Scaffold KSR2 Overexpression Is Associated With Melanoma A375 Cells Resistance to Vemurafenib

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1. Abstract

A large number of tumors show a deregulation of the pathway RAS-RAF-MEK-ERK. Most of cases of melanoma are caused by the mutation V600E of BRAF that leads to the constitutive activation of this kinase and of the MAPK pathway. One of the most important BRAF V600E inhibitors used against melanoma is vemurafenib. An extension study of melanoma patients with BRAF V600E tumors shows that vemurafenib treatment of these metastatic melanomas causes complete or partial tumor regression. However, the majority of patients eventually develop resistance or present intrinsic resistance against this drug, and the tumor becomes more aggressive. Several mechanisms of resistance to BRAF inhibitors have been described. In most of these mechanisms the resistance to BRAF inhibitors results from reactivation of MEK-ERK pathway. Scaffold KSR2 is an important modulator of the ERK-MAPK signalling pathway. In this study, we investigated the role of KSR2 in vemurafenib-treated melanoma cells. We found that the treatment with the BRAF-selective inhibitor vemurafenib induced the expression of KSR2 in A375 human melanoma cells. Interestingly, the KSR2 overexpression increased the melanoma cells' growth after treatment with vemurafenib. These results suggest that scaffold KSR2 could play an important role in the mechanism of resistance of melanoma against BRAF inhibitor vemurafenib.

Keywords: scaffold KSR2, ERK-MAPK, melanoma, drug resistance, Vemurafenib

2. Introduction

Malignant melanoma is the most deadly form of skin cancer. It has been estimated that there are >100,000 cases with 22,000 deaths in Europe (Forsea, Del Marmol, De Vries, Bailey, & Geller, 2012) and each year there are >76,000 cases of melanoma with >9,000 deaths in the U.S. (www.cancer.org; American Cancer Society).

Aberrant activation of the ERK-MAPK pathway is common in human tumors. This pathway consists of a three-tiered kinase module (comprising the kinases RAF, MEK and ERK). Critically, 45%-50% of melanomas carry somatic mutations in BRAF, and those in another 20% carry mutations in NRAS. The mutant proteins are active and constitutively activate the RAS-ERK pathway, driving cancer cells proliferation, survival, metastasis and, thereby, tumor progression (Albino, Le Strange, Oliff, Furth, & Old, 1984; Chudnovsky, Khavari, & Adams, 2005). Because this pathway is frequently dysregulated in human cancers, intense efforts are under way to develop selective inhibitors of the ERK pathway as anticancer drugs. Although promising results have been reported in early trials for inhibitors of RAF or MEK, resistance invariably occurs.

Vemurafenib is an orally available and clinically active small molecule inhibitor of BRAF that achieves increased progression-free and overall survival of patients with BRAF mutant melanoma, but not those with BRAF wild-type melanoma (Chapman et al., 2011; Flaherty et al., 2012; Sosman et al., 2012). However, most patients treated with vemurafenib develop acquired resistance after a relatively short period of disease control. Furthermore >20% of patients having BRAF mutant melanoma, present intrinsic resistance and do not respond to vemurafenib. Several mechanisms of resistance to BRAF inhibitors have been described. In the majority of these mechanisms, the resistance to BRAF inhibitors results from reactivation of the MEK-ERK pathway (Girotti et al., 2013; Nazarian et al., 2010; Wilson et al., 2012).

The ERK-MAPK signalling pathway is regulated by scaffold molecules that assemble multiple components of the signalling cascade in sequence (Burack & Shaw, 2000; Dhanasekaran, Kashef, Lee, Xu, & Reddy Fels, 2007; Kolch, 2005; Morrison & Davis, 2003; Shaw & Filbert, 2009). An important scaffold known to regulate the ERK signalling cascade is the KSR (Kinase Suppressor of Ras) family. The best characterized member of this family is KSR1 that promotes activation of Raf/MEK/ERK kinase cascade (Kornfeld, Hom, & Horvitz, 1995; Kortum & Lewis, 2004; Sundaram & Han, 1995; Therrien et al., 1995). It has been shown that KSR1 is required for Ras-mediated tumorigenesis in vitro and in vivo (Lozano et al., 2003; Xing et al., 2003).

Similar to KSR-1, the scaffold KSR-2 can interact with a number of signalling components of the Ras/MAPK pathway, including Ras, RAF-1, MEK-1, ERK-1/2 (Ohmachi et al., 2002), and kinases and phosphatases proteins (Costanzo-Garvey et al., 2009; Dougherty et al., 2009; Liu et al., 2009; Revelli et al., 2011) involved in ubiquitin– proteasome, apoptosis, insulin signalling and obesity. Due to the presence of additional 63 amino acids between CA2 and CA3 domains, KSR2 interacts with the Ser/Thr protein phosphatase calcineurin (CN) and AMPK (Costanzo-Garvey et al., 2009). Recently, a role of KSR2 in tumor transformation was analyzed (Fernandez, Henry & Lewis, 2012). However, the precise mechanism by which KSR molecules modulate the sensitivity of cells to anticancer drugs is still unknown. In this

study we provide evidence that PLX4720, an analogue of the BRAF-selective inhibitor vemurafenib, affects KSR2 expression in melanoma A375 cells. Notably, KSR2-overexpression reduces the sensitivity of A375 to PLX4720, indicating the ability of KSR2 to mediate resistance to BRAF inhibitor. These findings suggest that KSR2 expression levels may impact the therapeutic effect of vemurafenib.

3. Materials and Methods

3.1 Cell cultures

A375 malignant melanoma human cells were grown in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS). Cells were passaged every two-three days as required to maintain log phase growth for all experiments.

3.2 Real Time PCR

Total RNA was extracted from single cell suspension from A375 cells using RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RT-PCR was carried out on 0.5–1 mg total RNA using iScript cDNA Synthesis Kit (Bio-Rad) and oligo-dT (Promega Italia srl, Milan, Italy) as a first-strand primer. Real-time qPCR was performed using SsoFast Eva green SuperMix (Bio-Rad), according to the manufacturer's instructions, in an Opticon 2 continuous fluorescence detection system (MJ Research, Bio-Rad Laboratories, Waltham, MA, USA). All samples were run in triplicate on 96-well optical PCR plates (Roche Diagnostics, Milan, Italy). The cDNA fragments of indicated genes were amplified using specific pairs of primers (PRIMM, Milan, Italy): 5'-AGCAAGTCCCATGAGTCTCA and 5'-CAACCTGCAATGCTTGCACT (human KSR-1); 5'-CCGACACAGAGGAGGAT AAG and 5'-TCAAAGGCCCAGCAGA AG (human KSR-2);5'-CAAGGCCGCA GATGCAATCTT and 5'-AGTCAGACT CCTGGCTTTGCA-3 (human COT1); 5'-GAA GGTGAAGGTCGGAGT and 5'-GAAGATGGTGATGGGATTTC(human GAPDH). After an initial denaturation for ten minutes at 95°C, denaturation for the subsequent forty cycles was performed for 15 s at 95°C, followed by a 15 s primer annealing at 60°C and a final extension at 72°C for 30 s. The 2-ΔΔct method was applied as a comparative quantification method and the mean fold change in expression of the target gene in each condition was calculated, according to Livak and Schmittgen (Methods, 2001; 25, 402-408). Human KSR1, KSR2 and COT1 mRNA levels were normalized to human GAPDH, used as a housekeeping gene.

3.3 Transfection

A375 cells were transfected with the indicated DNA using Effectene Transfection Reagent (Qiagen). To generate GFP-KSR2 vector, mouse KSR2 full-length cDNA (a gift from AS Shaw) was digested with EagI and HindIII and subcloned into the pEGFP-C2 vector (Giurisato et al., 2014). To generate melanoma cells resistant to BRAF inhibitor PLX4720, A375 cells were incubated with 1 mM PLX4720 for 72 hrs. and death cells were removed by several washings.

3.4 Cell growth analysis

A375 cells (5x103/well into of a 24-wells plate) were incubated with 1 μM PLX4720 (Aurogene S.r.L) or with DMSO (as control) in completed DMEM. In some experiments, melanoma cells were transiently transfected with mammalian vector expressing 0.4 μg GFP (as control) or 0.4 μg GFP-KSR2.

After transfection, cells were incubated with 1μM PLX4720 or with DMSO (as control) for 72 hrs. A375 cell growth was analyzed by microscopy as described in Vermi et al., 2013.

3.5 Immunoblotting analysis

Expression level of KSR2 protein was assessed by immunoblotting. Cells were resuspended in ice-cold lysis buffer with the following composition: 20 mM Tris base, 137 mM NaCl, 0,2% Triton X-100, 1 mg/ml apoprotenin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and incubated on ice for twenty minutes. After centrifugation, proteins from cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were blotted onto activated nitrocellulose membranes (Amershan Pharmacia Biotechnology) and probed with antibodies as specified in each experiment. Cell lysates were analyzed by immunoblotting with anti-KSR2 antibody (Santa Cruz Biotechnology), anti-COT1 (Sigma), anti-pERK1/2 (Cell Signalling Technology), anti-GFP (Santa Cruz Biotechnology), anti-α-tubulin antibody (Sigma). The primary antibody was detected by incubating the membranes for one hour with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Promega, WI USA) followed by enhanced chemiluminescence system for detection (Bio-Rad).

3.6 Statistical analysis

All error bars represent mean ± SE based on several independent experiments. Statistical analyses were performed using a paired Student's t test. Statistically significant differences are indicated in the figure legends.

4. Results

4.1 Analysis of KSR2 expression in PLX4720 sensitive melanoma cells

To identify the role of KSR2 in melanomas, we used A375, a BRAF (V600E) mutant human malignant melanoma cell line that is sensitive to the BRAF-selective inhibitor PLX4720 (Johannessen et al., 2010). Since PLX4720 treatment affects gene expression (Johannessen et al., 2010), we investigated whether BRAF Kinase inhibitor PLX4720 affects KSRs expression. A375 cells were incubated with 1 μM PLX4720 for six hours and mRNA was quantified by RT-PCR. We observed that while KSR1 expression was slightly affected by BRAF inhibitor, PLX4720 treatment induced strong up-regulation of KSR2 transcript after six hours (Figure 1). As previously shown, PLX4720 treatment induced up-regulation of COT1 transcript, consistent with its role in drug resistance (Johannessen et al., 2010).

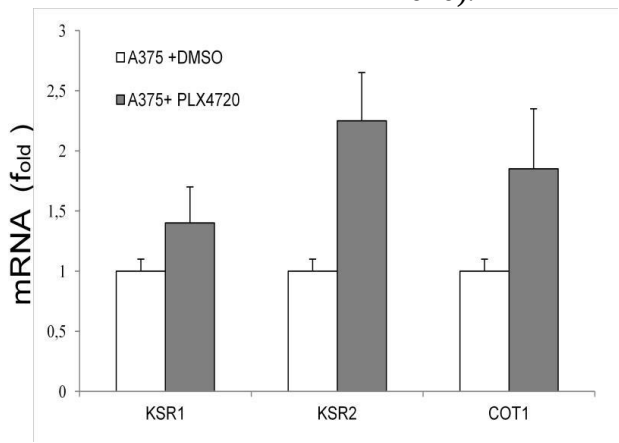


Figure 1. KSRs mRNA expression in A375 cells. Total RNA was extracted from A375 cells incubated with DMSO (control) or 1μM PLX4720 for six hours and the amounts of KSR1, KSR2 and COT1 transcripts were measured by quantitative real-time PCR. The data corresponds to the mean ± SE of three independent experiments.

That data was confirmed by protein analysis. We observed that endogenous KSR2 protein expression is not detectable in A375 melanoma cells by immunoblotting (Figure 2). A375 cells were treated with 1 μM PLX4720 for 72h and KSR2 protein expression in cells lysates was analyzed by immunoblotting. As shown in Figure 2, PLX4720 treatment induced KSR2 protein expression.

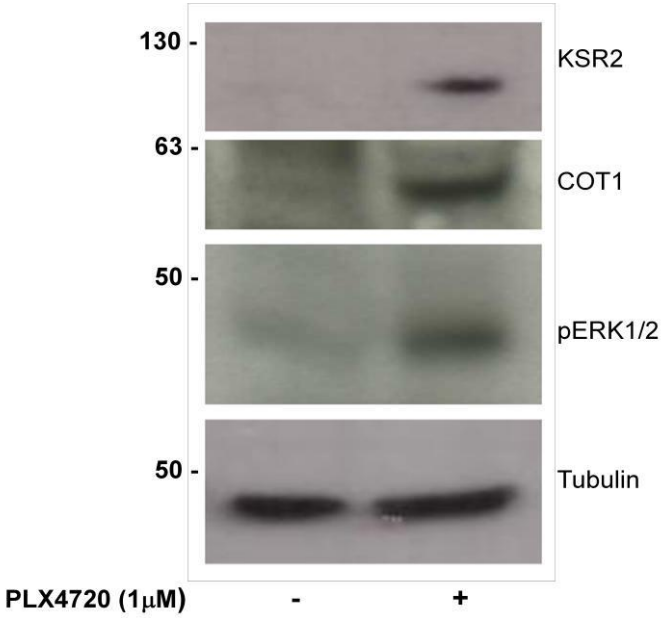


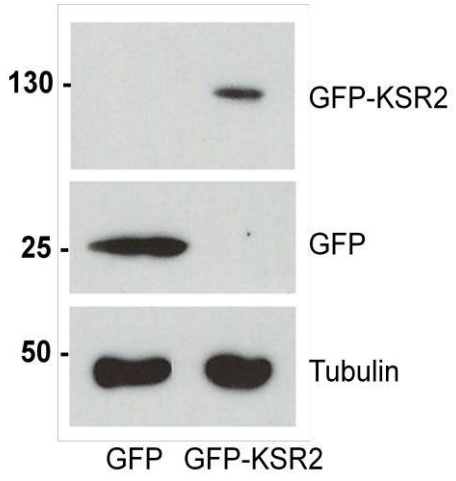
Figure 2. KSR2 protein expression is induced by PLX4720 in A375. Immunoblotting analysis of KSR2 expression in BRAF (V600E) melanoma cells after treatment with DMSO (as control) or 1 μM PLX4720 inhibitor for 72 hrs. COT1 and pERK1/2 expression was also induced after treatment with BRAF (V600E) inhibitor. Tubulin was used as loading control. Relative molecular mass (kDa) is reported on the left.

Notably, as previously reported (Johannessen et al., 2010), we observed that PLX4720 treatment induced COT 1expression and ERK1/2 activation (Figure 2). That data indicates that KSR2 expression is affected by PLX4720 and suggests that it might be involved in the sensitive/resistance mechanism of melanoma cells to the selective BRAF mutant inhibitor.

4.2 KSR2 overexpression confers resistance to RAF inhibition

To determine whether KSR2 overexpression was sufficient to affect the resistance of A375 to PLX4720, melanoma cells were transiently transfected with mammalian vector expressing GFP (as control) or GFP-KSR2. Transfection efficiency was analyzed by microscopy and GFP-KSR2 protein expression was determined by immunoblotting (Figure 3A). Eighteen hours after transfection cells were incubated with 1 μM PLX4720 or vehicle (DMSO, as control) for 72 hours and cells' growth was assessed by specific assay. As shown in Figure 3B, A375 cell growth was significantly increased in KSR2-overexpressing melanoma cells, indicating the ability of the scaffold KSR2 to confer resistance to RAF inhibition.

A



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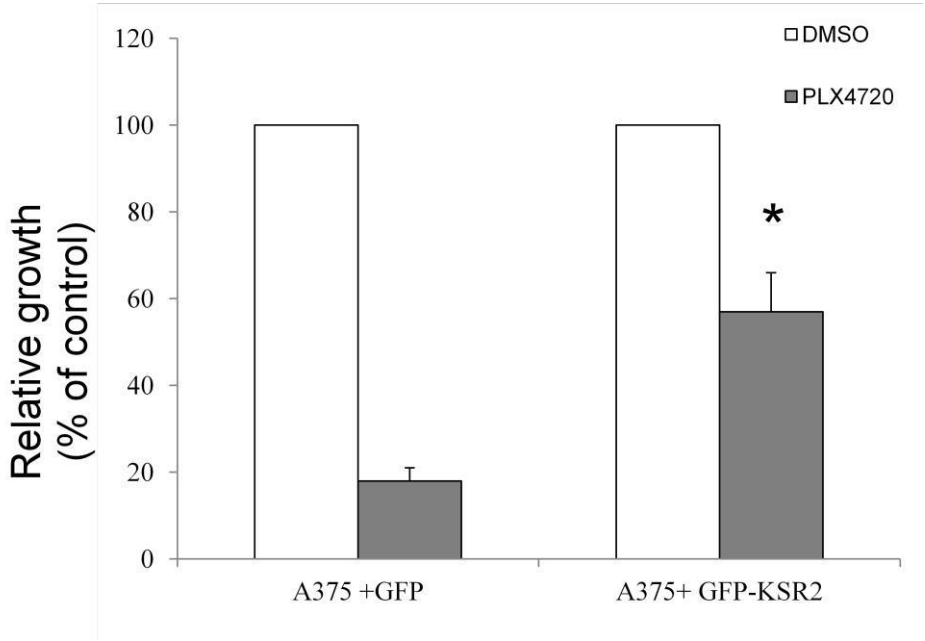

Figure 3. KSR2 overexpression induced resistance to B-RAF inhibition in melanoma cells. A375 cells were transiently transfected with mammalian vector expressing 0.4 μg of GFP (as control) or 0.4 μg GFP-KSR2. Transfection efficiency was analyzed by microscopy (more than 90% of transfected A375 were GFP positive). A) Immunoblot analysis of GFP and GFP-KSR2 in lysates from transfected A375 cells. Lysates were blotted for GFP. Tubulin protein was used as loading control. Relative molecular mass (kDa) is reported on the left. B) Eighteen hours after transfection, cells were incubated with 1 μM PLX4720 or vehicle (DMSO, as control) for 72 hours and cells growth was assayed. Quantitative analysis (mean ± SE) of growth of GFP or KSR2-GFP transfected cells incubated with DMSO or with 1 μM PLX4720 from three experiments is shown in the graph bars. The significance of values is compared with GFP-transfected PLX4720-treated cells. *$p<0.05$.

5. Discussion

Previous studies underlined that the most important mutation causing the melanoma is the V600E of BRAF (Davies et al., 2002). This mutation leads to the constitutive activation of this kinase protein which is involved in the MAPK pathway, responsible for the control of proliferation, differentiation and apoptosis of cells (Ascierto et al., 2013).

Vemurafenib is an oral, small-molecule kinase inhibitor that selectively targets activated BRAF V600E and has been approved for the treatment of advanced BRAF mutation-positive melanoma (Ravnan & Matalka, 2012; Vultur, Villanueva, & Herlyn, 2012). However, after few months, melanoma cells develop a resistance against this drug, and the tumor becomes more aggressive (Girotti et al., 2015). So, urgently, it is essential to discover the molecules responsible for the resistance, to target them for new therapies.

It has recently been reported that acquired resistance is mediated through constitutive signalling by receptor tyrosine kinases

(RTKs) (IGF1R and PDGFR-β), mutations in NRAS or MEK1, and the increased expression of COT as the result of BRAF truncations (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2010; Wagle et al., 2011).

In particular, melanoma cells with overexpression of COT showed signs of intrinsic resistance to vemurafenib. COT activates ERK primarily through MEK-dependent mechanisms that do not require RAF signalling. In addition, COT expression is associated with de novo resistance in BRAF (V600E) cultured cell lines and acquired resistance in melanoma cells and tissue obtained from relapsing patients, following treatment with MEK or RAF inhibitors (Johannessen et al., 2010). However, how COT expression and activation is regulated in melanoma cells remain to be clarified.

As BRAF, COT1 is a MAP3K8 that associates with scaffold molecule KSR2 (Channavajhala et al., 2003). It has been reported that COT function might be regulated by KSR2 in a dose-dependent manner. The scaffold KSR2 is responsible for the modulation of RAS-MAPK signalling. Similar to KSR1, the scaffold KSR2 can interact with a number of signalling components of the RAS/MAPK pathway, including RAS, RAF-1, MEK-1, ERK-1/2 (Ohmachi et al., 2002), kinases and phosphatases proteins (Costanzo-Garvey et al., 2009; Dougherty et al., 2009; Fernandez et al., 2012; Liu et al., 2009; Revelli et al., 2011) involved in ubiquitin–proteasome, apoptosis, insulin signalling, obesity, cell cycle control and microtubule association. In our study, we investigated on the role of KSR2 in melanoma pathogenesis.

First of all, we evaluated the KSR2 expression in A375 human melanoma cell line which is very sensitive to PLX4720. We observed KSR2 mRNA expression increased after PLX4720 treatment and become more upregulated than KSR1 after six hours treatment. In parallel, PLX4720 treatment induced the expression of COT1 transcript. Similar results were observed by immunoblotting. While KSR2 proteins were not detectable in steady state condition, our result showed that KSR2 expression increased after treatment with the specific BRAF inhibitor, vemurafenib. As previously observed (Johannessen et al., 2010), we found that PLX4720 treatment induced COT1 protein expression. Interestingly, overexpression of KSR2 increased A375 cells growth after treatment with PLX4720 which is correlated with ERK1/2 activation, indicating that this scaffold molecule might be involved in the drug-resistance mechanism.

Additional studies are required to understand the signalling mechanism underlying the KSR2-mediated resistance to BRAF inhibitors. The suppression of endogenous KSR2 by siRNA will provide important evidence on the molecular mechanism by which scaffold KSR2 could affects melanoma tumor progression and drug resistance. In addition, whether KSR2 is involved in the COT1-mediated resistance of melanoma cells to PLX4720 remains to be clarified. Furthermore, since RAF kinase inhibitors treatment can paradoxically induce ERK-MAPK cascade signalling in activated RAS tumor cells (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010), will be challenging to investigate the role of KSR2 in PLX4720-treated NRAS mutant melanoma.

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