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Original Article

Novel mechanisms of resistance to vemurafenib in melanoma – V600E B-Raf reversion and switching VEGF-A splice isoform expression

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Abstract: Targeting activating mutations in the proto-oncogene B-Raf, in melanoma, has led to increases in progression free survival. Treatment with vemurafenib, which inhibits the most common activating-mutated form of B-Raf (B-Raf^{V600E}), eventually results in resistance to therapy. VEGF-A is the principal driver of angiogenesis in primary and metastatic lesions. The bioactivity of VEGF-A is dependent upon alternative RNA splicing and pro-angiogenic isoforms of VEGF-A are upregulated in many disease states dependent upon angiogenesis, including cancers. Using techniques including RT-PCR, Western blotting, ELISA and luciferase reporter assays, the effect of vemurafenib on proliferation, ERK1/2 phosphorylation and the levels of pro- and anti-angiogenic VEGF-A isoforms was investigated in melanoma cell types expressing either wild-type B-Raf or B-Raf^{V600E}, including a primary melanoma culture derived from a highly vascularised and active nodule taken from a patient with a V600E mutant melanoma. The primary melanoma culture was characterised and found to have reverted to wild-type B-Raf. In B-Raf^{V600E} A375 cells ERK1/2 phosphorylation, pro-angiogenic VEGF-A mRNA, total VEGF-A protein expression and VEGF-A 3'UTR activity were all decreased in a concentration-dependent manner by vemurafenib. Conversely vemurafenib treatment of wild-type B-Raf cells significantly increased ERK1/2 phosphorylation, pro-angiogenic VEGF-A mRNA and total VEGF-A expression in a concentration-dependent manner. A switch to pro-angiogenic VEGF-A isoforms, with a concomitant upregulation of expression by increasing VEGF-A mRNA stability, may be an additional oncogenic and pathological mechanism in B-Raf^{V600E} melanomas, which promotes tumor-associated angiogenesis and melanoma-genes. We have also identified the genetic reversal of B-Raf^{V600E} to wild-type in an active melanoma nodule taken from a V600E-positive patient and continued vemurafenib treatment for this patient is likely to have had a detrimental effect by promoting B-Raf^{WT} activity.

Keywords: Melanoma, vemurafenib, A375, 92.1, VEGF-A, VEGF-A_{xxx}b, mechanism of resistance

Introduction

A substantial proportion of all melanomas contain activating mutations in the proto-oncogene B-Raf (50-70%) [1]. Vemurafenib, the most common inhibitor of the activating-mutated form of B-Raf (B-Raf^{V600E}) increases progression-free survival in melanoma patients but eventually resistance to therapy develops and disease progression occurs [2].

Angiogenesis that results in a functional tumor vasculature must develop, in addition to tumor

cell proliferation, for cancer progression. Angiogenesis is driven by the upregulation of pro-angiogenic molecules of which the vascular endothelial growth factor isoform, VEGF-A₁₆₅ (herein referred to as VEGF-A₁₆₅a), is the principal molecule in primary and metastatic lesions [3]. The bioactivity of VEGF-A is dependent upon alternative RNA splicing [4, 5] and changes in the splicing pattern of VEGF-A are observed in many disease states dependent upon angiogenesis, including cancers [6-8]. Anti-angiogenic VEGF-A proteins that contain an alternative C-terminus (VEGF-A_{xxx}b family), in particular

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VEGF-A₁₆₅_b, are endogenously expressed in normal tissues and downregulated in metastatic melanoma and other cancers [6, 8, 9]. These VEGF-A_{xxx}_b family proteins competitively antagonise the pro-angiogenic signalling of VEGF-A_{xxx}_a and are upregulated in systemic sclerosis [10] and obesity [11].

Activated B-Raf results in constitutive activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K) signalling [12]. Inhibition of ERK1/2 signalling decreases VEGF-A expression and inhibits splicing of VEGF-A to pro-angiogenic VEGF-A_{xxx}_a [13] indicating that constitutive B-Raf activity may drive tumor-associated angiogenesis in melanoma through an effect on the expression and/or splicing pattern of VEGF-A. We tested the hypotheses that inhibiting B-Raf activity in melanoma cells would A) decrease VEGF-A expression and B) alter the splicing to favour anti-angiogenic VEGF-A. A primary human melanoma culture was established from an active nodule that had developed resistance to vemurafenib therapy. We investigated the effect of vemurafenib in these cells and we propose a novel mechanism of resistance.

Materials and methods

Ethics

Primary human melanoma cells were isolated with ethical approval from the Local Ethics Committee.

Cell culture

A highly vascularised and active nodule resistant to vemurafenib therapy was removed from the anterior chest of a B-Raf^{V600E}-positive patient and dissociated within 12 h. In brief, the tumor was washed, the fat tissue and epidermis were removed and discarded and placed into DMEM:F12. The melanoma tissue was cut into small (2-5 mm) pieces and enzymatically digested (0.125% collagenase P) for 2 h at 37°C with occasional agitation. Trypsin was added (final concentration 0.01% w/v) and incubated for 15 minutes. Using a 1 mL pipette tip the cells were mechanically dispersed, centrifuged (250 × g-force for 5 minutes) and the cell pellet resuspended in DMEM:F12 + 10% FBS media. Cells were loaded onto 3 × 15% BSA cushion (1 mL 30% BSA in PBS added to 1

mL DMEM:F12) and centrifuged at 200 × g-force for 8 minutes. The cell pellet was resuspended in serum-free DMEM:F12 and plated into T25 flasks with 2% pen/strep for 48 h, following which media was changed to 10% FBS DMEM:F12. A375 (vemurafenib sensitive cutaneous melanoma, ATCC), 92.1 vemurafenib resistant ocular melanoma and primary melanoma cells were grown to ~80% confluence in DMEM + 10% FBS before passaging or vemurafenib treatment. All treatments were performed in DMEM + 0.5% FBS.

Reverse transcription PCR

Following 24h vemurafenib treatment, RNA was extracted using Trizol as described previously [14]. ~1 mg RNA was DNase treated and reversed transcribed with M-MLV reverse transcriptase in the presence of 500 ng Oligo d(T) and 250 ng random primers. VEGF-A primers (forward 5-GAGCGGAGAAAGCATTGT-3, reverse 5-TCAGTCTTCTGCTGGTGAGAGAT-3) are designed to amplify different exon 8-containing VEGF-A mRNA transcripts [15]. Pro-angiogenic (exon 8a-containing) and anti-angiogenic (exon 8b-containing) isoforms produce 201 and 135 bp PCR products respectively.

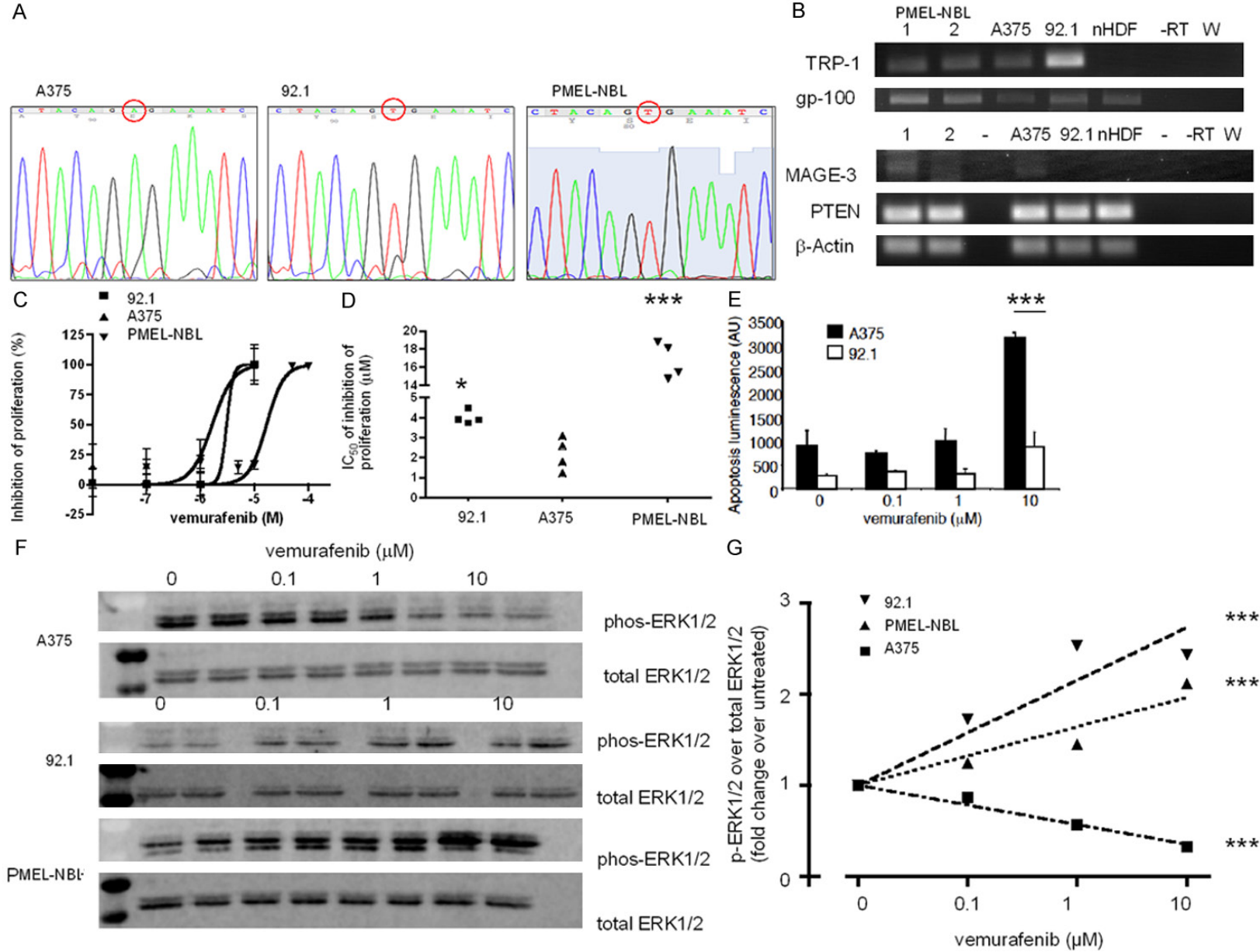
Cell proliferation and apoptosis assays

Melanoma cells were treated with vemurafenib concentrations for 24 or 48 h in DMEM + 0.5% FBS. For proliferation analysis Prestoblu® (Life Technologies) was added at the same time as vemurafenib and absorbance was read at multiple time points over 24 h. Absorbance values, taken from the logarithmic phase of Prestoblu® colour development, were used to determine IC₅₀ values. Apoptosis was measured using the commercially available Caspase-3/7-Glo® (Promega, G8090).

Transfection

10,000 cells were seeded in a 96 well plate in antibiotic-free media and allowed to attached (6h). 100 ng plasmid DNA was added per well for 24 h (60 ng firefly construct of interest, 40 ng renilla) following the Effectene transfection reagent protocol (Qiagen, 301425). The media was removed and vemurafenib concentrations were added for 24 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay (Promega, E1910) following the manu-

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Figure 1. Differing effects of vemurafenib on proliferation, apoptosis and ERK1/2 phosphorylation. A. B-Raf exon 15 was sequenced in three different melanoma cell-types. Cutaneous malignant melanoma-derived A375 contains B-Raf^{V600E} sequence while uveal melanoma-derived 92.1 and PMEL-NBL, a primary culture derived from an active and vascularised nodule contain wild-type B-Raf. B. Expression of melanocyte/melanoma mRNA markers by the three melanoma cell types. C&D. The effect of vemurafenib on proliferation was significantly greater on A375 cells compared to the wild-type melanoma cell types. E. The effect of vemurafenib on caspase activity was investigated in V600E (A375) and wild-type cells (92.1) cells. Vemurafenib had a cytotoxic effect on A375 cells at 10 μ M and no significant effect on 92.1 cells at any concentration. F&G. Levels of phosphorylated ERK1/2 were detected by Western Blotting in A375, 92.1 and PMEL-NBL cells. Vemurafenib significantly reduced ERK1/2 phosphorylation in a concentration-dependent manner in A375 while conversely significantly increased ERK1/2 phosphorylation in 92.1 and PMEL-NBL. Abr: TRP-1, tyrosinase-related protein-1; gp-100, glycoprotein-100; MAGE-3, melanoma-associated antigen-3; PTEN, phosphatase and tensin homolog; ERK1/2, extracellular-signal regulated kinase1/2.

facturers protocol. For each well the Firefly luciferase activity (construct of interest) was normalised to renilla luciferase activity to control for differences in transfection and cell number.

Western immunoblotting and ELISA

Protein was extracted in radioimmunoprecipitation assay buffer with protease inhibitor cocktail plus 2 mM NaVO₄ and 20 mM NaF. For immunoblotting, protein was separated by SDS-PAGE (12%) and blotted onto polyvinylidene difluoride membrane. This was blocked in 2% BSA, TBS + Tween 20 (0.05%) for 1 hour, and anti-ERK1/2 (rabbit pAb, no.9102, 1 in 500; Cell Signaling) and anti-phospho-ERK1/2 (mouse mAb, no.9106, 1 in 250; Cell Signaling) were added in blocking solution and incubated overnight at 4°C. Fluorescent secondary antibodies (LI-COR, Lincoln, NE) were added 1 in 10,000 in blocking solution for 1 h at room temperature and fluorescence visualized on a LI-COR Odyssey. VEGF-A protein was assayed in cell lysate extracts by commercially available ELISAs using a VEGF-A_{xxx}b-specific capture antibody and a pan-VEGF-A_{xxx} detection antibody for VEGF-A_{xxx}b isoforms or two pan-VEGF-A antibodies for total VEGF-A, as previously described [16]. As the pan-VEGF-A ELISA has a reduced affinity for VEGF-A_{xxx}b compared with VEGF-A_{xxx}a total VEGF-A levels were determined as described previously [16].

Results

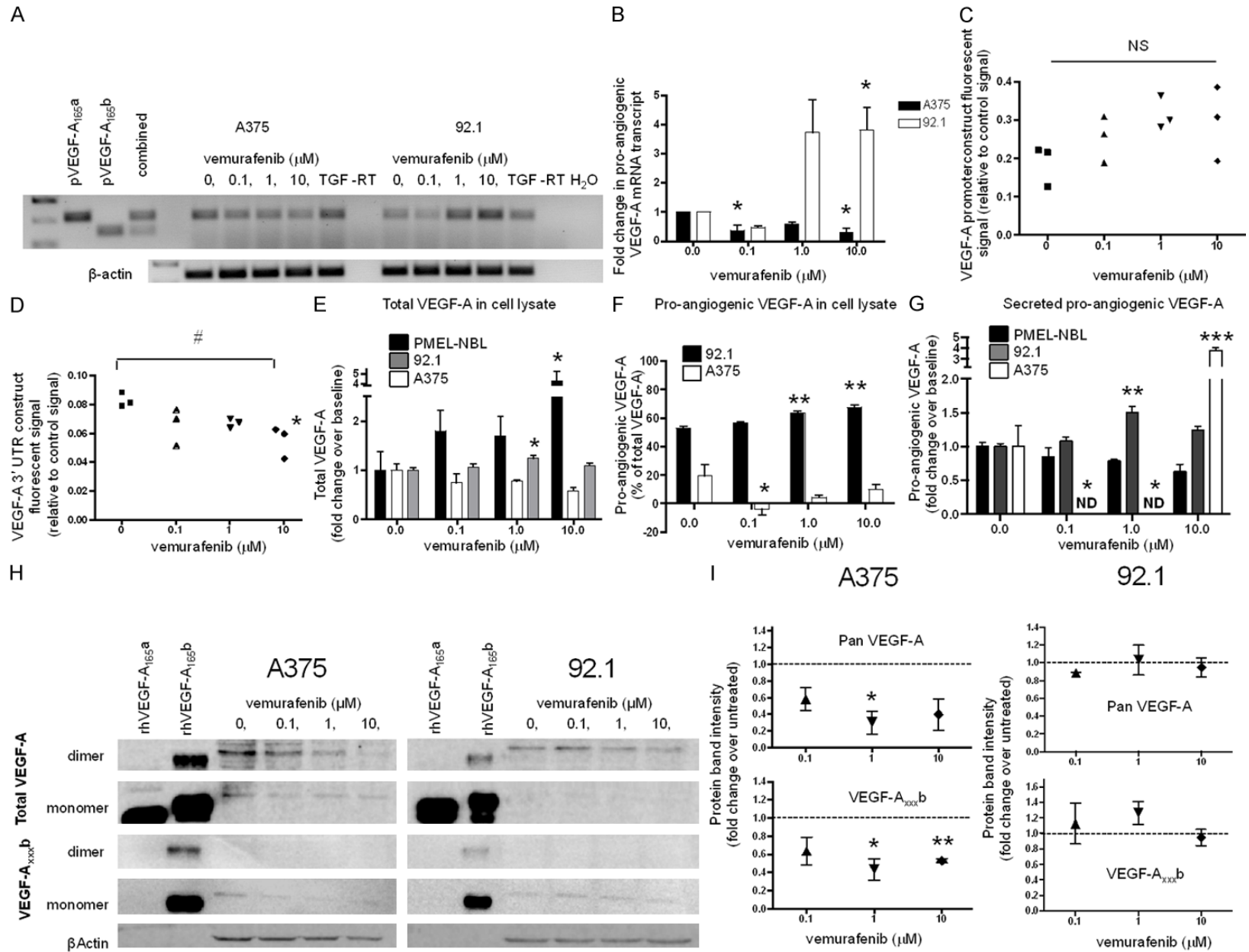
The B-Raf exon 15 genetic sequence was determined for two commercially available melanoma cell lines: A375 (primary cutaneous tumor-derived), 92.1 (uveal melanoma cell line) and for a human primary culture (PMEL-NBL) derived from a highly vascularised and active melanoma nodule resistant to vemurafenib thera-

py (**Figure 1A**). As previously reported A375 and 92.1 cell lines contained V600E and wild-type sequences for exon 15 respectively. PMEL-NBL contained wild-type B-Raf exon 15 sequence. RT-PCR confirmed that the primary cultured cells retained expression of melanocyte/melanoma-associated markers tyrosinase-related protein-1 (TRP-1), glycoprotein-100 (gp100 also known as PMEL-17) and melanoma-associated antigen-3 (MAGE-3) (**Figure 1B**). TRP-1 and MAGE-3 mRNAs were not detected in normal human dermal fibroblasts (nHDF). PTEN mRNA was detected in all three melanoma cell types.

The effect of vemurafenib on cell proliferation was determined by Prestoblu[®] analysis (**Figure 1C** and **1D**). The proliferation of the two wild-type B-Raf cultures (PMEL-NBL culture and 92.1) was significantly less affected by vemurafenib in comparison with the constitutively active B-Raf^{V600E} A375 (1-way ANOVA + Bonferroni * $p < 0.05$, *** $p > 0.001$). Vemurafenib induced significant apoptosis in A375 cells at 10 μ M but not in wild-type 92.1 (**Figure 1E**, 2-way ANOVA + Bonferroni *** $p > 0.001$). ERK1/2 lies downstream of B-Raf signalling. Vemurafenib significantly decreased ERK1/2 phosphorylation in A375 cells, while conversely significantly increased ERK1/2 phosphorylation in the two wild-type cultures (**Figure 1F** and **1G**, linear regression sig. diff. than 0, *** $p > 0.001$).

The effect of vemurafenib on VEGF-A exon 8-containing mRNA was investigated in the A375 and 92.1 using competitive RT-PCR. Increasing concentrations of vemurafenib significantly decreased the level of exon 8a-containing mRNA transcripts as detected by RT-PCR (**Figure 2A** and **2B**). Conversely, 1 or 10 μ M vemurafenib significantly increased the level of exon 8a-containing VEGF-A mRNA transcripts in 92.1 cells (**Figure 2A** and **2B**, 1-way

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Figure 2. Differing effects of vemurafenib on VEGF-A mRNA and protein. A&B. The effect on vemurafenib on VEGF exon 8-containing mRNA transcript expression in A375 and 92.1 cells. Vemurafenib reduced exon 8a containing transcripts in a concentration-dependent manner in A375 cells while conversely increased exon 8a containing transcripts in 92.1 cells. C. A375 cells were transfected to overexpress a 4 kb VEGF promoter-luciferase construct. Vemurafenib treatment had no significant effect on luciferase activity. D. A375 cells were transfected to overexpress a VEGF 3'UTR-luciferase construct. Vemurafenib significantly decreased luciferase activity in a concentration dependent manner. E. Vemurafenib significantly increased total VEGF levels in 92.1 and PMEL-NBL cells but decreased it in A375 cells. F. The level of pro-angiogenic VEGF was determined using both pan-VEGF and VEGF-A_{xxx}b ELISAs. In wild-type cells vemurafenib increased pro-angiogenic VEGF in a concentration-dependent manner while conversely pro-angiogenic VEGF was decreased in V600E A375 cells. G. Vemurafenib significantly increased secreted pro-angiogenic VEGF in 92.1 cells but significantly decreased secreted pro-angiogenic VEGF in A375. At 10 μ M pro-angiogenic VEGF was significantly increased in cell media due to increased cell death and lysis. H&I. The effect of vemurafenib on VEGF protein was investigated by Western blotting in A375 and 92.1 cells. Vemurafenib decreased total VEGF and VEGF-A_{xxx}b protein in A375 cells. In 92.1 cells vemurafenib treatment did not significantly affect total VEGF or VEGF-A_{xxx}b protein. Abr: 3'UTR, 3' untranslated region.

ANOVA + Bonferroni $*p > 0.05$). No transcripts containing exon 8b were detected by RT-PCR (**Figure 2A**) in any of the melanoma cell types tested.

To investigate the mechanism by which vemurafenib decreased the level of pro-angiogenic VEGF-A mRNA in constitutively active A375 cell line, cells were transfected with a plasmid DNA construct that contains the 4 kbp promoter sequence of VEGF-A upstream of the reporter gene, luciferase [17]. In a separate experiment A375 cells were transfected with a luciferase construct that contains the 3' untranslated region (3'UTR) of the VEGF-A mRNA sequence downstream of the luciferase sequence. The cells were treated with vemurafenib for 24 h, and a change in VEGF-A promoter activity or VEGF-A 3'UTR mRNA stability was detected by bioluminescence. Increasing concentrations of vemurafenib did not significantly affect the VEGF-A promoter activity (**Figure 2C**, 1-way ANOVA + Bonferroni, NS not significant). Vemurafenib did however significantly decrease luciferase activity in cells expressing the 3'UTR luciferase construct, in a concentration-dependent manner, indicating that vemurafenib affects VEGF-A 3'UTR stability in A375 B-Raf^{V600E} cells (**Figure 2D**, 1-way ANOVA, vs. 0 μ M, $*p > 0.05$. Linear reg. sig. diff. from 0 $\#p > 0.01$).

Total VEGF-A detected in cell lysate protein, as detected by ELISA, was decreased by vemurafenib treatment in A375 cells whereas in the wild-type cell-types, 92.1 and PMEL-NBL, total VEGF-A detected was significantly increased (**Figure 2E**, 2-way ANOVA + Bonferroni, $*p > 0.05$). The proportion of total VEGF-A that consisted of pro-angiogenic VEGF-A_{xxx}a was determined. Vemurafenib significantly decreased

the proportion of pro-angiogenic VEGF-A in A375 cells but significantly increased VEGF-A_{xxx}a in 92.1 (**Figure 2F**, 2-way ANOVA + Bonferroni, vs. 0 μ M, $*p > 0.05$, $**p > 0.01$). Following 0.1 and 1 mM vemurafenib treatment of A375 cells, no VEGF-A was detectable in the media (**Figure 2G**). Conversely 1 mM vemurafenib treatment of 92.1 caused a significant increase in VEGF-A detected in the media. 10 mM vemurafenib treatment of A375 cells caused a significant increase in VEGF-A detected in the media presumably as at that concentration the cell were apoptotic (see **Figure 1E**) and VEGF-A would have been released into the media from the compromised cells, (**Figure 2G**, 2-way ANOVA + Bonferroni, vs. 0 μ M, $*p > 0.05$, $**p > 0.01$, $***p > 0.001$).

The effect of vemurafenib on total and VEGF-A₁₆₅b cell lysate protein was investigated by Western immunoblotting (**Figure 2H**) and the fluorescence intensity of bands was quantified, normalised to respective loading controls and expressed as a fold increase over untreated. Both total VEGF-A and VEGF-A_{xxx}b were significantly decreased in A375 cells by vemurafenib treatment (**Figure 2I**, 1-way ANOVA + Bonferroni, vs. con, $*p > 0.05$). In 92.1 cells, vemurafenib did not significantly affect either the total VEGF-A or VEGF-A_{xxx}b protein levels detected by Western immunoblotting.

Discussion

Mutant B-Raf-driven ERK1/2 MAPK activity is implicated in the sustained cancer cell proliferation that can drive tumor growth. Vemurafenib selectively inhibits the constitutively active form of B-Raf, B-Raf^{V600E} [18] while paradoxically the drug stabilises and promotes the

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activity of wild-type B-Raf [19]. Our results demonstrate that ERK1/2 phosphorylation, pro-angiogenic VEGF-A mRNA and total VEGF-A protein expression are decreased by vemurafenib treatment in B-Raf^{V600E} A375 cells. 3'UTR mRNA sequences are known to regulate mRNA stability and consequently protein production [20]. Vemurafenib treatment significantly decreased VEGF-A 3'UTR activity in A375 cells indicating an effect on VEGF-A mRNA stability. ERK1/2 promotes VEGF-A expression [21] and stability through the RNA-binding proteins tristetraprolin and TIA-1 [20]. Vemurafenib may therefore downregulate VEGF-A mRNA levels in B-Raf^{V600E} cells through inhibition of ERK1/2- TTP or TIA-1 activity leading to a decrease in VEGF-A mRNA stability. Conversely vemurafenib treatment of wild-type B-Raf cells significantly increased ERK1/2 phosphorylation, pro-angiogenic VEGF-A mRNA and total VEGF-A expression as detected by ELISA. These data support the previous published finding that vemurafenib, instead of having the desired inhibitory effect, stabilises and promotes the activity of wild-type B-Raf. Our ELISA data indicate that inhibiting constitutively active B-Raf caused a decrease in the proportion of pro-angiogenic VEGF-A while vemurafenib did not affect anti-angiogenic VEGF-A_{xxx} b in wild-type cells.

No anti-angiogenic VEGF-A mRNA transcripts (exon 8b-containing transcripts) were detected. It is worth noting that the competitive nature of the PCR will favour one product over the other. When 1ng of plasmid VEGF-A₁₆₅ a DNA (pVEGF-A₁₆₅ a) and 1ng of plasmid VEGF-A₁₆₅ b DNA (pVEGF-A₁₆₅ b) were combined for competitive PCR, pVEGF-A₁₆₅ a clearly produced a greater PCR product than pVEGF-A₁₆₅ b (**Figure 2A**). Therefore the competitive nature of this particular PCR provides a possible explanation for exon 8b containing transcripts not being detected. An alternative explanation has been provided by the recent work demonstrating that translational repression of stop codon usage in exon 8a can also result in anti-angiogenic VEGF isoforms containing the C-terminal sequence of VEGF-A_{xxx} b [22].

We generated a primary human melanoma culture (PMEL-NBL) from a nodule that had developed resistance to vemurafenib. TRP-1 and gp-100 are expressed by melanocytes and melanoma cells [23, 24] and melanoma-associated antigen-3 (MAGE-3) expression is swi-

tched on in many incidence of melanoma [25]. PMEL-NBL expressed these 3 mRNAs, and TRP-1 and MAGE-3 mRNAs were not detected in normal human dermal fibroblast cells, indicating that the primary melanoma culture was of melanocyte/melanoma and not from a fibroblastic origin. The tumor-suppressor gene PTEN is downregulated in many cancers, a process thought to be an oncogenic event in melanomagenesis [26]. PTEN mRNA expression was detected in the PMEL-NBL, indicating that the original B-Raf^{V600E}-associated oncogenic mechanism was not associated with a decrease in PTEN mRNA expression. Multiple mechanisms of resistance to vemurafenib therapy developed by cutaneous melanomas in B-Raf^{V600E} patients have been described but to date none have attributed the resistance to a reversal of the B-Raf^{V600E} genetic sequence to wild-type. Our results indicate that the acquired mechanism of resistance to vemurafenib therapy was likely due to a genetic reversal of B-Raf from V600E to wild-type, or a selection of wild-type clones from the original melanoma. With the genetic reversal we hypothesise that continued treatment with vemurafenib in this patient may have the opposite effect to that desired by promoting B-Raf/ERK1/2-driven pro-angiogenic VEGF-A signalling in any further undetected B-Raf wild-type nodules. Our data indicates that a switch to pro-angiogenic VEGF-A isoforms, with a concomitant upregulation of expression by increasing VEGF-A mRNA stability, may be a major oncogenic and pathological mechanism in B-Raf^{V600E} melanoma that promotes tumor-associated angiogenesis and melanomagenesis.

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Disclosure of conflict of interest

The authors have no conflicts of interest.

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