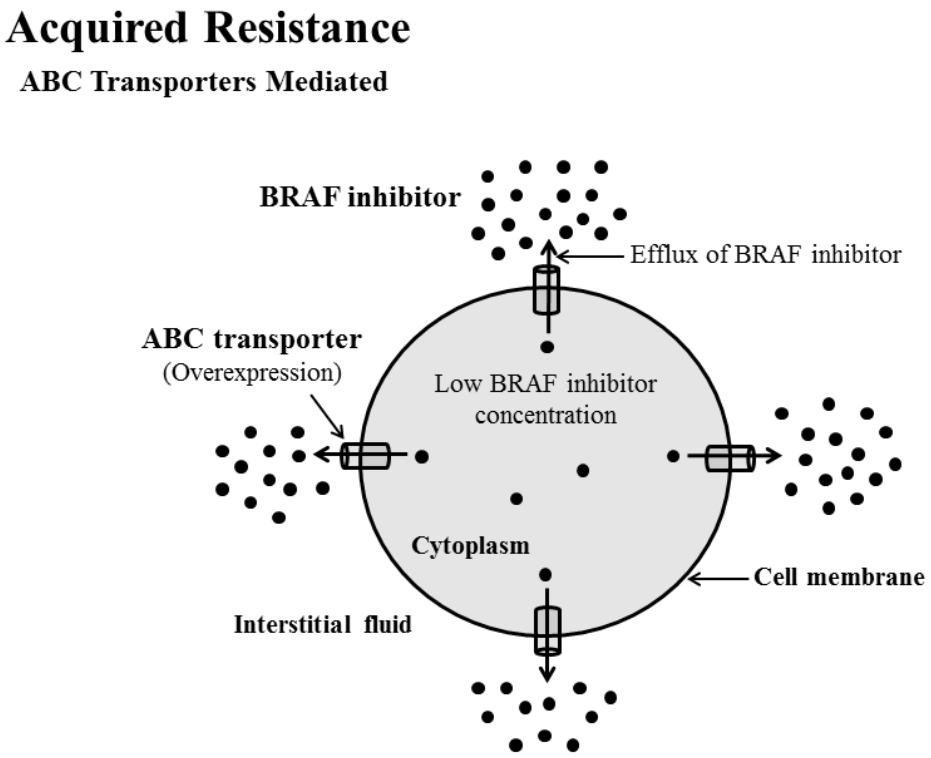

Acquired Resistance
ERK-independent
Epidermal Growth Factors (EGF)
EGFR
Cell membrane
RAS
JAK
SFK
BRAF
STAT3
MEK
ERK
Nuclear membrane
Cellular proliferation, survival, invasion and metastasis

Figure 7:


Acquired Resistance
ABC Transporters Mediated
BRAF inhibitor
Efflux of BRAF inhibitor
ABC transporter
(Overexpression)
Low BRAF inhibitor
concentration
Cytoplasm
Cell membrane
Interstitial fluid