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# Study of molecular mechanism of vemurafenib resistance via PIN1 in malignant melanoma

Chosun University Graduate School

Department of Pharmacy

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악성흑색종에서 **PIN1** 에 의한 **vemurafenib** 내성의 분자기전 연구

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# Contents

# List of figures

# List of abbreviations and acronyms

| | |
|---|---|
| ATRA | All trans retinoic acid |
| Bcl2 | B-cell lymphoma 2 |
| DMSO | Dimethylsulfoxide |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal regulated kinase |
| FACS | Fluorescence activated cell sorting |
| MEK | MAPK/ERK Kinase (also known as MAP2K) |
| PARP | Poly (ADP-ribose) polymerase |
| p-ERK | Phosphorylated ERK Kinase |
| PIN1 | Peptidyl prolyl cis-trans isomerase NIMA-interacting factor1 |
| p-MEK | Phosphorylated MAPK/ERK Kinase |
| RAF-1 | Rapidly accelerated fibrosarcoma kinase (also known as cRAF) |
| siRNA | small interefering RNA |
| TUNEL | Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labeling assay |
| VMR | vemurafenib |
| XP | xpress peptide tag |

# 국문 초록

## 악성흑색종에서 PIN1 에 의한 vemurafenib 내성의 분자기전 연구

포산 유갈 밧타라이

지도교수: 최홍석

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악성흑색종은 피부암 중에서도 가장 치명적인 암으로 악성도가 높고 진행속도가 빨라 전이에 의한 사망 위험이 높다. 악성흑색종 환자에 사용되고 있는 vemurafenib 는 BRAFV600E 를 표적으로 하는 항암제로 환자의 생존기간을 2배 이상 늘리며 획기적인 치료제로 평가받았다. 하지만 vemurafenib 의 저항성이 빠르게 발생하여 악성흑색종 환자의 근본적인 치료가 제한되어 문제되고 있다. 현재까지 저항성을 유도하는 가장 중요 기전으로는 MAPK 재활성이 보고 되었으며, 저항성을 극복하기 위하여 다양한 조절 기전에 대한 이해와 연구가 필요한 실정이다.

본 연구에서는 vemurafenib 저항성을 유도하는 매커니즘 및 조절단백질을 규명하고자 vemurafenib 를 지속적으로 처리하여 저항성 세포를

구축하였다. 획득된 저항성 세포에서 EGFR 발현 및 MAPK 활성을 확인하였고 이와 함께 PIN1 의 발현이 증가되는 것을 볼 수 있었다. PIN1은 vemurafenib 저항성을 유도하는 RAF/MEK, CyclinD1 과 같은 단백질을 직접적으로 조절한다고 보고 되었기에 저항성 세포내에서 발현되는 PIN1 은 vemurafenib 저항성에 밀접하게 관련되어 있을 것이라고 보았다. 저항성 세포에서 siRNA-PIN1을 이용하여 PIN1을 저해시켰을 때 MAPK 재활성이 억제되는 것을 볼 수 있었다. 또한 유방암, 급성골수성 백혈병을 포함하여 여러 암세포에서 다양한 약물의 효과를 나타내었던 PIN1의 저해제인 ATRA 는 저항성 세포에서 vemurafenib 의 민감도를 증가시켜 줄 뿐만아니라 세포사멸 신호를 증가시켜 vemurafenib 저항성을 극복하였다.

본 연구를 통해서 PIN1 에 의해 재활성화되는 RAF/MEK/ERK 신호의 활성은 vemurafenib 저항성을 유도하는 것을 관찰하였고 PIN1 저해제 ATRA 와 병용처리에 의해 저항성이 극복되는 것으로 PIN1 은 저항성 형성에 중요한 역할을 하는 것으로 제안한다.

# I. Introduction

Melanoma is the most aggressive form of skin cancer and around 50% of cutaneous melanoma harbors mutations in *BRAF* gene (Ascierto et al., 2012; Cantwell et al., 2011). The gene encodes protein BRAF, a serine/threonine-specific protein kinase belonging to the Raf kinase family. BRAF, along with two other isoforms ARAF and CRAF, participates in RAS-RAF-MEK1/2-ERK1/2 signaling pathway. Around 66 % of melanoma patients are reported to have mutations in *BRAF* gene. (Davies et al., 2002). Substitution of valine by glutamic acid at position 600 (BRAFV600E) alone accounts for 90% of all BRAF mutations found in malignant melanoma (Forbes et al., 2008). Mutated BRAFV600E protein has eleveated kinase activity and is independent of upstream regulations. (Wan et al.,2014; Tsavachidou et al., 2004). Both of which culiminates in constitutive activation of RAF/MEK/ERK signaling leading to the robust progression of melanoma tumorigenesis.

Advanced melanoma patients with BRAFV600E mutation are treated with vemurafenib (Ascierto et al., 2012). vemurafenib is a specific

inhibitor of BRAFV600E mutated protein and significantly reduces the melanoma tumors. However, improved clinical outcomes obtained with vemurafenib are often temporary due to the rapid development of resistance to the drug (Piro et al., 2013). Several mechanisms are reported to be involved in the development of vemurafenib resistance in melanoma such as: MAPK reactivation; activation of RTKs, more specifically IGF-1R and PDGFR; and activation of PI3K/AKT signaling (Chan et al., 2017; Lito et al., 2013). The precise molecular mechanisms, which guides vemurafenib-resistant melanoma cell through these alternative survival pathways, are poorly described. Clinical trials for combinatorial targeted chemotherapy with an emphasis on dual inhibition of BRAFV600E and some other molecular targets, such as MEK, has recently gained a momentum to recover the resistance from BRAF mutant inhibitors (Chabbra et al., 2017; Lu, H et al.,2017; Sullivan et al., 2011). However, resistance was developed against the combination as well (welsh et al., 2016). At the same time, little attention has been paid towards the identification and characterization of novel targets, which might facilitate the development of resistance to BRAF inhibitors.

PIN1 is a peptidyl prolyl cis-trans isomerase which promotes the

tumorigenesis in different cancers including melanoma (Khanal et al., 2010; Lu, Z et al., 2016). Since PIN1 induces conformational change in specific pSer/Thr-Pro motifs, it can regulate the outcome of protein phosphorylation and thereby is a key regulator of oncogenic signaling pathways including classical MAPK pathway (Min et. al., 2016). In addition, PIN1 is reported to promote melanoma tumor progression through its interaction with FOXM1 (Kruiswijk et al., 2016). In other study, RNA interefernce-based knockdown of PIN1 reduced the tumorogenicity of A375 cell (Jin et al., 2013). PIN1 was also reported to enhance the phosphorylation of MEK 1/2 to mediate tamoxifen resistance in breast cancer (Namgoong et al., 2010). However, direct role of PIN1 in mediating vemurafenib resistance isn't yet established.

Recent study shows that that all trans retinoic acid (ATRA) is a potent inhibitor of PIN1, where it binds with PIN1 leading to its degradation. Encouraged by such finding, in this study, we first assessed the role of PIN1 in mediating vemurafenib resistance. Then, we evaluated the efficacy of ATRA in the recovery of vemurafenib resistance.

# II. Materials & Methods

**Cell culture and establishment of resistant cell line**

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen. A375 cells were purchased from American Type Culture Collection (ATCC). To generate cell lines with *in vitro* resistance, the melanoma cell were treated with 10 μM vemurafenib for 1 hour, and then surviving cells were cultured with 10 μM vemurafenib until a vemurafenib-resistant sub-line was established. Both the A375 and A375R cell cultures were cultured in DMEM supplemented with 10% featel bovine serum, at 37 °C in humidified air containing 5 % CO2.

**Antibodies and reagents**

The antibodies against p-MEK (1:1000), p-ERK (1:1000), cyclin D1(1:1000), p-c-RAF (1:1000), cleaved caspase 3(1:1000) from were purchased from Cell Signaling Technology, Inc.; antibodies against EGFR (1:1000) and cleaved PARP (1:5000) were from Santa Cruz Biotechnology; β-actin (1:10000) was from Sigma-Aldrich; anti-Xpress (1:5000) was from Invitrogen. The ratio given inside parentheses is the dilution ratio used. The MTT reagent was from Do-gen (south korea). The

jetPEI® cationic polymer transfection reagent was from Polyplus-transfection. Lipofectamine Plus reagent transfection reagent was from Invitrogen. The Dual-Luciferase® Reporter assay kit was purchased from Promega.

**Mammalian expression vectors and small interfering RNA**

The cDNA of full sequence of PIN1, a gift from Dr. Kun Ping Lu, was subcloned to the pcDNA4/xpress vector (Invitrogen). The silencing of human PIN1 (accession number: NM_005221) was carried out by transfecting the ONTARGET plus siRNA SMART pool-specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon, Chicago, IL) using Lipofectamine 2000(Invitrogen)

**MTT assay**

The MTT assay was performed to check cell viability. In brief, cells ($1 \times 10^4$) were seeded in 96-well plates with 100 μL of cellsuspension in each well and incubated at 37°C in humidified air containing 5% $CO_2$. After 24 h in culture, cells were incubated with different concentrations and combination of drugs for 72 h. The cells were then treated with 5 $mg \cdot mL^{-1}$ MTT solution (10 μL per well) and incubated for 4 h, the purple formazan

formed by the live cells was dissolved in 0.04 N HCl in isopropanol (100 μL per well), and the absorbance was measured at 450 nm.

**Protein immunoblotting**

For immunoblotting cell grown in monolayer were harvested, washed in PBS and lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH7.4), 0.25% Sodium deoxycholate, 1 mM EDTA, 1% NP40, 1 mM NaF, 0.2 mM PMSF, 0.1 mM Sodium orthovanadate and protease inhibitor cocktail. (Roche) protein lysate was resolved using SDS-PAGE and blotted onto the PVDF membrane, immunoblots were probed with indicated antibodies. The immunoblots were visualized using a SuperSignal West Femto chemiluminesecene substrate (Pierce) and detected by LAS4000-mini (FUJIFILM, Tokyo, Japan).

**Anchorage-independent growth assay (soft agar assay)**

Cells were exposed to the indicated drug in 1 ml of 0.3% basal medium Eagle's (BME) containing 10% FBS. The cultures were maintained at 37°C in 5% $CO_2$ incubator for 14 days. The cell colonies were scored using an Axiovert 200M fluorescence microscope and Axio Vision software (Carl Zeiss, Thornwood, NY, USA).

**TdT-mediated dUTP nick end labeling (TUNEL) assay**

The induction of apoptosis was assessed by TUNEL staining and detected with an *in Situ* Cell Death Detection Kit (Roche Life Science, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, $2 \times 10^5$ cells were cultured for 24 h in six-well plates. The cells were then starved for 24 h and treated with sitagliptin for 6 h. Treated cells were washed with PBS and fixed with Cytofix/Cytoperm™ (BD Biosciences, San Diego, CA, USA) at 4°C for 20 min. Cells were stained with 50 μL TUNEL solution at 37°C for 1 h, then washed twice with PBS and fixed. DNA fragmentation was detected using an Axiovert 200 M fluorescence microscope and quantified using the axiovision software (Carl Zeiss).

**Cell cycle analysis**

A375 (seeding density: $5\times10^5$) and A375R (seeding density: $5\times10^5$) cells were seeded and treated with vemurafenib and combination of ATRA and vemurafenib. After cells were washed, fixed with 70% ethanol, and 200 μl of Muse™ Cell cycle reagent (EMD Millipore Corp. Billerica, MA, USA) was added. Then, cells were incubated at RT for 30 min in the dark. Samples were measured with Muse Cell cycle kit (Merck Millipore,

Billerica, MA, USA)

**Statistical analysis**

Data from soft agar assays, sub G1 assays and TUNEL assay were statistically analyzed using unpaired t-tests, and *P*-values < 0.05, 0.01, or 0.001 were considered significant.

# III. Results

## 1. Enhanced activation of RAF1-MEK1/2-ERK1/2 pathway promotes cell survival and anchorage-independent transformation of vemurafenib-resistant A375R cell

To study the mechanism of resistance to vemurafenib, we established a vemurafenib resistant melanoma cell line, designated as A375R, by continuous exposure of A375 cells to increasing concentrations (up to 10 μM) of vemurafenib. Cell viability assays showed that the A375R cells were resistant to vemurafenib ($IC_{50}$ > 10 μM) compared to parental A375 cells ($IC_{50}$ = 0.8 μM) (Figure 1A). Consistent with the results, we found that A375R cells displayed a considerable resistant to vemurafenib treatment as evident by enhanced colony formation compared to A375 cellssoft agar assay (Figure 1B). As previous reports indicated that EGFR overexpression and MAPK reactivation is responsible for the development of vemurafenib resistance (Yadav et al., 2012), we examined the protein level of EGFR and RAF1/MEKs/ERKs in A375 and A375R cells by immunoblotting. It was observed that treatment of parental A375 cells with vemurafenib caused a robust dose dependent inhibition of MEK and ERK activities and decreased EGFR level. However, EGFR expression and level

of phosphorylated MEK and ERK remained relatively unchanged in A375R cells, even in the presence of vemurafenib (Figure 1C). We then studied the cell cycle distribution using a fluorescence-based cell cycle analyser. As expected, 63.8% of parental A375 cells arrested in the $G_0$-$G_1$ phase upon vemurafenib treatment, but A375R cells abrogated vemurafenib-induced cell cycle arrest $G_0$–$G_1$ (Figure 1D). Taken together, these results suggest that reactivation of RAF/MERK/ERK pathway is responsible for enhanced survival and anchorage-independent proliferation of vemurafenib resistant A375R cell.

# Figure 1

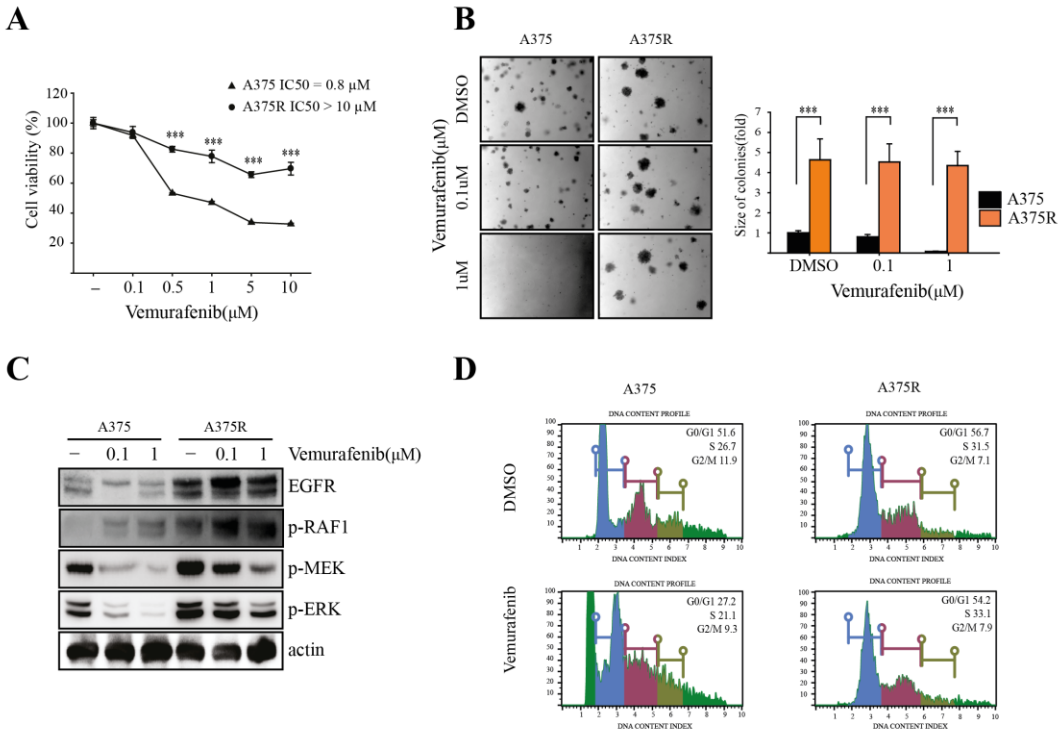

**Figure 1. Role of distinct RAF1-MEK1/2-ERK1/2 activation in mediating vemurafenib resistance in A375R cells**

**(A)** A375 and A375R cell were seeded and cultured for 24 h at 37°C in a 5% CO2 atmosphere. Then, cells were treated with various concentrations of vemurafenib, as indicated. Cell viability was estimated using a MTT assay. **(B)** A375 and A375R cell were exposed to indicate concentrarion of vemurafenib in soft agar matrix and incubated at 37°C in a 5% $CO_2$ atmosphere for 14 days. The colonies from three separate experiments are photographed. The average number of colonies was calculated, and colony size was measured under a microscope. Columns, geometrical mean of triplicate measurements; bars, standard deviation. *, $P<0.05$ **(C)** A375 and A375R cell were seeded and after 24h, cells were treated with vemurafenib for 24 h. Cells were harvested and proteins in whole-cell lysates were separated by SDS-PAGE and immunoblotted. **(D)** A375 and A375R cell were either treated or not treated with vemurafenib. After 24 h, cells were harvested and used for FACS analysis as described in materials and methods.

**2. PIN1 mediates vemurafenib resistance through the activation of RAF1-MEK1/2-ERK1/2 signaling in A375R cells**

Previous study indicates that PIN1 can bind with and promote the activation of MEK1/2 to mediate tamoxifen resistance in breast cancer (Namgoong et al., 2010). In other hand, RNA intereference-based knockdown of PIN1 supressed the tumorigenicity of A375 cells. (Jin et al., 2013). We, therefore, speculated that PIN1 might play a role in the development of vemurafenib resistance as well. To determine the possible role of PIN1 in vemurafenib resistance, we first compared the protein levels of PIN1 in between A375 and A375R cells by immunoblotting. Result showed that PIN1 level is elevated in A375R and is positively correlated with the expression of EGFR and with the levels of phosphorylated c-RAF, MEK and ERK (Figure 2A). To further evaluate the role of PIN1 in mediating the resistance to vemurafenib, we overexpressed PIN1 in A375 cells. Overexpression of PIN1 in A375 causes a significant increase in EGFR level with a concomitant increase in the phosphorylation of c-RAF, MEK and ERK (Figure 2B). In contrast, PIN1 silencing in A375R cell line decreased the EGFR level, and attenuated the phosphorylation of -RAF1, MEK and ERK (Figure 2C). Furthermore, overexpression of PIN1 in A375 cells decreased the

sensitivity to vemurafenib, whereas knockdown of PIN1 in A375R cell restored the sensitivity to an increasing dose of vemurafenib, as shown by the EGFR expression and phosphorylation of MAPK signaling proteins (Figure 2D and 2E). Taken together, these results suggest that PIN1 positively regulates the development of vemurafenib resistance in melanoma through the modulation of MAPK reactivation.

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# Figure 2

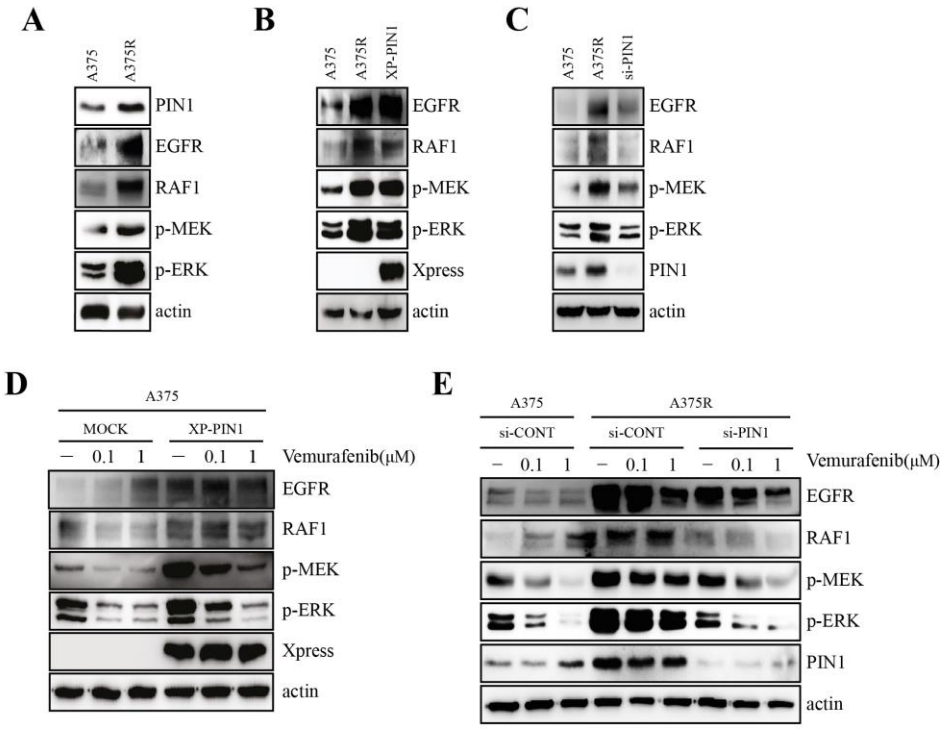

**Figure 2. PIN1 overexpression in A375R cells and its role in the reactivation of RAF1-MEK1/2-ERK1/2 signaling pathway**

**(A)** A375 and A375R cell were seeded and cultured for 48 h and then cell were harvested, lysed and immunoblotted using respective antibodies. **(B)** A375 cell were transfected with XP-PIN1 and incubated for 48 h and then cell were harvested, lysed and immunoblotted using respective antibodies **(C)** A375 and A375R cells were transfected with siRNA-control or siRNA-PIN1 and incubated for 48 h and then cell were harvested, lysed and immunoblotted using respective antibodies **(D)** A375 cells were transfected with MOCK or XP-PIN1 and then treated with indicated concentration of vemurafenib. After 24 h, cell were harvested and whole cell lysates were immunoblotted. **(E)** A375 and A375R cell were transfected with siRNA-control or siRNA-PIN1and then treated with indicated concentration of vemurafenib. After 24 h, cell were harvested and whole cell lysates were immunoblotted.

### 3. ATRA induced inhibition of PIN1 restores vemurafenib sensitivity in A375R cells

The role of PIN1 in conferring resistance to vemurafenib treatment lead us to speculate whether it is possible to recover such resistance with a PIN1 inhibitor. So, we performed cell viability assay to examine the growth inhibitory effects of the selective PIN1 inhibitor, ATRA, on vemurafenib-resistant cells. Notably, treatment with a combination of vemurafenib and ATRA was more effective than vemurafenib alone in reducing cell viability (Figure 3A). Next, we measured the level of phosphorylated C-RAF, MEK and ERK by immunoblotting after treatment with ATRA. It was found that co-treatment of A375R cells with vemurafenib and ATRA reduced the level of EGFR as well as phosphorylated RAF1, MEK and ERK (Figure 3C) suggesting the downregulation of MAPK pathway by PIN1 inhibition. Accordingly, combination of vemurafenib and ATRA also reduced the anchorage-independent colony forming capacity of vemurafenib resistant melanoma (Figure 3B). Taken together, these results suggest that inhibition of PIN1 restores the vemurafenib sensitivity in an otherwise resistant cell line through the downregulation of RAF/MEK/ERK pathway.

# Figure 3

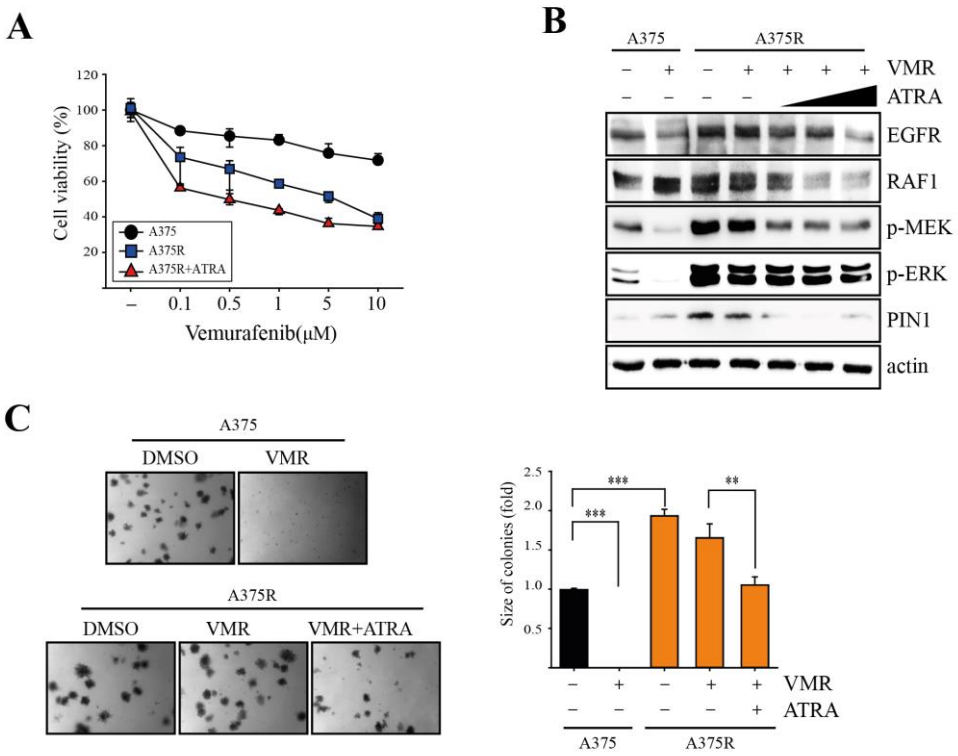

**Figure 3. Inhibitory effects of ATRA on the survival and colony forming capacity of A375R cells**

**(A)** A375 and A375R cell were treated with indicate concentrarion of vemurafenib and combination of ATRA and vemurafenib. Cell viability was measured using MTT assay. **(B)** A375 and A375R cell were treated with vemurafenib and combination of ATRA and vemurafenib. After 24 h, cell were harvested and whole cell lysates were immunoblotted using respective antibodies. **(C)** A375 and A375R cell were exposed to vemurafenib (1 μM) alone or in combination with ATRA (20 μM) in soft agar matrix and incubated at 37°C in a 5% $CO_2$ atmosphere for 14 days. The colonies from three separate experiments are photographed. The average number of colonies was calculated, and colony size was measured under a microscope. Columns, geometrical mean of triplicate measurements; bars, standard deviation. *, $P<0.05$

**4. PIN1 inhibition induces apoptosis in vemurafenib-resistant A375R cells**

To investigate the effect of vemurafenib and ATRA combinations in the induction of apoptosis, we detected cleaved caspase-3 and PARP levels by immunoblotting. Simultaneous treatment of A375R cells with vemurafenib and ATRA showed increased levels of cleaved caspase 3 and cleaved PARP (Figure 4A). To determine whether combination of vemurafenib and ATRA also influences the progression of cell cycle, we carried out cell-cycle analysis. Fluorescence-based cell cycle analysis showed that treatment with vemurafenib and ATRA caused cell-cycle arrest at $G_0$–$G_1$ and induced apoptosis in A375R cells (Figure 4B). Consistently, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining were in agreement with the above results and showed much more nuclear fragmentation of cells in the vemurafenib and ATRA combination-treated group in A375R cells (Figure 4C). These results suggest that PIN1 inhibition restores the pro-apoptotic and anti-proliferative effects of vemurafenib in vemurafenib-resistant melanoma.

# Figure 4

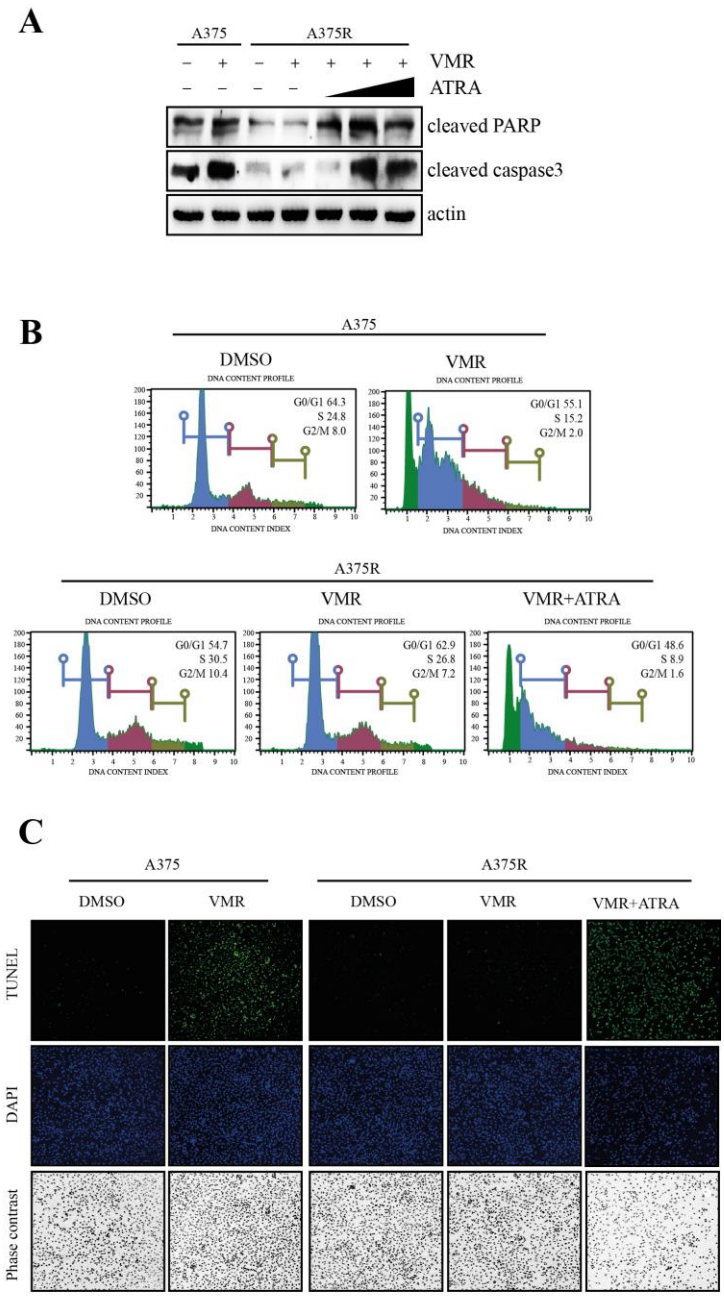

**Figure 4. Pro-apoptotic role of vemurafenib and ATRA combination in A375R cells *in vito***

(A) A375 and A375R cell were treated with vemurafenib and combination of ATRA and vemurafenib. After 24 h, cell were harvested and whole cell lysates were immunoblotted using respective antibodies. (B) A375 and A375R cell were treated with vemurafenib and combination of ATRA and vemurafenib. After 24 h, cell were harvested, washed in PBS and stained samples were analyzed using cell cycle analyzer. (C) A375 and A375R cell were treated with either vemurafenib alone or a combination of ATRA and vemurafenib for 24 h. After 24 h of incubation DNA fragmentation was measured by TUNNEL asssay.

# IV. Discussion

A large proportion of melanoma patients harbor BRAFV600E mutation, an oncogenic variant of B-RAF gene. Inhibition of BRAFV600E was therefore an obvious strategy in the chemotherapy of melanoma (Cantwell et al., 2011). Accordingly, vemurafenib was developed as a specific inhibitor of BRAFV600E mutant (Flaherty, 2011). However, resistance was shortly developed against vemurafenib. Mechanism for the development of resistance appears to be complex as it involves several signaling pathways (Lito et al., 2013). But, in majority of the cases vemurafenib resistance is accompanied by paradoxical activation of MEK/ERK signaling by RAF1, and overexpression of EGF receptors (Wan et al., 2004; Montagut et al., 2008). Here, we reported that Peptidyl-prolyl cis-trans isomerase - PIN1 positively regulates the development of resistance, through the reactivation of RAF1/MEK/ERK signaling, against vemurafenib.

PIN1 selectively binds to a subset of phosphorylated proteins at pSer/Thr-Pro motif and induces cis/trans isomerization around the proline residue. Together with proline-directed kinases and phosphatase, PIN1 can dynamically regulate several oncogenic pathways. It is already recognized for its tumor promoting role

in different cancer models including melanoma (Lu, Z et al., 2014; Min et al.,2016). Upon EGF stimulation, PIN1 was reported to bind with MEK1 to promote MEK/ERK signaling pathway (Khanal et al., 2010). In addition, RNA-interference-mediated downregulation of PIN1 was reported to reduce the tumorigenicity of A375 cell (Jin et al., 2013). However, direct role of PIN1 in mediating vemurafenib resistance was not established.

In our study, PIN1 expression was found to be increased in vemurafenib resistant A375R cell. The expression of PIN1 was positively correlated with the level of EGFR and phosphorylated RAF1. PIN1 together with PP2A is required in the recycling of RAF1 from their hyperphosphorylated and desensitized state to signaling-competent state (Dougherty et al., 2005). Therefore, it was, tempting to speculate that PIN1 possibly promotes the development of resistance through the recycling of RAF1 to active state. Previous study has also shown that elevated level of RAF1 is responsible for the acquisition of vemurafenib resistance (Montagut et al., 2008). In agreement with our hypothesis, overexpression of PIN1 in A375 cell increased the level of Phosphorylated RAF1 whereas siRNA mediated knock down of PIN1 in A375R resulted in the decreased level of Phosphorylated RAF1. In terms of the activation of MEK/ERK pathway, effects produced by PIN1 overexpression in A375 cell were comparable to vemurafenib-

resistant A375R cells. This indicates the crucial role of PIN1 in the reactivation of MAPK signaling pathway.

Recent study shows that all trans retinoic acid (ATRA) is a potent inhibitor of PIN1 (Wei et al., 2015). ATRA is a retinoid that has been used in the treatment of acute myeloid leukema(AML) for a long time, although its molecular target and mechanis of acion was unknown. Wei et al. reported that ATRA binds with PIN1 leading to its degradation. So, we seek to overcome vemurafenib resistance with ATRA-induced PIN1 ablation. In our study, knockdown of PIN1 produced effects that were similar to the effects obtained with ATRA treatment. Tretament of A375Rcells with a combination of ATRA and vemurafenib reduced the level of p-RAF1, p-MEK1/2 and p-ERK1/2. Therefore, it can be speculated that ATRA might work by inhibiting the dependency of resistant cells to RAF1-MEK1/2-ERK1/2 signaling pathway for survival, on the face of ongoing BRAFV600E inhibition. In addition to RAF1, other targets might also exist for PIN1. For instance, recent study reported that PIN1, through the stabilization of pro-proliferative transcription factor FOXM1, promoted 3D-cultured melanoid tumorigenesis (Kruiswijk et al., 2016). BRAFV600E mutation reportedly enhanced the interaction between PIN1 and FOXM1. FOXM1 activity is regulated by MEK1.(Ma et al., 2010). Based on our study, ATRA and vemurafenib combination ablated PIN1 level and reduced MEK1 activity

suggesting that same combination might reduce the melanoma tumorigenesis through the destabilization of FOXM1-PIN1 interaction.

There are stll many questions remaining to be answered. For instance, how the PIN1 level is regulated on vemurafenib resistant melanoma is still unknown. Co-occuring mutation in RAF1 might make them irresponsive to ERK-mediated negative feedback, eliminating the whole need of PIN1. Efficacy of this combination is, therefore, largely determined by how the C-RAF activity is regulated in resistant melanoma. Similarly, how the EGFR level is regulated by PIN1 is still unknown.

Resistance to B-RAF inhibitors is one of the major obstacle in the chemotherapy of melanoma (chabbra et al., 2017). In this context, a combination of ATRA and vemurafenib holds a strong promise for clinical translation. Since, both the compounds are already approved for clinical use, a comprehensive clinical trial will produce a more clear picture of clinical efficacy for this combination.

# V. References

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# ABSTRACT

## Study of molecular mechanism of vemurafenib resistance via PIN1 in malignant melanoma

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A substantial fraction of melanoma patients contain mutations in *BRAF* gene. BRAF mutation in which valine at position 600 is substituted by glutamic acid (BRAFV600E) is by far the most common BRAF mutation. BRAFV600E is responsible for the constitutive activation of RAF/MEKs/ERKs signaling pathway leading to the robust progression of melanoma. vemurafenib is a specific inhibitor of BRAFV600E and is used in the treatment of melanoma patients having this mutation. Despite showing improved clinical outcomes at the beginning of chemotherapy, most of the patient who respond to the drug developed acquired resistance to vemurafenib. Several mechanisms are reported to be involved in the

development of resistance and one of the key mechanism appears to be the reactivation of RAF1-MEK1/2-ERK1/2 signaling pathway. However, what molecular events regulate the reactivation of RAF1/MEKs/ERKs pathway is poorly known. Here, we report that peptidyl/prolyl cis-trans isomerase NIMA interacting-1 (PIN1) positively regulate the activation of RAF1-MEK1/2-ERK1/2 pathway to develop vemurafenib resistance in A375 melanoma cells *in vitro*. PIN1 level were found to be increased in vemurafenib-resistant A375 (A375R) cells, where it regulated the overexpression of EGFR and the level of phosphorylated RAF1, MEK1/2 and ERK 1/2. We also showed that vemurafenib in combination with a PIN1 inhibitor all trans retinoic acid (ATRA), downregulates both EGFR expression and RAF1/MEKs/ERKs signaling in A375R cells. Furthermore, ATRA potently induced apoptosis, suppressed cell viability, and abrogated cell transformation of A375R cells. Our results suggest the potential value of PIN1 inhibitor, ATRA, as an adjuvant, in the chemotherapy of vemurafenib resistant melanoma.