ORIGINAL ARTICLES

The gene set enrichment analysis of BRAF^{V600E}-mutated HT-29 colorectal cancer cells treated of Selumetinib

Duong Hong Quan[1*], Trinh Thi Hai[2], Nguyen Phuong Thoa[1], Nguyen Huyen Trang[1], Dinh Thi Thanh[1], Bui Thi Ngoc Ha[1], Dang Vu Phuong Linh[1], Dang The Hung[1]

ABSTRACT

Objective: BRAF activating mutations represents approximately 10% colorectal cancer (CRC) patients who are associated with a poor prognosis in stage II, III and IV. Therefore, to better understand the antitumor effects of Selumetinib in BRAF^{V600E}-mutated CRC, gene set enrichment analysis (GSEA) of gene expression profile of BRAFV^{600E}-mutated HT-29 cells treated of Selumetinib is proposed in this study**.**

Methods: The differentially expressed genes across the comparison of groups of non-treated and treated of Selumetinib in BRAF^{V600E}-mutated HT-29 cells were analyzed by GSEA.

Results: We identified the significant pathways of DNA dependent DNA replication, DNA replication, regulation of DNA replication, cell activation, leukocyte activation, receptor complex, endoplasmic reticulum membrane, RNA processing, RRNA processing, amino acid transmembrane transporter activity, nucleolar part, ribonucleoprotein complex, ribosome biogenesis and assembly and regulation of protein modification process for the upregulated genes and the significant pathways of response to hypoxia, response to extracellular stimulus, ATPase coupled to transmembrane movement of ions, ATPase coupled to transmembrane movement of ions phosphorylative mechanism, lipase activity, MAP kinase activity, phospholipase activity and actin filament for the downregulated genes relevant to resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells. Our data revealed that GLI2, IL8, LAT2, ICOSLG, TLR4, SPINK5, HDAC4, LAT and TGFB1 are the upregulated important genes and PDIA2, TGFB2, GIPR and GCGR are the importantly downregulated genes associated with resistance of BRAF^{V600E}-mutated HT-29 cells to Selumetinib.

Conclusion: We identified the upregulated and/or downregulated genes significantly associated with the resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells. These top upregulated and/or downregulated genes have significant value in prediction of sensitivity of CRC patients to Selumetinib and in identification of effective combination strategy for treatment of CRC patients.

Keywords: *Gene set enrichment analysis (GSEA), BRAF^{V600E} mutation, Selumetinib, Colorectal cancer, Targeted therapy, Treatment.* .

INTRODUCTION

Colorectal cancer (CRC) is major cause of morbidity and mortality throughout the world (1). CRC is the third most common malignant tumor worldwide and the fourth most common cause of death with an estimate of 1,880,725 new cases and 915,880 deaths occurring in 2020 (1). In Vietnam, CRC is the fourth leading cause of cancer death with an estimate of 16,426 new cases and 8,203 deaths occurring in 2020 (2). Recently, the

Corresponding author: *Duong Hong Quan*
Email: dhq@huph.edu.vn
[1]Hanoi University of Public Health
[2]Me Linh Hospital

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response rates and overall survival in CRC have improved with the introduction of FDA-approved targeted therapeutic agents as (Cetuximab, Panitumumab) and/or in combination with FOLFIRI/FOLFOX (3-5). However, the efficacy of these drugs is limited because many CRC patients are resistant to them.

The MAPK (mitogen-activated protein kinase) pathway, the key important signaling network, is constitutively activated in several cancers, leading to cancer cell resistance to targeted- and chemotherapy in several types of cancers including CRC, pancreatic, lung, thyroid and breast cancer (6,7). As the biological function of *RAS* isoforms, $BRAF^{V600E}$ mutation presented approximately 18% of CRC patients can activate the MAPK pathway (8). Inhibitor of MEK1/2 as Selumetinib is well known as novel class of targeted therapeutic drugs for CRC treatment because MEK1/2 is the exclusive substrate for RAS. Especially, Selumetinib is a potent, orally available, highly specific, non-ATP competitive MEK1/2 inhibitor with nanomolar activity against MEK1/2 in cell culture and mouse models (9,10).

The most important aspect of anticancer drugs is that many patients exhibit inherent and/or acquired drug resistance. Especially, the molecular and genetic mechanisms significantly driven drug resistance remain poorly understood. Therefore, a critical component of cancer drug development is the identification of potential biomarkers and significant signaling pathways to predict effective responses to novel targeted therapeutic drugs thus enhancing individualization of cancer treatment. The novel tool, Gene Set Enrichment Analysis (GSEA), requires knowledge-based databases accumulating large-scale expression sets (11). Furthermore, the GSEA is important tool to apply for gene expression data to select gene sets of functionally related groups of genes by comparison with the selected gene sets from available databases (11). Therefore, the GSEA will be powerful tool to investigate the molecular pathways processing for gene expression data associated with resistance of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells.

In the present study, identification of molecular signaling pathways processing for significantly upregulated and/or downregulated genes is associated with the resistance of $BRAF^{V600E}$-mutated HT-29 cells to Selumetinib by GSEA. These results could be investigated potential biomarkers of sensitivity to Selumetinib and identified rational combination strategy to effectively treat of colorectal cancer patients with BRAF mutation.

METHODS

Cell culture and reagents

$BRAF^{V600E}$ mutated colon cancer cell lines (HT-29) were purchased from American Type Culture Collection (ATCC, Manassas, VA). $BRAF^{V600E}$-mutated HT-29 cells were cultured in McCoy's 5A supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, Naerum, Denmark) and 100 units/ml penicillin/streptomycin. Selumetinib (AZD6244) were purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO). Small molecule compounds were stored at -20°C in small aliquots.

MTS assay

$BRAF^{V600E}$-mutated HT-29 cells, counted by the TC20™ Automated Cell Counter (Bio-Rad, Pleasanton, CA, USA) were plated in 96-well flat bottom plates at a density of 2000

cells per well in triplicate and then treated with various concentrations of Selumetinib for 72 hours. Cell viability was determined using the MTS assay reagent (CellTiter 96 AQueous one Solution Cell proliferation Assay; Promega, Madison, WI, USA) according to the manufacturer's protocol. The absorbance was measured at 490 nm using a Wallac Victor^2 1420 Multilabel counter (Perkin Elmer, Wellesley, MA). Absorbance of untreated cells was designated as 100% and the number of viable cells in other experimental conditions were relative to that (10).

RNA-Seq expression analyses

Total RNAs were extracted using the E.Z.N.A Total RNA kit (Promega). Gene expression profiling of Control- or Selumetinib-treated BRAF^{V600E}-mutated HT-29 cells was carried out by RNA-Seq Analyses. Total RNAs integrity was verified on the Bioanalyser 2100 with RNA 6000 Nano chips and RIN scores were >9 for all samples. The Illumina Truseq RNA Sample Preparation kit V2 was used to prepare librairies from 500 nanograms of total RNAs. Poly-A RNAs were purified with polyT-coated magnetic beads, chemically fragmented and used as template for cDNA synthesis using random hexamers. cDNA ends were subsequently end-blunted, adenylated at 3'OH extremities and ligated to indexed adaptors. Finally, the adapters-ligated library fragments were enriched by PCR following Illumina's protocol and purified. Libraries were validated on the Bioanalyser DNA 1000 chip and quantified by qPCR using the KAPA library quantification kit. Sequencing was performed on HiSeq2000 in paired-end 2x100 base protocol, 6 libraries were multiplexed per lane. An average of 57.7M of reads per sample was obtained. For Data analysis, Fastq files were not trimmed for adaptor sequences. The reads were aligned with Tophat 2.0.9 to the human genome mm10. Cufflinks 2.1.1 suite was used to generate FPKM values and CuffDiff was used to identify significantly differentially expressed genes. Significant genes were selected with a corrected p value < 0.05. Raw data was processed and expression data was normalized by using TopHat and Cufflinks Software (10).

Gene set enrichment analysis

Gene Set Enrichment Analysis (GSEA) is a computational method for exploring whether a given gene set is significantly enriched in a group of gene markers ranked by their relevance with a phenotype of interest. The curated KEGG pathway V5.2 data set was used to compare the impaired pathway in control and selumetinib-treated HT-29 cells. In addition, the gene sets with fewer than 15 genes or more than 500 genes were excluded. The phenotypes lables was set as colon cancer versus control. The t-statistic mean of the genes was computed in each KEGG pathway using a permutation test with 1000 replications. The upregulated pathways were defined by a normalized enrichment score (NES) > 0, and the downregulated pathways were defined by an NES < 0. Pathways with an P value ≤1 were considered significantly enriched (11).

Statistical analysis

The two-tailed Student's t test was used for two groups' comparison and the One-way ANOVA test was used for more than two groups' comparison. Data are expressed as the mean ± SD, and P<0.05 was considered to indicate a statistically significant difference.

RESULTS

Selumetinib induces cellular proliferation

In CRC, BRAF^{V600E} mutation constitutively activates signaling through MAPK pathways and targeting these oncogenic pathways

has shown efficacy against CRC both in vitro and vi vivo. In order to investigate effective inhibitors against these pathways in our model of drug resistance, we selected a MEK1/2 inhibitor as Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells and screened with various concentrations of Selumetinib (0, 0.01, 0.1, 1 and/or 10 μM) for 72 h and the cell viability of $BRAF^{V600E}$-mutated HT-29 cells were assessed by an MTS assay. The MTS results showed that Selumetinib did not significantly reduced the number of viable cells in cells in a dose-dependent manner (Fig. 1) and showed the resistance in $BRAF^{V600E}$-mutated HT-29 cells. Therefore, it is necessary to investigate the signaling pathways involved resistance of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells.

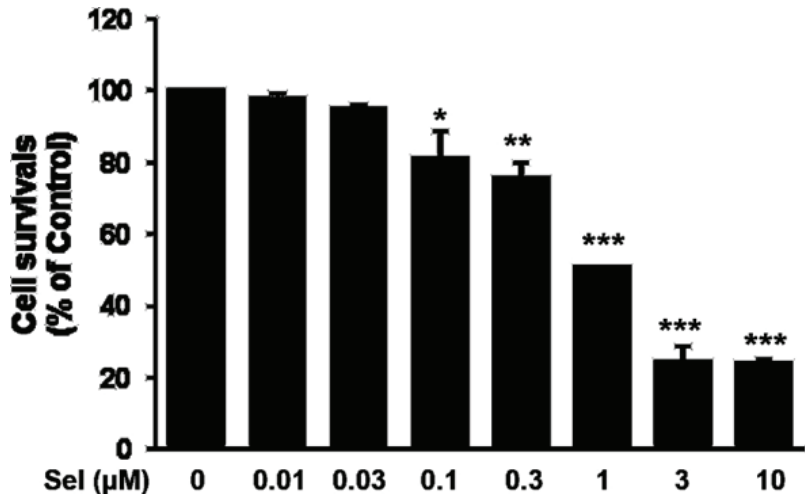

Figure 1. Selumetinib (Sel) inhibits cellular proliferation

$BRAF^{V600E}$-mutated HT-29 cells were treated with 0.01, 0.1, 1 or 10 μM Sel for 72 h, and cell viability was assessed using an MTS assay. Representative data from three independent experiments performed in triplicate, presented as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. the associated untreated control.

Gene set enrichment analysis (GSEA) of upregulated genes in $BRAF^{V600E}$-mutated HT-29 cells treated of Selumetinib

Next, a pathway controlling a particular cellular function if regulated by multiple genes therefore GSEA is specific tool to determine whether members of a genes set are enriched at the top or the bottom of the genes expression data in order to investigate the molecular mechanism of Selumetinib resistance in $BRAF^{V600E}$-mutated HT-29 cells. In Figure 2, the GSEA results identified the significant pathways of DNA dependent DNA replication, DNA replication, regulation of DNA replication, cell activation, leukocyte activation, receptor complex, endoplasmic reticulum membrane, RNA processing, RRNA processing, amino acid transmembrane transporter activity, nucleolar part, ribonucleoprotein complex, ribosome biogenesis and assembly and regulation of protein modification process for the upregulated genes relevant to resistance of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells. Furthermore, among these significant pathways relevant to resistance of Selumetinib in these cells, KEGG pathway

analysis revealed that two top modules were mainly associated with DNA replication (DNA dependent DNA replication, DNA replication, regulation of DNA replication) and cell activation/leukocyte activation processing for the upregulated genes relevant to resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells (Fig. 2).

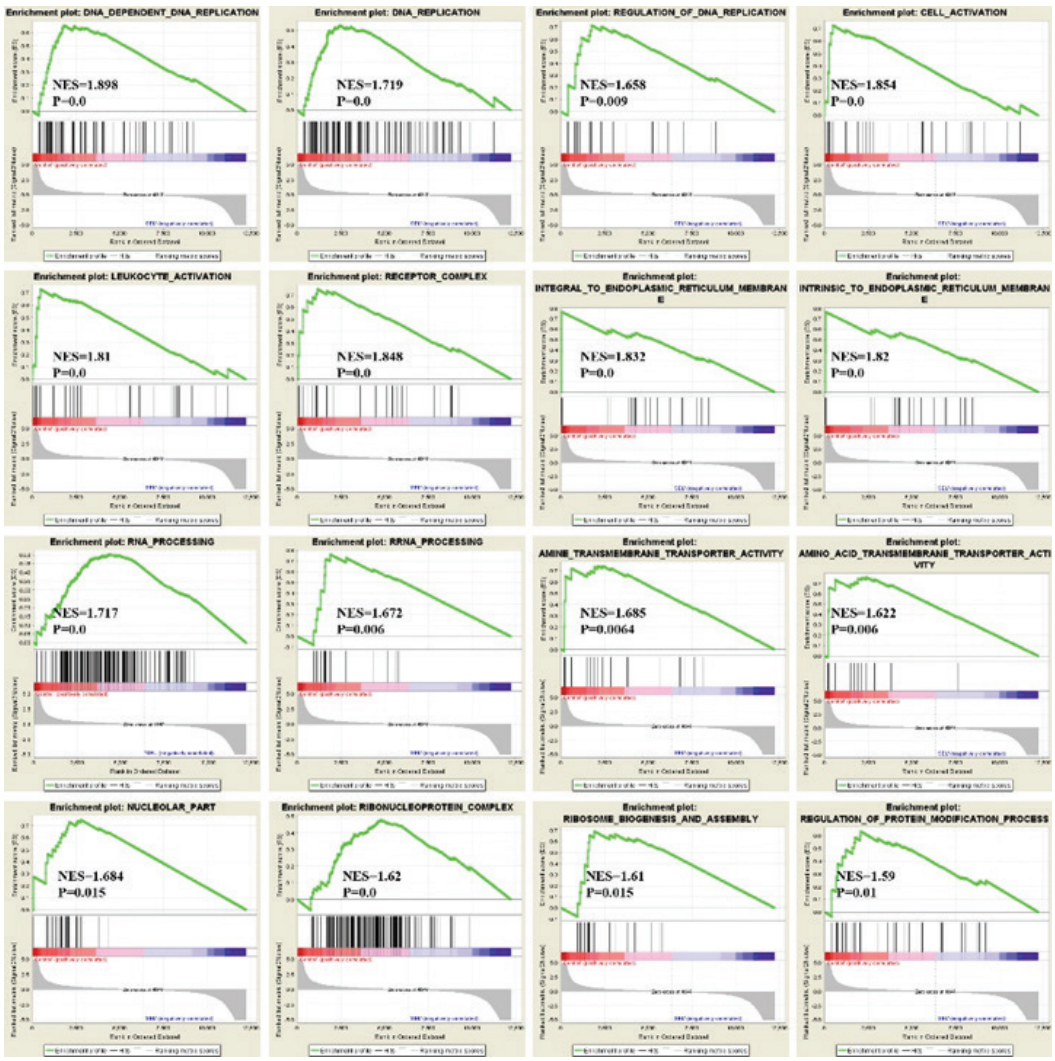

Figure 2. Gene set enrichment analysis (GSEA). Listed pictures are upregulated gene sets enriched in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib (Sel).

Gene set enrichment analysis (GSEA) of downregulated genes in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib

To identify the significant pathways for the process of downregulated genes relevant to Selumetinib resistance in BRAF^{V600E}-mutated HT-29 cells, in Figure 3, the GSEA results identified that the significant pathways of response to hypoxia, response to extracellular stimulus, ATPase coupled to transmembrane movement of ions, ATPase coupled to transmembrane movement of ions phosphorylative mechanism, lipase activity, MAP kinase activity, phospholipase activity and actin filament for the downregulated

genes relevant to resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells. KEGG pathway analysis also applied two top modules were mainly associated with response to hypoxia and response to extracellular stimulus processing for the downregulated genes relevant to resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells (Fig. 3).

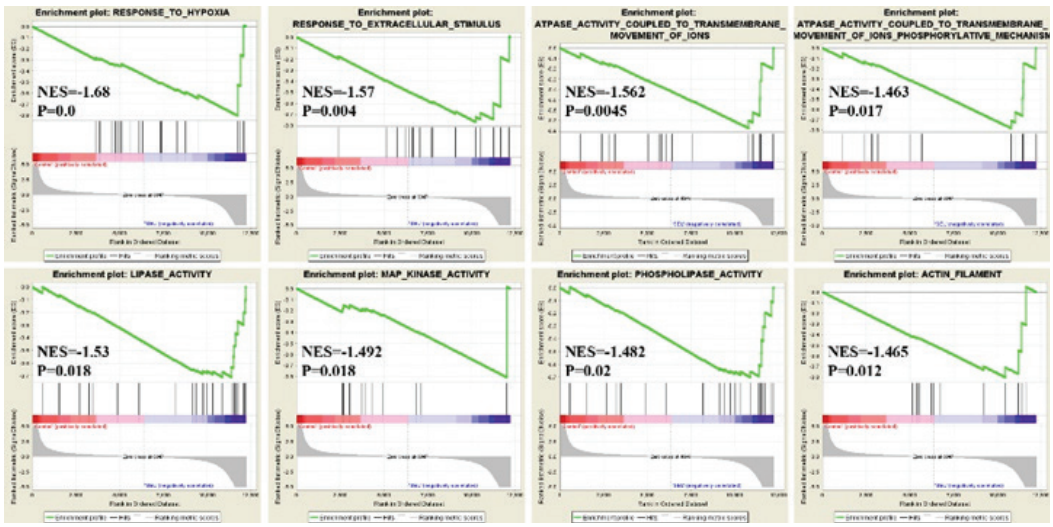

Figure 3. Gene set enrichment analysis (GSEA). Listed pictures are downregulated gene sets enriched in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib.

Identification of the top upregulated genes in regulation of Selumetinib resistance in BRAF^{V600E}-mutated HT-29 cells

To investigate the most upregulated genes in the pathways of DNA replication (DNA dependent DNA replication, DNA replication, regulation of DNA replication) and cellular activation/leukocyte activation relevant to resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells, GSEA was applied to identify the top upregulated genes in these pathways by treatment of Selumetinib in HT-29. The GSEA showed that GLI2 (GLI family zinc finger 2) is the top upregulated gene in DNA replication pathway (DNA dependent DNA replication, DNA replication, regulation of DNA replication) significantly regulated the resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells (Fig. 4). Moreover, the GSEA also showed that eight top upregulated genes as IL8 (Interleukin 8), LAT2 (Linker for Activation of T cells family member 2), ICOSLG (Inducible T cell Costimulatory Ligand), TLR4 (Toll Like Receptor 4), SPINK5 (Serine Peptidase Inhibitor Kazal type 5), HDAC4 (Histone deacetylase 4), LAT (Linker for Activation of T cells) and TGFB1 (Transforming Growth Factor Beta 1) in cellular activity/leukocyte activity pathway were significantly regulated the resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells (Fig. 5). Collectively, GLI2, IL8, LAT2, ICOSLG, TLR4, SPINK5, HDAC4, LAT and TGFB1 are the upregulated important genes associated with Selumetinib resistance in BRAF^{V600E}-mutated HT-29 cells.

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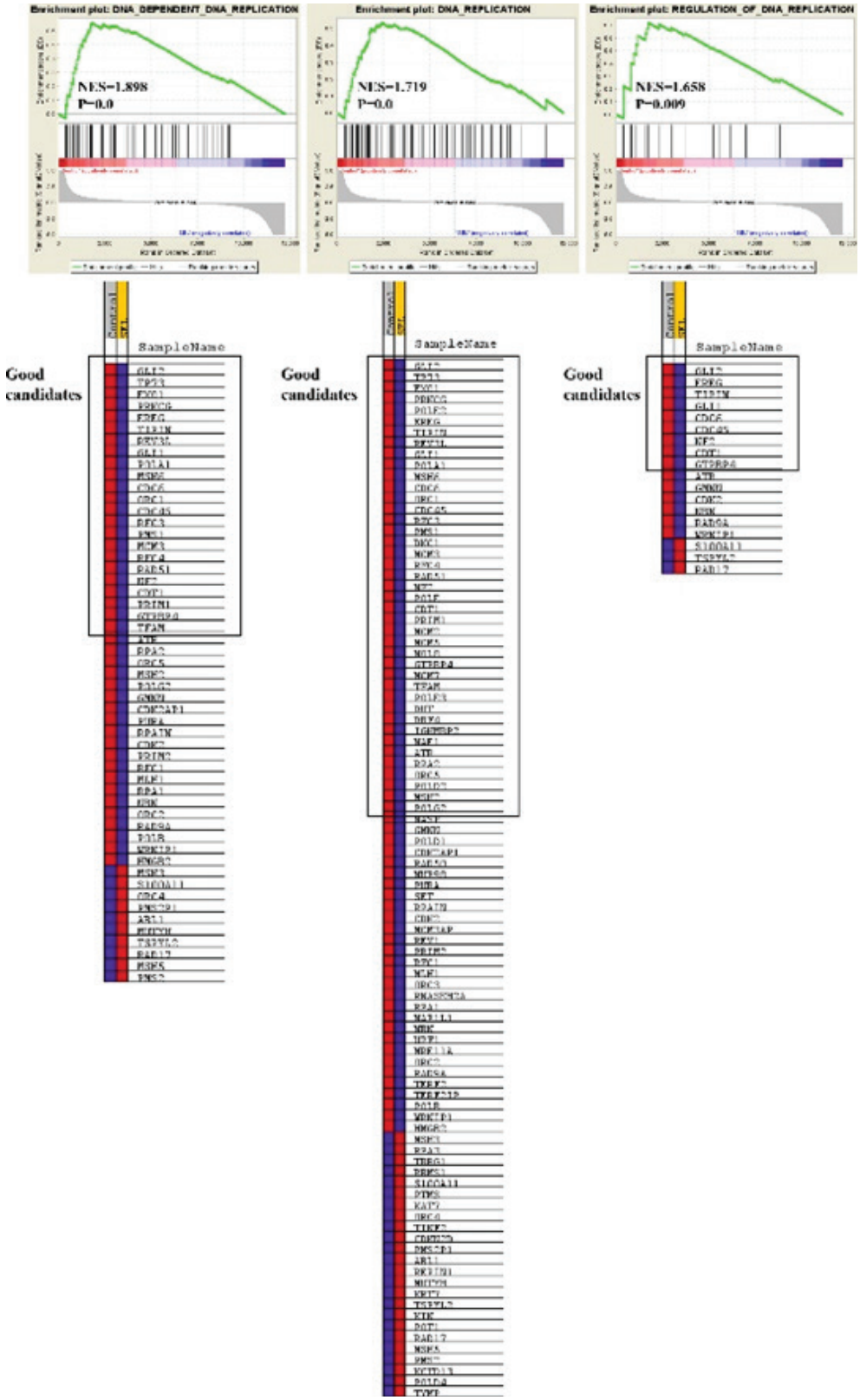

Figure 4. Gene set enrichment analysis (GSEA). Listed pictures are functional gene sets enriched in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib with GLI2 highly expressed.

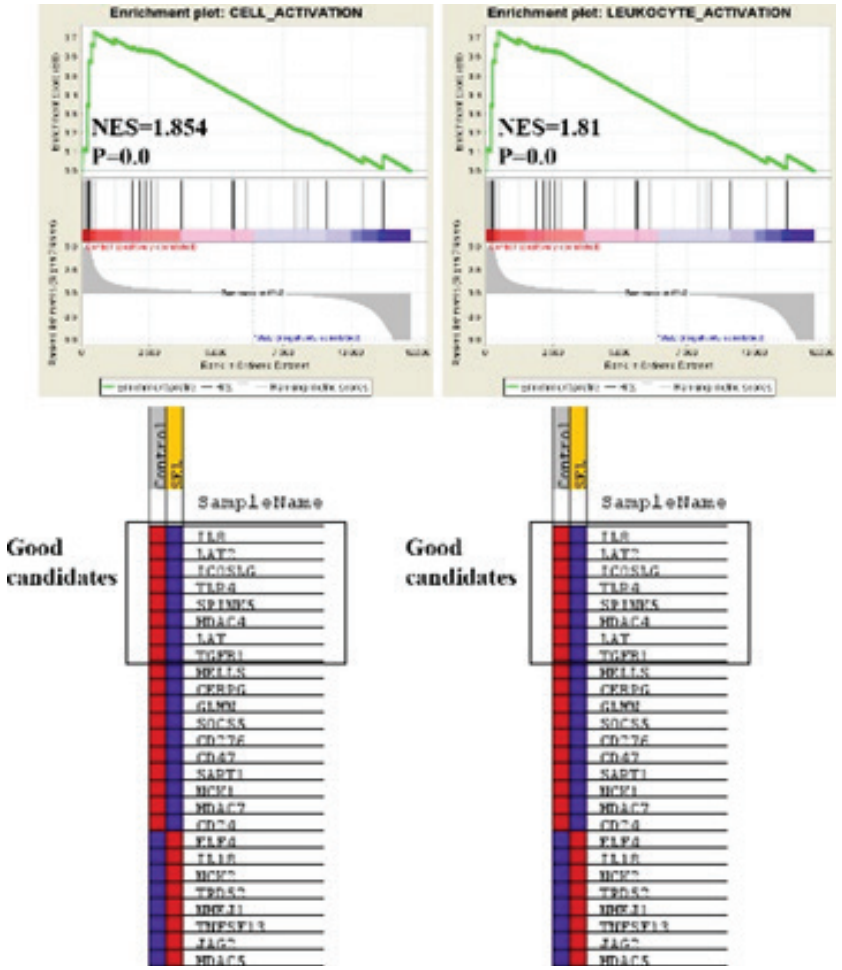

Figure 5. Gene set enrichment analysis (GSEA). Listed pictures are two functional gene sets enriched in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib with IL8, LAT2, ICOSLG, TLR4, SPINK5, HDAC4, LAT and TGFB1 highly expressed.

Identification of the top downregulated genes in response to hypoxia and response to extracellular stimulus in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib

To investigate the most downregulated genes in the pathways of response to hypoxia and response to extracellular stimulus relevant to resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells, GSEA was applied to identify the top downregulated genes in these pathways in these cells treated of Selumetinib. The GSEA showed that PDIA2 (Protein disulfide isomerase family A member 2) and TGFB2 (Transforming Growth Factor Beta 2) were the top downregulated genes in response to hypoxia significantly regulated the resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells (Fig. 6). Moreover, the GSEA also showed that two top upregulated genes as GIPR (Gastric Inhibitory Polypeptide Receptor) and GCGR (Glucagon Receptor) in response to extracellular stimulus pathway were significantly regulated the resistance of Selumetinib in BRAF^{V600E}-mutated HT-29 cells with BRAF mutation (Fig. 7). Collectively, PDIA2, TGFB2, GIPR and GCGR are the importantly downregulated genes associated with Selumetinib resistance in BRAF^{V600E}-mutated HT-29 cells.

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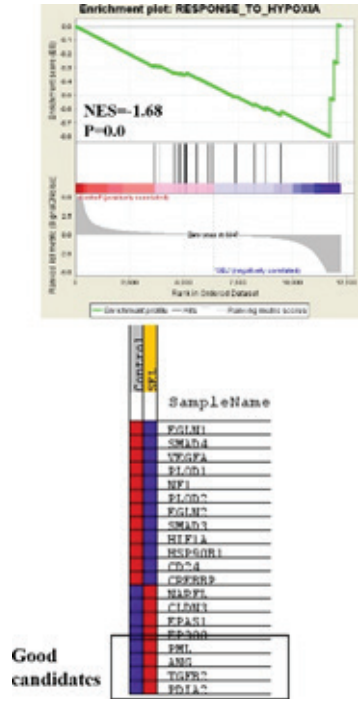

Figure 6. Gene set enrichment analysis (GSEA). Listed pictures are functional gene sets enriched in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib with PDIA2 and TGFB2 weakly expressed.

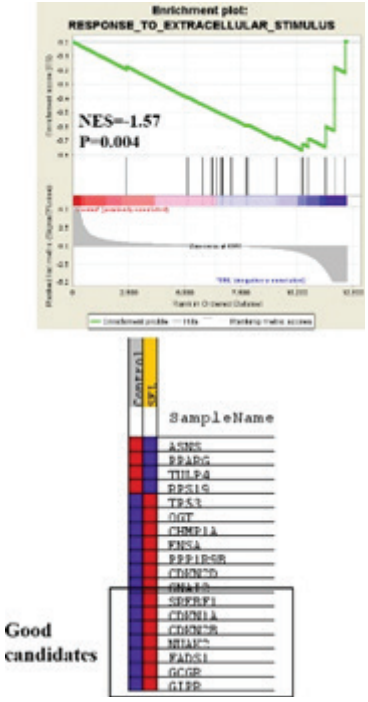

Figure 7. Gene set enrichment analysis (GSEA). Listed pictures are functional gene sets enriched in BRAF^{V600E}-mutated HT-29 cells treated of Selumetinib with GIPR and GCGR weakly expressed.

DISCUSSION

Even with a gradual decline in the past few years, CRC remains the third most common malignant tumor and the fourth most common cause of death worldwide (1). The occurrence and development of resistance of CRC to chemotherapy, radiotherapy and targeted therapy are a dynamic process. Furthermore, The MAPK signaling pathway is constitutively activated in several cancers including CRC leading to cancer cell resistance to targeted therapy and chemotherapy (6,7) and $BRAF^{V600E}$ mutation constitutively activates signaling through MAPK pathways (12). Therefore, it is necessary to identify accurate and meaningful biomarkers of these resistances in CRC. Our study systematically focused on the upregulated and downregulated gene profiles obtained from NGS results of non-treated and treated of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells. Our results identified the significant pathways of DNA dependent DNA replication, DNA replication, regulation of DNA replication, cell activation, leukocyte activation, receptor complex, endoplasmic reticulum membrane, RNA processing, RRNA processing, amino acid transmembrane transporter activity, nucleolar part, ribonucleoprotein complex, ribosome biogenesis and assembly and regulation of protein modification process for the upregulated genes and the significant pathways of response to hypoxia, response to extracellular stimulus, ATPase coupled to transmembrane movement of ions, ATPase coupled to transmembrane movement of ions phosphorylative mechanism, lipase activity, MAP kinase activity, phospholipase activity and actin filament for the downregulated genes relevant to resistance of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells.

Gene expression profiling is used predominantly in the assessment of disease diagnosis, prognosis and treatment in CRC (13,14,15). Especially, the development of a predictive genomic classifier for KRAS mutated CRC by the gene expression profiling related to resistance or sensitivity of Selumetinib and comparing to sensitive KRAS mutated CRC, members of the Wnt signaling pathway were highly upregulated in associated to resistance of Selumetinib in KRAS mutated CRC (16). GSEA also showed that dual therapy downregulated DNA replication and cell-cycle drivers and upregulated lysosomal gene set associated with resistance of Selumetinib in high-grade serous ovarian cancer (17). Then, GSEA of gene expression profile of $BRAF^{V600E}$ CRC in exposure to Selumetinib has been conducted to better understand its molecular mechanism of resistance to Selumetinib. Here, the upregulated and downregulated genes are associated with resistance of $BRAF^{V600E}$-mutated HT-29 cells to Selumetinib. Our results investigated GLI2, IL8, LAT2, ICOSLG, TLR4, SPINK5, HDAC4, LAT and TGFB1 are the top upregulated important genes and PDIA2, TGFB2, GIPR and GCGR are the top downregulated important genes associated with resistance of $BRAF^{V600E}$-mutated HT-29 cells to Selumetinib.

GLI2 is the top upregulated gene relevant to DNA replication pathway which significantly regulated the resistance of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells. GLI2 has been shown to control the invasive and metastatic potential and to contribute the epithelial-to-mesenchymal transition (EMT) in melanoma (18) and high GLI2 expression was achieved through the Hedgehog (Hh) and TGF-β/SMAD pathway that is associated with resistance of inhibitors of BRAF and MAPK pathway in melanoma (19,20). Furthermore, targeting the Hh pathway in BRAF inhibitor-resistant melanoma may represent a viable therapeutic strategy to

restore vemurafenib sensitivity reducing the acquired chemoresistance in melanoma patients (21). Therefore, GLI2 is the potential to be a significant biomarker for diagnosis and therapy in CRC, inclusive $BRAF^{V600E}$-mutated CRC.

PDIA2 is a member of the disulfide isomerase family which is a group of multifunctional endoplasmic reticulum enzymes that mediate the formation of disulfide bonds, catalyze the cysteine-based redox reactions (22). Especially, the expression of PDIA2 in cancer cells is linked to tumor progression and prognosis of several human cancers as breast cancer, hepatocellular carcinoma, gallbladder cancer (23-25). Furthermore, the expression of PDIA2 is significant for tumor cell resistance to chemotherapy in lung cancer A549 and UO31 cells (26). Therefore, the expression level of PDIA2 is an important potential biomarker for rapid progression and poor prognosis of CRC, inclusive $BRAF^{V600E}$-mutated CRC.

CONCLUSION

Our study demonstrates that identification of the upregulated and/or downregulated genes is significantly associated with the resistance of Selumetinib in $BRAF^{V600E}$-mutated HT-29 cells. These top upregulated and/or downregulated genes could be potential biomarkers to predict sensitivity of CRC patients to Selumetinib and to identify effective combination strategy for treatment of CRC patients. However, Further studies are necessary to verify these results of our study.

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