Sensitivity of Melanoma Cells to EGFR and FGFR Activation but Not Inhibition is Influenced by Oncogenic BRAF and NRAS Mutations

Tamás Garay · Eszter Molnár · Éva Juhász · Viktória László · Tamás Barbai · Judit Dobos · Karin Schelch · Christine Pirker · Michael Grusch · Walter Berger · József Tímár · Balázs Hegedűs

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Abstract BRAF and NRAS are the two most frequent oncogenic driver mutations in melanoma and are pivotal components of both the EGF and FGF signaling network. Accordingly, we investigated the effect of BRAF and NRAS oncogenic mutation on the response to the stimulation and inhibition of epidermal and fibroblast growth factor receptors in melanoma cells. In the three BRAF mutant, two NRAS mutant and two double wild-type cell lines growth factor receptor expression had been verified by qRT-PCR. Cell proliferation and migration were determined by the analysis of 3-days-long time-lapse videomicroscopic recordings. Of note, a more profound response was found in motility as compared to proliferation and double wild-type cells displayed a higher sensitivity to EGF and FGF2 treatment when compared to mutant cells. Both baseline and induced activation of the growth factor signaling was assessed by immunoblot analysis of the phosphorylation of the downstream effectors Erk1/2. Low baseline and higher inducibility of the signaling pathway was characteristic in double wild-type cells. In contrast, oncogenic BRAF or NRAS mutation did not influence the response to EGF or FGF receptor inhibitors in vitro. Our findings demonstrate that the oncogenic mutations in melanoma have a profound impact on the motogenic effect of the activation of growth factor receptor signaling. Since emerging molecularly targeted therapies aim at the growth factor receptor signaling, the appropriate mutational analysis of individual melanoma cases is essential in both preclinical studies and in the clinical trials and practice.

Keywords Melanoma · BRAF · NRAS · Mutation · EGF · FGF2 · EGFR inhibitor · FGFR inhibitor

Introduction

Malignant melanoma is characterized by steadily increasing incidence and dismal prognosis due to its high metastatic potential. This metastatic behavior of melanoma cells relies on...
the interplay of cell migration and proliferation which are in part regulated by growth factors including EGF and FGF2.

EGF and FGF2, stimulating EGFR and FGFR receptors, respectively [1–6], activate extensively overlapping downstream signaling cascades [7–10] that are affected by the two most common oncogenic driver mutations in human malignant melanoma, namely BRAF and NRAS mutations. BRAF mutation could be identified in 40 to 70 % of the melanoma cases while NRAS mutation is detected in 10 to 30 % of the cases [11–17]. EGF and FGF2 as well as BRAF and NRAS oncogenic mutations activate the RAS-RAF-MEK-ERK pathway that is linked to cell growth and inhibition of apoptosis [18, 19], and implicated in cell differentiation and proliferation [20, 18] and to migratory processes through the ERK-FAK interplay [21].

Earlier studies showed correlation between EGFR expression [22] and EGFR gene amplification with tumor progression [23–25]. However, there is a varying degree of expression of EGFR in melanoma cells and some cell lines lack expression [26]. Recently the importance of the EGF-pathway in melanoma and metastasis formation was supported by a meta-analysis and gene-expression microarray investigations [27] and EGF was shown to facilitate melanoma lymph node metastases by affecting lymphangiogenesis [28].

Normal melanocytes and malignant melanoma cells express predominantly FGFR1 [29]. Nevertheless an increase in overall GF receptor expression and melanoma specific FGFR4 expression was detected in malignant melanoma cells [30, 31]. Furthermore, the expression of FGFR4 is thought to be a potential prognostic marker for melanoma [32]. Impaired FGFR1 function in melanoma cells results in reduced cell proliferation and survival in vitro and in decreased tumorigenic potential in vivo [31]. Of note, several loss-of-function mutations in FGFR2 have been identified in melanoma [33]. While normal melanocytes do not express FGF2, FGF2 is expressed in melanoma cells [34] and forced FGF2 expression resulted in autonomous and increased growth in vitro but not in increased tumor forming capacity in vivo [35, 36]. Similarly, inhibition of FGF2 signaling by either specific neutralizing antibodies or by antisense oligonucleotides resulted in decreased migration and proliferation in vitro and in prolonged survival time and suppression of tumor growth in animal models [37–39, 29, 40, 31, 41, 34].

In the last few years several novel drugs emerged that can efficiently target receptor tyrosine kinases [42, 43]. The EGFR inhibitor gefitinib inhibited proliferation of malignant melanoma cells harboring wild-type BRAF and NRAS in vitro [44] but failed to show significant clinical efficacy as a single-agent therapy for unselected patients with metastatic melanoma [45]. Erlotinib administered as single therapy failed to reduce proliferation of melanoma cells but in combination with bevacizumab, a VEGF-A binding antibody, the decrease in proliferation was significant in vitro and in vivo [46]. Further, the pan-EGFR tyrosine kinase inhibitor canertinib (PD183805, CI-1033) was shown to be effective in inhibiting proliferation in vivo and tumor growth in vitro in malignant melanoma harboring wild-type BRAF and NRAS [47]. The irreversible EGFR inhibitor pelitinib (EKB-569) was efficient against hepatocellular carcinoma cells in vitro [48] and against gefitinib- and erlotinib-resistant non-small cell lung cancer cells [49]. Furthermore it delivered clinical benefit in combination with temsirolimus in a phase I study [50]. However, effect of pelitinib on melanoma cells is currently unexplored.

FGF receptor inhibitors are also promising treatment modalities for melanoma patients. Antitumor effect of FGFR inhibitors (SU5402 and PD166866) in combination with BRAF inhibitors was shown in vitro and in vivo in a set of BRAF mutant melanoma cells [51]. The small molecule multitarget kinase inhibitor BIBF-1120 acting on FGF, VEGF and PDGF receptors inhibited the proliferation of a large panel of tumor cells including kidney, pharyngeal, ovary, lung, colon, pancreatic cancer and glioma cells in vitro and antitumor effect in vivo [52–54]. Similarly ponatinib (AP24534), with affinity to FGFR but to VEGFR and ABL as well, decreased proliferation of breast, lung, gastric, endometrial, bladder, colon cancer cells and reduced growth of tumor xenografts and prolonged survival of host mice in vivo [55, 53, 56]. BGJ-398, a highly selective inhibitor for FGFRs effectively reduced proliferation of bladder cancer cells in vitro and reduced the amount of circulating tumor cells and lymph node as well as distant metastases in vivo [57, 58]. Recently, a phase II clinical study has started with the FGFR inhibitor BGI-398 in combination with the RAF inhibitor LGX818 on BRAF-mutant advanced melanoma (http://clinicaltrials.gov; NCT01820364). The FGFR selective inhibitor AZD-4547 reduced the proliferation of breast cancer, multiple myeloma, acute myeloid leukemia and myeloproliferative syndrome-derived cells and demonstrated antitumor effect on colon cancer xenografts in vivo [53, 59]. Although EGF and FGF receptor inhibitors show promising results in a great variety of cancers, their effect on melanoma cells are quite unexplored.

Altogether, EGF-FGF2 signaling plays an important role in melanoma progression and the most common oncogenic mutations in melanoma result in the constitutive activation of the EGF-FGF signaling, downstream of the receptors. The importance of the EGF-FGF signaling cascades is underlined also in the involvement of the acquired resistance against the recently introduced V600E BRAF inhibitor vemurafenib [60, 61]. Even so, the effect
of these oncogenic mutations on the activation and inhibition of EGF and FGF receptors is widely anticipated nevertheless not yet systematically investigated.

Accordingly our aim was to evaluate the in vitro effect of BRAF and NRAS mutations on the activation and inhibition of EGFR and FGFR signaling in human melanoma cells.

Methods

Compounds/Drugs

The following compounds were used in the experiments: EGF (human recombinant; Life Technologies, Carlsbad, CA), FGF2 (human recombinant; Life Technologies, Carlsbad, CA), the FGFR inhibitors ponatinib, BGJ-398, BIBF-1120, AZD-4547, the EGFR inhibitors gefitinib, erlotinib, canertinib, pelitinib (all from Selleckchem, Housten, TX, USA).

Cell Lines

Seven human melanoma cell lines were used in this study. A2058, A375 and MEWO as well as CRL-2066 small cell lung carcinoma and CRL5885 lung adenocarcinoma cell lines were from ATCC. The M24met melanoma line, established from an invaded lymph node of a nude mouse [62], was kindly provided by B. M. Mueller (Scripps Research Institute, La Jolla, CA). The HT199 melanoma cell line was developed in the National Institute of Oncology, Hungary [63]. Cell lines VM-15 and VM-47 were established at the Institute of Cancer Research at Medical University of Vienna [64]. Cell cultures were maintained in DMEM media (Lonza, Switzerland; with 4500 mg/dm³ glucose, pyruvate and L-glutamine) supplemented with 10 % fetal calf serum (Lonza, Switzerland) and 1 % penicillin-streptomycin-amphotericin (Lonza, Switzerland) in tissue culture flasks at 37°C in a humidified 5 % CO2 atmosphere. Cells were kept in media with 5 % FCS for 24 h before GF treatment.

Mutational Analysis

After isolating DNA from the cell lines and performing PCR reaction, samples were purified with Applied Biosystems BigDye® XTerminator™ Purification Kit and mutations were verified through sequencing on ABI 3130 genetic Analyser System with BigDye® Terminator v1.1 Kit.

Quantitative Real-Time PCR

Messenger RNA levels of EGFR and FGFRs were determined by qRT-PCRs. In a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems) 1 µl cDNA and 11 µl TaqMix qRT-PCR Master Mix containing the appropriate TaqMan probe were mixed. TaqMan probes used were as follows: FGFR1 - Hs00915135 m1, FGFR2 - Hs01552926 m1, FGFR3 - Hs00179829 m1, FGFR4 - Hs00608751 g1, EGFR - Hs01076078 m1, GAPDH - Hs99999905 m1. PCR was performed in an ABI Prism 7000 SDS Thermocycler (Applied Biosystems) and fluorescence was measured after every cycle. As references for normalization the housekeeping gene GAPDH was used. Additionally, the size of PCR products was verified by agarose gel electrophoresis. Each preparation and measurement was performed twice.

Sulforhodamine B (SRB) Proliferation Assay

SRB assay was performed to analyze cell proliferation, based on the measurement of cellular protein content. Briefly, melanoma cells were plated in the inner 60 wells of a 96-well plate and left 24 h to adhere. After 48 h treatment with EGF and FGF2 (50 ng/ml each) or both, cell monolayers were fixed with 10 % trichloroacetic acid and stained for 15 min with SRB. Excess dye was removed by repeated washing with 1 % (vol/vol) acetic acid, and then the protein-bound dye was dissolved in 10 mM Tris and OD determined at 570 nm using a microplate reader (EL800, BioTec Instruments, USA). Proliferation data are averages of six independent experiments and effect of treatment was expressed as control to treated ratio.

Videomicroscopy

Videomicroscopy measurements were carried out as described previously [65, 66]. Briefly, melanoma cells were plated in the inner 8 wells of 24-well plates (Corning Incorporated, USA) in DMEM medium supplemented with 10 % FCS. After the overnight cell attachment, culture medium was changed to CO2-independent medium (Gibco-BRLLife Technologies, UK) supplemented with 5 % FCS and 4 mM glutamine. The reduction of evaporation from the inner wells was achieved by filling the outer wells with medium. Cells were kept for a custom designed incubator built around an inverted phase-contrast microscope (World Precision Instruments, USA) at 37 °C and room ambient atmosphere. Images of 3 neighboring microscopic fields were taken every 5 min for 1 day before and 2 days after the treatment with.
EGF and FGF2 (50 ng/ml each), or both. For migration data the captured phase contrast microscope pictures were analyzed individually with a cell-tracking program enabling manual marking of individual cells and recording their position parameters into data files. The parameter migrated distance is calculated by averaging for each cell the displacement for the first 24 h interval after treatment, in two independent experiments and 3 microscopic fields. Cell proliferation was also assessed by counting the number of cell divisions in the first 2 days after treatment and normalized to the initial number of cells on the first picture taken after the treatment. For proliferation data two independent experiments and 3 microscopic fields were evaluated.

**Immunoblot Analysis**

Immunoblot analysis was performed to quantify the phosphorylation of Erk1/2 and S6 proteins, two downstream components of the RAS pathway. Cells were plated in six-well dishes and maintained as mentioned above. Following 2 days of incubation time cells were treated in two independent replicates for 1 h with either EGF or FGF2 (50 ng/ml each) or both and collected in RIPA Buffer (Thermo Scientific) supplemented with 1 % Halt Protease Inhibitor Single-Use Cocktail (Thermo Scientific). Total protein concentrations were measured using Pierce BCA Protein Assay kit (Thermo Scientific). Following denaturation, equal amounts of protein were loaded on SDS-PAGE (12 %) and transferred to nitrocellulose membrane (Whatman). Incubation with anti p-Erk/Erk, anti p-S6/S6 and as loading control anti tubulin (Cell Signaling; polyclonal, rabbit) was performed overnight at 4 °C in a dilution of 1:2000. Secondary, HRP labeled anti-rabbit antibody was applied 1:2000 for 0.5 h at room temperature. Visualization was achieved with Amersham ECL Advance Western Blotting Detection kit (GE HealthCare). Activation of signaling was quantified as the ratio of phosphorylated and total protein densitometry measurements using ImageJ software.

**Statistics**

To determine statistical differences between groups ANOVA and post hoc Dunnett’s Multiple Comparison test was computed. Statistical significance was determined at \( p < 0.05 \). Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc, USA, San Diego, CA).

**Results**

**Mutational Analysis**

Mutational status of the examined cell lines was confirmed by direct sequencing. The most common V600E mutation in the BRAF gene and wild-type NRAS was detected in A375, A2058 and HT199 cells. The VM-15 and M24met cell lines were found BRAF wild-type and NRAS mutant with Q61K and Q61R mutations, respectively. Two cell lines, MEWO and VM-47 carried no mutations in these two genes.

**Expression of Growth Factor Receptors**

To see the whether a treatment with EGF and FGF has the potential to affect the examined cells, expression of EGF and FGF receptors were investigated by quantitative real-time PCR. Transcription of EGFR, FGFR1 and FGFR4 was confirmed in each of the investigated cell lines (Fig. 1). Interestingly, FGFR2 and FGFR3 were not expressed in the two NRAS mutant cell lines. Of note,
on average the lowest expression of growth factor receptors was found in the double wild-type cells.

Effect of EGF/FGF2 Treatment on Proliferation

Effect of EGF and/or FGF2 treatment on proliferation and cell viability was measured by videomicroscopy and SRB assay, respectively, essentially yielding comparable results. Importantly, both BRAF and NRAS activating mutations resulted in elevated levels of proliferation compared to the double wild-type cells (Supplementary figure 1A). Proliferation of the double wild-type cell line VM-47 was significantly increased upon GF treatment. There was no significant change in the proliferation of cells with BRAF or NRAS oncogenic mutations (Fig. 2a–c).

Effect of EGF/FGF2 Treatment on Cell Morphology and Migration

Next, the effect of addition of EGF and/or FGF2 on cell morphology and migration was investigated by videomicroscopy. Clear morphological changes could be observed after EGF or FGF2 treatment in the double type cell lines and the majority of cells obtained an elongated morphology. A modest alteration was found in NRAS mutant cells following the addition of FGF2 (Fig. 3a). A more profound response was found in cell migration as compared to proliferation (Fig. 3b). Remarkably, without any treatment both BRAF and NRAS activating mutations resulted in elevated levels of migration compared to double wild-type cells (Supplementary figure 1B). Significant increase in cell migration was observed in the two double wild-type cell lines following GF treatment. In both cases the increase in migratory activity was higher after EGF treatment as compared to FGF2 treatment. Importantly, the combined treatment resulted in greater increase than treatment with EGF or FGF2 alone. Significant increase in migration was measured after combined EGF and FGF2 treatment in VM-15 and M24met NRAS mutant cells, respectively. Of note, this increase was considerably smaller than those in double wild-type cells. In average, NRAS mutant cells showed a modest increase in migration in response to FGF2 or combined growth factor treatment, whereas BRAF mutant cells failed to show changes in cell migration after growth factor treatment (Supplementary figure 2).

![Fig. 2 Effect of EGF and/or FGF2 treatment on cell proliferation measured by videomicroscopy and SRB assay. Melanoma cells harboring oncogenic BRAF (a), NRAS (b) mutations and wild-type for these genes (c). Evaluation of changes in cell proliferation by videomicroscopy or SRB assay yielded comparable results. No changes in proliferation were observed in BRAF and NRAS mutant cells. In contrast, double wild-type VM-47 cells showed significantly increased proliferation upon EGF and/or FGF2 treatment. Colors blue, red and green indicate BRAF, NRAS mutation and wild-type, and striped and plain columns stand for videomicroscopy and SRB assay, respectively. Data shown as average ± SEM are results of more independent measurements, 6 in case of SRB assays and 4 in case of videomicroscopy measurements. Asterisks indicate significance of p<0.05 by ANOVA and Dunnett’s post hoc test. (E = EGF; F = FGF2; E + F = EGF and FGF2 treatment)
Activation of the EGFR and FGFR Pathway

Activation of the growth factor receptor pathway was measured by the immunoblot investigation of the phosphorylation of the downstream effectors Erk1/2.

BRAF and NRAS oncogenic mutations resulted in a 2.2 and 2.6 times higher phosphorylation of Erk1/2, as compared to wild-type cells, respectively (Fig. 4a). Treatment with growth factors resulted in a notably higher level of phosphorylation of Erk1/2 in double wild-type cells and in a modest elevation of phosphorylation in either BRAF or NRAS mutant cells (Fig. 4b). A 1.4 to 2.3 fold increase in phosphorylation of Erk1/2 was measured in double wild-type cells. In contrast, the highest increase in the mutant cells was only about 20% in the phosphorylation of Erk1/2 measured in mutant cells after GF treatment (Fig. 4). Altogether, the alteration of Erk1/2 phosphorylation measured in cells with oncogenic mutations was rather modest when compared to the double wild-type cells’ response.
Pharmacological Inhibition of EGFR and FGFR

In order to examine the mutation dependence of melanoma cells to growth factor receptor inhibition, four EGFR and four FGFR inhibitors were tested on the seven melanoma cell lines with BRAF, NRAS mutation or wild-type for these genes. The treatment with EGFR inhibitors failed to show mutation dependence in melanoma cells (Fig. 5). However, the two irreversible inhibitors (canertinib and pelitinib) showed higher growth inhibiting potential compared to the reversible inhibitors (gefitinib and erlotinib). Similarly, the effect of FGFR inhibitors on proliferation of melanoma cells was independent of the BRAF and NRAS mutational status of the melanoma cells (Fig. 6).

Discussion

The majority of novel targeted therapies are inhibiting the growth factor receptors or their downstream signaling cascades. Importantly, the major oncogenic driver mutations in melanoma are part of these signaling pathways. Accordingly, we determined the mutation dependent activation and inhibition of GF receptor signaling cascades. Reflecting the prevalence of these mutations we included three BRAF-, two NRAS-mutant and two double wild-type cell lines. In line with the finding, that an increase in the EGFR gene copy number possibly correlates with tumor progression [24, 25] the majority of melanoma cell lines are EGFR positive [26]. Expression of a variety of FGFR receptors was demonstrated in recent studies on melanoma cell lines [51, 67]. Interestingly, in our study none of the NRAS mutant cell lines showed FGFR2 and FGFR3 expression, and of particular importance wild-type cell lines showed the lowest level of GF receptor expression.

Clinical studies indicate that BRAF or NRAS mutant melanoma has a worse prognosis [68, 69]. Of note, in our in vitro assays we found that BRAF and NRAS
mutant melanoma cells display higher proliferation and migration compared to melanoma cells lacking these driver mutations. Our finding suggests that these in vitro biological characteristics may correlate with the dismal clinical course.

Although the correlation of BRAF mutation and downstream activation of the GF receptor pathway has not been demonstrated in clinical studies \[70, 71\], our in vitro experiments demonstrate that BRAF or NRAS mutant cells display higher phosphorylation of the downstream effectors Erk1/2, surrogate marker of the activation of the RAS/RAF/MEK pathway. According to the high baseline phosphorylation of Erk1/2 in BRAF and NRAS mutant cells, the treatment with growth factors resulted only in a modest activation of the downstream targets. In contrast, a notably higher level of phosphorylation of Erk1/2 was measured after the treatment with GFs in wild-type cells.

The responsiveness of wild-type melanoma cells towards GF treatment was seen not only in the phosphorylation of the downstream target of the RAS/RAF/MEK but also in proliferation and cell migration. The highest significant increase in cell migration was found in the double wild-type cell lines VM-47 and MEWO following either EGF or FGF2 treatments. Of note, FGF2 treatment increased the migration of NRAS mutant cells, whereas BRAF mutant cells failed to show changes in cell migration after either growth factor treatment. Interestingly, more profound migratory response was found when compared to proliferation. The comparable results from the videomicroscopy and protein measurement-based cell viability assay demonstrate that cell proliferation can be estimated by viability assays in this setting. A significant increase in proliferation was found in the double wild-type cell line VM-47, whereas MEWO cells, the other wild-type cell line, failed to show increase in proliferation. The smaller responsiveness of MEWO cells, compared to VM-47 cells could be explained with the NF1 loss, an intrinsic inhibitor of RAS activation, in MEWO cells that can lead...
In line with the lack of further activation of the EGFR and FGFR pathway, there was no remarkable increase in the proliferation or migration of cells with BRAF or NRAS oncogenic mutations. Despite differences in the response of melanoma cells with different oncogenic mutations there was no similar mutation dependence in their sensitivity to EGFR and FGFR inhibitors. Our findings are in line with previous studies where inhibition of EGFR failed to show antitumor effect in melanoma cells in vitro [46] and in a clinical trial [45]. Similarly, inhibition of FGFR-VEGFR had minimal effect on melanoma cells in patients [73].

In conclusion, we demonstrated that in NRAS and BRAF mutant melanoma cells the activation of growth factor receptor signaling is essentially different compared to wild-type cells. In contrast, the effect of EGFR and FGFR inhibition seems to be rather independent from BRAF and NRAS oncogenic mutations. However, in order to establish the predictive role of oncogenic mutations in molecularly targeted therapy of melanoma, appropriate mutational analysis of melanoma cases is indispensable in both preclinical studies and in clinical trials and practice.

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