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THE CORRELATION BETWEEN BRAFV600E EXPRESSION AND INTEGRIN $\beta 3$ IN MELANOMA CELLS

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Abstract

This study investigated the relationship between ITGB3 and BRAFV600E expression in melanoma. It specifically focused on how the overexpression of BRAFV600E and Integrin $\beta 3$ impacts wound healing and the TGF-β pathway. The findings provide valuable insights into melanoma progression and highlight potential therapeutic targets.

Homozygous BRAFV600E-carrying A375 and heterozygous BRAFV600E-carrying M14 melanoma cell lines were utilized. ITGB3 mRNA levels were measured in both A375 and M14 cells, while Integrin $\beta 3$ protein levels were analyzed in BRAFV600E-overexpressing A375 cells. The impact of increased BRAFV600E and Integrin $\beta 3$ on cell migration was assessed using an in vitro wound healing assay. Additionally, the influence of BRAFV600E and Integrin $\beta 3$ overexpression on the TGF-β pathway was evaluated through luciferase reporter assays and real-time PCR.

ITGB3 levels were significantly higher in A375 cells compared to M14 cells. Overexpression of BRAFV600E in A375 cells resulted in a marked increase in Integrin $\beta 3$ levels. Cells overexpressing both BRAFV600E and Integrin $\beta 3$ demonstrated enhanced wound healing rates and elevated TGF-β activity. These findings suggest a strong correlation between BRAFV600E expression and Integrin $\beta 3$ levels in melanoma cells.

This study uncovers a significant relationship between BRAFV600E expression and Integrin $\beta 3$ levels in melanoma cells. The overexpression of both BRAFV600E and Integrin $\beta 3$ enhances wound healing and promotes TGF-β

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signalling. These findings suggest that Integrin $\beta 3$ may contribute to melanoma progression and poor prognosis by influencing cell migration and the TGF-β pathway. Targeting integrins, the TGF-β pathway, and BRAFV600E present potential therapeutic strategies for melanoma treatment.

Key words: BRAFV600E, Integrin $\beta 3$, melanoma, TGF-β

Introduction. Many cancer types harbour the BRAF V600E mutation [1]. Interestingly, the BRAFV600E mutation is found in 50% of melanomas, a deadly skin cancer caused by mutated melanocyte cells initially localized in the epidermis. Beyond melanoma, this mutation is also common in hairy cell leukemia, non-Hodgkin lymphoma, thyroid cancer, ovarian cancer, lung adenocarcinoma, colorectal cancer, and glioblastoma [1].

BRAF is a protein serine/threonine kinase that phosphorylates MEK in the MAPK pathway. The Val600Glu mutation results in the overactivation of MAPK signalling, leading to excessive cell proliferation, enhanced survival, and poor prognosis in carcinogenesis [2].

A significant correlation has been established between elevated BRAF levels and tumourigenesis [3]. Within the scope of PAN-Cancer studies, BRAF expression was compared across various types of tumour and normal tissue samples. Increased BRAF expression was observed in 14 different cancers, including cervical, colon, oesophageal, liver, lung, stomach, breast, and renal cell carcinomas. In line with RNA results, an analysis of 370 melanoma patient tissue samples revealed that high BRAF expression in melanoma is associated with poor prognosis and overall survival [4]. These findings suggest that, beyond the mechanism of the BRAFV600E mutation, elevated BRAF RNA and protein levels may also contribute to carcinogenesis and poor prognosis. However, it is important to note that the analyses could not differentiate between mutant and wild-type BRAF; the BRAF levels analysed included both forms.

BRAFV600E contributes to tumourigenesis by overstimulating the MAPK pathway, leading to changes in the expression and transcription of various proteins [2]. Integrins are key players in tumour development and cancer progression, primarily facilitating cell attachment and managing intracellular pathways for signal transmission and cytoskeletal organization. These functions are crucial for invasion and metastasis. A previous study identified a link between integrins and the BRAFV600E mutation, showing that ITG$\alpha 3$ and ITG$\alpha 6$ expressions are higher in homozygous BRAFV600E-carrying 8505c human thyroid carcinoma cells than in heterozygous BRAFV600E-carrying SW1736 cells [5]. This finding suggests that increased BRAFV600E expression affects integrin levels. However, no correlation between BRAFV600E expression and integrin $\beta 3$, an RGD-binding integrin involved in molecular signalling, has been demonstrated. Therefore, our study aimed to investigate the correlation between the BRAFV600E mutation and ITGB3.

Integrin αIIb forms a complex with β3 to create integrin αIIbβ3, which is involved in cancer growth, invasion, metastasis, and resistance development [6]. In ovarian cancer cell lines SKOV-3 and OVCAR3, as well as the renal cell carcinoma cell line 786-O, CDH6 interacts with integrin αIIbβ3 to regulate cell adhesion, epithelial-mesenchymal transition [7], migration, invasion, and proliferation by decreasing phosphorylated FAK, SRC and AKT levels [8]. Another example is αIIbβ3's contribution to metastasis through circulating tumour cells. During extravasation, platelet-derived CAMs (cell adhesion molecules) promote the production of P-selectin and E-selectin, which activate platelet αIIbβ3 to recruit immune cells that protect circulating tumour cells [9].

The role of the integrin β3 subunit has been extensively studied, and its collaboration with αv in melanoma has been established. High *ITGB3* levels are found in melanoma cells, and increased integrin β3 expression is correlated with malignant features [10–12]. For instance, αvβ3 overexpression induces both anchorage-dependent and independent growth in skin, increases invasion from the epidermis to the dermis, and prevents apoptosis of melanoma cells. Another study demonstrated that silencing integrin β3 decreases the motility and metastatic behaviour of melanoma cells [13]. Additionally, interactions between platelet cells and melanoma cells suggest that inhibiting αIIbβ3 on platelets and αvβ3 on melanoma cells reduces metastasis by preventing platelet-melanoma interactions [14]. These findings indicate a correlation between poor prognosis and β3 expression, although the underlying mechanisms require further investigation. In this context, we investigated the correlation between the BRAFV600E mutation and *ITGB3* in melanoma cells.

Materials and methods. ***Cell culture, in vitro wound healing assay and transfection.*** The A375 and M14 ATC cell lines, harbouring homozygous and heterozygous BRAFV600E mutations, respectively, were graciously provided by the Institute of Cancer Therapeutics at the University of Bradford. The cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% sodium pyruvate, and 2 mM L-glutamine. A375 cells were seeded into six-well plates at a density of 2.2×10^5 cells per well, reaching 40–60% confluency. They were then transfected 24 hours later using Lipofectamine 2000, following the manufacturer's instructions. For Integrin β3 overexpression, the pcDNA3.1-beta-3 plasmid (a generous gift from Timothy Springer, Addgene plasmid #27289; `http://n2t.net/addgene:27289`; RRID: Addgene_27289) was utilized. For the in vitro wound healing assay, cells were scratched with a pipette tip 8 hours post-transfection and recorded 20 hours later.

Real-time PCR. A total of 1×10^7 cells were harvested for RNA extraction using trypsin/EDTA, with RNA isolated using the Qiagen RNeasy kit according to the manufacturer's instructions. RNA concentrations were determined using a NANODROP 1000 Spectrophotometer. RT-qPCR was conducted with the Bio-Rad iScript Reverse Transcription Supermix, using 1 µg of RNA for cDNA syn-

thesis per reaction, in accordance with the manufacturer's guidelines. Real-time PCR primers were purchased from Sigma.

Site-directed mutagenesis. To introduce the mutation at the 600th amino acid of BRAF, primers were designed to include a T > A transition at the 1999th base in both the forward and reverse complementary sequences. Two distinct DNA fragments were amplified using the primers listed in Table 1. The first fragment, upstream of the mutation, ends with the sequence containing the mutation, while the second fragment, downstream of the mutation, starts with the primer that includes the mutation. For the first fragment, an attB1-tagged forward primer was utilized, and for the second fragment, an attB2-tagged reverse primer was employed. These primers were designed to create complementary sequences, facilitating homologous recombination with the cloning vector according to the manufacturer's instructions (Invitrogen Gateway Cloning System).

T a b l e 1

Primer sequences to gain fragments for site directed mutagenesis, underlines sequences are attB tags, bold **A** and **T** complementer sequences to create the mutation in 1999th base T > A transition (BRAFV600E)

Fragment name	Primer name	Sequence (5′3′)
First fragment	BRAF_att_B1_F	**GGGGACAAGTTTGTACAAAAAAGCAGGCTTC** ATGGCGGCGCTGAGCGGTGG
	Braf_R_mt	GCTACAG**A**GAAATCTCGATGGAG
Second fragment	Braf_F_mt	CTCCATCGAGATTTC**T**CTGTAGC
	BRAF_att_B2_R	**GGGGACCACTTTGTACAAGAAAGCTGGGTC** TCATCAGTGGACAGGAAACGCACC

PCR reactions to obtain the fragments were performed using Thermo Phusion High-Fidelity DNA polymerase, known for its high proofreading activity. The PCR conditions for amplifying the fragments were as follows: initial denaturation at 98 °C for 30 s; 30 cycles of 98 °C for 10 s, 68 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 8 min. The PCR products were hybridized under the same conditions, but with the PCR mix prepared using the amplified fragments instead of primers.

Cloning. To overexpress the BRAFV600E protein, the PCR product obtained through site-directed mutagenesis was inserted into the p3XFLAG-CMV/pDEST mammalian expression vector using the Invitrogen Gateway cloning system, following the manufacturer's instructions. Plasmids were amplified and collected using the *E. coli* DH5α system. After cloning, the plasmids were sequenced with the Applied Biosystems 9700 Thermocycler. These plasmids were then transfected into A375 cells to confirm protein-level overexpression.

Western blot. A375 cells transfected with BRAFV600E were collected by scraping, avoiding trypsin-EDTA treatment to prevent potential damage to in-

tegrins, and lysed in TNTE (Tris-NaCl-Triton X-100-EDTA) buffer. Samples were then loaded onto a 7.5% acrylamide gel in running buffer containing 10% SDS, with Precision Plus Protein™Dual Color Standards from Bio-Rad serving as a marker. Proteins were transferred onto a PVDF membrane, which had been pre-soaked in methanol, using 1X cold transfer buffer on ice.

The PVDF membranes were subsequently blocked in 5% skimmed milk in TBST. Following blocking, primary antibodies, including mouse monoclonal anti-ITGB3 (Santa Cruz) and mouse anti-β-actin (Sigma), were diluted in 5% skimmed milk in TBST at a 1:1000 ratio and incubated with the membrane overnight at 4 °C. The secondary antibody, goat anti-mouse IgG-HRP (Santa Cruz), was applied at a 1:7500 dilution in 5% skimmed milk in TBST and incubated for one hour. Protein bands were detected using chemiluminescence with Clarity™Western ECL Substrate from Bio-Rad and visualized using the Bio-Rad ChemiDoc MP system. Data normalized by β-actin expression.

Reporter assay. The 3TP-LUX (PAI-1 driven promoter) and SBE-LUC (Smad binding element) plasmids were utilized as TGF-β responsive reporter plasmids to demonstrate TGF-β activation. To ensure equal plasmid amounts in each well, the total amount was balanced by adding the p3XFLAG-CMV/pDEST vector, which also served as the mock control. The SBE-LUC and pCMV-β-Gal plasmids were generously provided by Talat Nasim at the University of Bradford, UK. The p3TP-lux plasmid was a gift from Joan Massagué and Jeff Wrana (Addgene plasmid #11767) [15].

Cells were lysed with reporter lysis buffer (Promega, Cat. No. E4030) 24 hours post-transfection, and luciferase activity was measured immediately after adding the luciferin substrate using a luminometer (Fluoroskan Ascent FL, Thermo Scientific). Luciferase activity was normalized for transfection efficiency, which was assessed via β-galactosidase activity. β-galactosidase activity was quantified by measuring absorbance at 405 nm after incubation with ONPG (4 mg/ml) in Z buffer ($Na_2HPO_4{\cdot}7H_2O$ (0.06 M) + $NaH_2PO_4{\cdot}H_2O$ (0.04 M) + 0.5 mL 1 M KCl (0.01 M) + 1 M $MgSO_4$ (0.001 M)) supplemented with β-mercaptoethanol, followed by termination with 1 M Na_2CO_3 buffer.

The reporter assay data were then normalized by dividing the measured luciferase activity by the β-galactosidase activity results.

Data analysis. To analyze the RT-qPCR data, the threshold cycle (Ct) values of cDNAs were normalized to the housekeeping gene GAPDH. The data were calculated using the following formula: the average of technical repeats and ΔCt = Ct (average of target gene) – Ct (average of housekeeping gene). The 2 ($-\Delta$Ct) value was calculated, and 2 ($-\Delta\Delta$Ct) = 2 ($-\Delta$Ct(sample))/2 ($-\Delta$Ct(control)) was calculated to show fold changes.

Statistical analysis was conducted using an unpaired, two-tailed Student's t-test, with significance set at $p < 0.05$. Error bars represent the mean ± SD of three independent experiments, each performed in triplicate. Western blot bands were

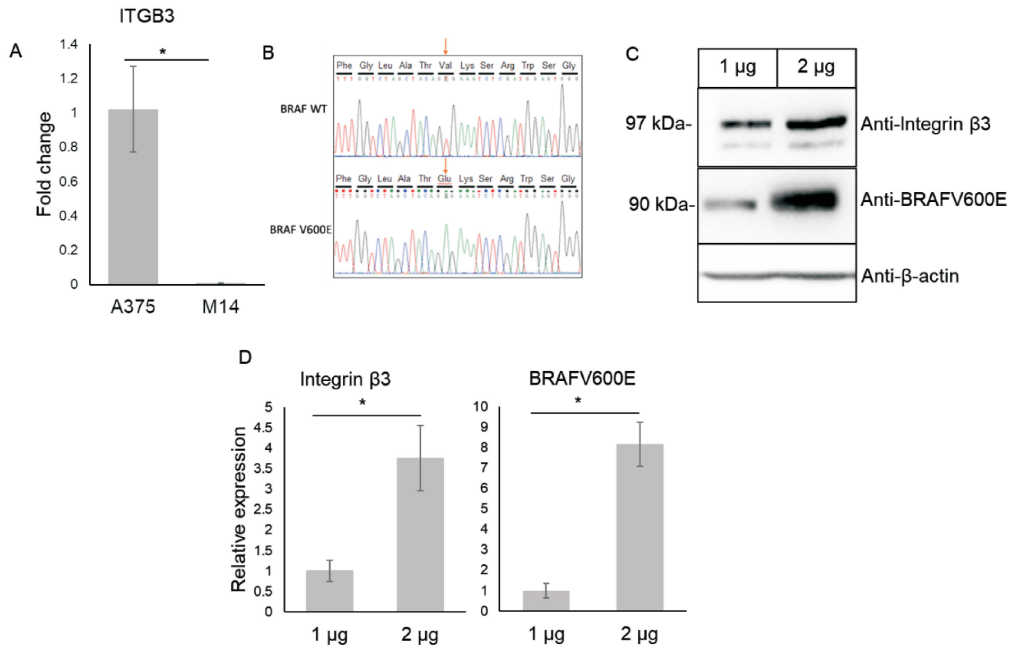

Fig. 1. ITGB3 levels are high in the A375 cell line, and overexpression of BRAFV600E is correlated with increased Integrin $\beta 3$ levels; A) mRNA expression of *ITGB3* in A375 and M14 cell lines; B) Sanger sequencing of WT BRAF and BRAFV600E plasmid; C) Protein expression changes of Integrin $\beta 3$ and αIIb in the presence of BRAFV600E overexpression; D) Quantification of Integrin $\beta 3$ and BRAFV600E expression, WT: Wild type. Results show average $\pm$ SD of three independent experiments, Student's t test $^{*}p < 0.05$

quantified using Bio-Rad's Image Lab software, with a Student's t-test used to determine significance. In vitro wound assay images were analyzed with ImageJ, and significance was assessed using a Student's t-test.

Results. ***Increased expression of Integrin β3 subunit due to BRAFV600E overexpression.*** A375 cells, which carry a homozygous BRAFV600E mutation, exhibit an approximately 100-fold increase in ITGB3 expression levels compared to M14 cells, which carry a heterozygous mutation. This significant increase in ITGB3 expression correlates with their BRAFV600E mutation status (Fig. 1A).

To mimic the effects of BRAFV600E in melanoma cells, the BRAFV600E mutation was introduced using site-directed mutagenesis, and the plasmid was sequenced to confirm the mutation (Fig. 1B). When BRAFV600E was overexpressed in A375 cells using 1 µg and 2 µg plasmids, there was an approximately 8-fold increase in BRAFV600E expression with the higher plasmid concentration. This 8-fold increase in BRAFV600E expression was associated with a significant 4-fold increase in the expression of the Integrin $\beta 3$ subunit (Fig. 1C, D).

Overexpression of BRAFV600E and Integrin β3 accelerates wound healing. Our study identified a positive correlation between the increased expression of BRAFV600E and Integrin $\beta 3$. However, the impact of elevated Integrin $\beta 3$ expression on cellular behaviour was not fully understood. To explore this, we conducted an in vitro wound healing assay to assess the effects of overexpressing Integrin $\beta 3$ and BRAFV600E. Notably, the simultaneous overexpression of Integrin $\beta 3$ and BRAFV600E significantly accelerated the wound healing rate in A375 cells (Fig. 2).

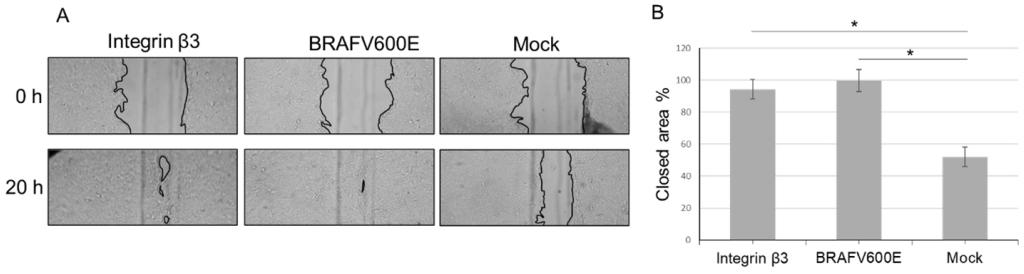

Fig. 2. Overexpression of Integrin $\beta 3$ and BRAFV600E significantly increased cell migration in A375 cells; A) The effect of Integrin $\beta 3$ and BRAFV600E overexpression on A375 cells by in vitro scratch-wound assay; B) The analysis of scratch-wound assay by imageJ and percentage of closed area after 20 h presented in graph; Results show average ± SD of three independent experiments, Student's t test $^{*}p < 0.05$

Overexpression of BRAFV600E and Integrin β3 induce TGF-β signalling. Previous studies support the role of TGF-β in melanoma [7, 11]. Additionally, the crucial role of TGF-β in wound healing is well-documented [16]. To investigate the response of the TGF-β pathway to the overexpression of BRAFV600E and Integrin $\beta 3$, luciferase reporter assays and real-time PCR were conducted using TGF-β responsive elements. The findings revealed that the overexpression of BRAFV600E and Integrin $\beta 3$ increased TGF-β activity (Fig. 3A, B) and induced the expression of TGF-β responsive genes (Fig. 3C, D). Notably, the combined overexpression of BRAFV600E and Integrin $\beta 3$ demonstrated a cumulative effect (Fig. 3A, B, C, D).

?IIb?3

Discussion. The BRAF V600E mutation is found in approximately 50% of melanoma cases, a deadly form of skin cancer originating from mutated melanocyte cells in the epidermis [4]. While the BRAFV600E mutation is not exclusive to melanoma, studying this cancer is essential due to its high incidence rate, aggressive nature, and the significant impact of the BRAF V600E mutation on its development and progression.

The correlation between BRAFV600E expression and Integrin $\beta 3$ levels in melanoma cells has not been extensively studied. We observed a significant increase in ITGB3 mRNA levels in the A375 cell line, which carries the homozygous

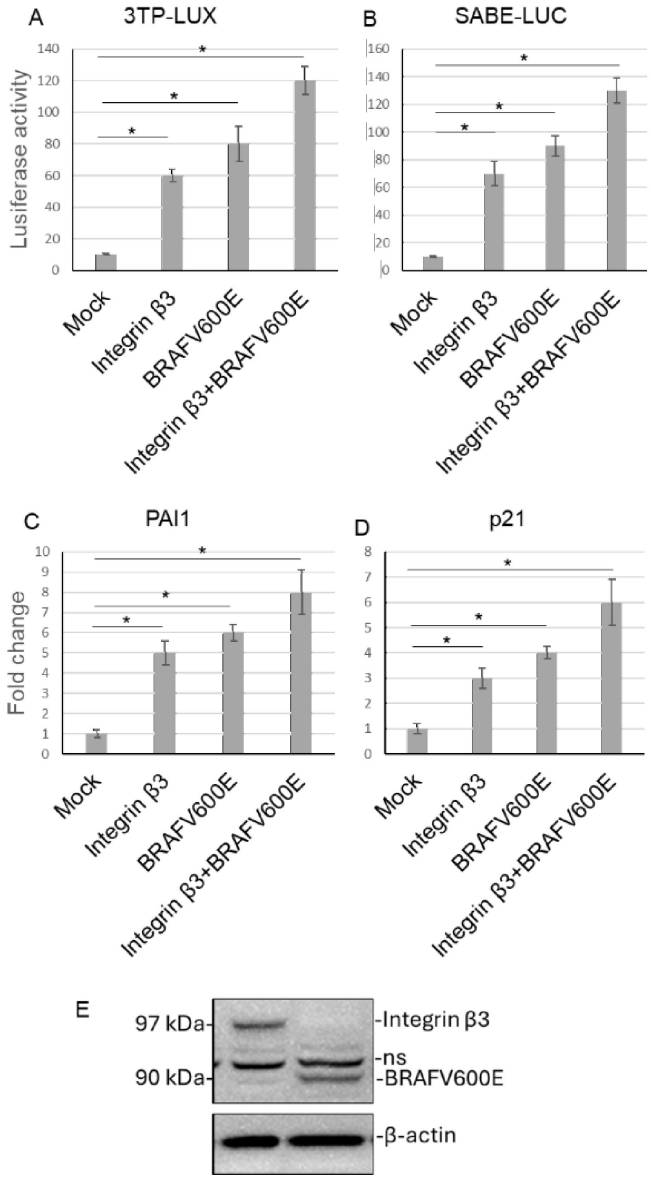

Fig. 3. Overexpression of BRAFV600E and Integrin $\beta 3$ activates TGF-β signalling in A375 cells; A) The response of 3TP-LUX to overexpression of integrin $\beta 3$, BRAFV600E, integrin $\beta 3$+ BRAFV600E and mock; B) the response of SABE-LUC to overexpression of integrin $\beta 3$, BRAFV600E, integrin $\beta 3$+ BRAFV600E and mock; C) Cells transfected with integrin $\beta 3$, BRAFV600E, integrin $\beta 3$+ BRAFV600E and mock, and C) PAI1; D) p21 levels determined by real time PCR; E) Overexpression of integrin $\beta 3$ and BRAFV600E in A375 melanoma cell line. Results show average $\pm$ SD of three independent experiments, Student's t test $^{*}p < 0.05$

BRAFV600E mutation (Fig. 1A). Although we did not analyze the MAPK pathway activity, existing literature indicates that BRAFV600E hyperactivates the MAPK signalling pathway by constitutive phosphorylation of the MEK protein. This suggests that the BRAFV600E protein expressed from both alleles activates the MAPK pathway in A375 cells to a greater extent than in M14 cells, which express BRAFV600E from one allele and wild-type BRAF from the other. The increase in ITGB3 mRNA levels in A375 cells may be due to the overactivity of the MAPK pathway caused by the mutated BRAF protein. However, it is essential to consider that the crosstalk between the MAPK pathway and the unique characteristics of different cell lines may also influence these results. Although both cell lines are melanoma-derived, they have other genotypic differences. For instance, M14 cells harbour the 150 + 2T > C [17] and 455insCdel26 mutations in the p16 tumour suppressor gene, whereas A375 cells carry the Glu61Ter and Glu69Ter [18] mutations in the p16 gene. To eliminate the effect of differing cell line characteristics, the BRAFV600E mutation was overexpressed in varying amounts in the A375 cell line. We found that increased BRAFV600E expression led to a significant rise in both Integrin β3 and Integrin αIIb subunit expression (Fig. 1C, D). These results demonstrate a strong connection between the increase in integrins and BRAFV600E expression, suggesting a potential relationship with the overactivation of MAPK signalling.

A study involving 370 melanoma patients revealed that high BRAF expression is associated with poor overall survival [4]. To investigate the impact of increased levels of BRAFV600E and Integrin β3 on cell migration, a crucial process linked to metastasis, we conducted an in vitro wound healing assay. Our findings revealed a significant increase in cell migration upon overexpression of BRAFV600E and Integrin β3 (Fig. 2). Given the known involvement of ITGB3 in tumourigenic processes, this increase in migration may contribute to the poor prognosis associated with metastasis. Therefore, targeting BRAFV600E expression while considering ITGB3 a potential drug target could be promising for future treatment strategies.

The crucial role of TGF-β in melanoma is well established in the literature. Gautron et al. [19] showed that SMAD3 expression increases during acquisition of BRAFi resistance. The upregulation of the SMAD3 transcription switches phenotype from epithelial to mesenchymal which is associated with migratory and invasive properties. Additionally, the interaction between TGF-β and integrin is responsible for epithelial-mesenchymal transition (EMT). It is understood that TGF-β1-mediated EMT induces the formation of the integrin β3-TGFβ receptor 2 (TβRII) complex on the cell surface, activating the MAPK pathway to induce invasion in normal mouse mammary epithelial cells [20].

Supporting previous findings, our results indicate that the overexpression of BRAFV600E and Integrin β3 enhances TGF-β activity (Fig. 3A, B) and induces the expression of TGF-β responsive genes in A375 cells (Fig. 3C, D). Moreover, the co-overexpression of BRAFV600E and Integrin β3 demonstrates a cumulative

effect (Fig. 3A, B, C, D). These findings highlight the involvement of the TGF-β pathway in the association between BRAFV600E and Integrin β3, underscoring their role in wound healing within melanoma cells.

Conclusion. In conclusion, the BRAF V600E mutation, which is frequently observed in melanoma as well as other cancer types, plays a pivotal role in tumour development and progression. Our study reveals a significant correlation between BRAFV600E expression and integrin levels, particularly ITGB3, in melanoma cells. This correlation suggests a key involvement of integrins in the TGF-β signalling pathway and tumourigenic processes.

We demonstrated that elevated BRAFV600E expression results in increased levels of both integrin β3 and integrin αIIb subunits, indicating a robust connection between BRAFV600E overactivation and integrin upregulation. Additionally, our findings show that overexpression of BRAFV600E and integrin β3 enhances cell migration, emphasizing the importance of these factors in promoting metastasis through TGF-β signalling.

These results not only deepen our understanding of the molecular mechanisms driving melanoma progression but also highlight potential therapeutic targets. Future strategies aimed at targeting BRAFV600E and integrins, particularly in the context of TGF-β-mediated metastasis, could lead to improved patient outcomes and offer new avenues for treatment.

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