Bioinformatics analysis of gene expression alterations conferring drug resistance in tumor samples from melanoma patients with EGFR-activating BRAF mutations

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Abstract. Melanoma is a highly malignant tumor of the skin melanocytes. Patients with this cancer have a high frequency (~50%) of oncogenic BRAF mutations, particularly BRAF V600E. Treatments for melanoma often target BRAF mutations or involve mitogen-activated protein kinase kinase/extracellular signal-regulated kinase inhibitors. A major challenge in melanoma treatment is resistance to BRAF inhibitor treatment, which may be enhanced by the BRAF mutation itself and/or epidermal growth factor receptor (EGFR) activation, leading to poor prognosis. However, no effective clinical treatment exists for patients with EGFR-activating BRAF V600E mutations. The aim of the present study was to analyze gene expression changes in tumors from patients with EGFR-activating BRAF mutations during development of drug resistance. RNA-seq data was downloaded from the Gene Expression Omnibus (GEO) database for pre- and post-treatment tumor samples from three melanoma patients with EGFR-activating BRAF V600E mutations, and from The Cancer Genome Atlas (TCGA) melanoma database for tumor or non-tumor samples from patients with the BRAF V600E mutation and unknown EGFR activation status. Using functional enrichment and KEGG pathway analyses, the present study analyzed differentially expressed genes (DEGs) between pre- vs. post-treatment data from the GEO database and tumor or non-tumor sample data from the TCGA database. The results of the present study indicated that functional and structural changes to the plasma membrane may be associated with drug resistance. The present study identified 9 DEGs that were significantly different between tumor and non-tumor samples and also between prior to and following treatment. Thus, it was confirmed that patients with EGFR-activating BRAF V600E mutations undergo gene expression changes during disease development, and during therapy. These findings may provide potential directions for melanoma-specific therapy.

Introduction

Melanoma is a highly malignant tumor of the skin melanocytes that is insensitive to chemotherapy or radiotherapy (1). The majority of patients with melanoma exhibit a poor prognosis, and the disease is associated with a high mortality rate (2). Patients with melanoma frequently exhibit activation of the BRAF gene due to somatic mutations, with up to 50% of patients exhibiting BRAF oncogenic mutations (3,4). The most common BRAF mutation in melanoma, BRAF V600E, accounts for ~79% of BRAF mutations (5). BRAF is a member of the RAF kinase family, which includes ARAF, BRAF and CRAF (6). BRAF mutations can lead to the constitutive activation of downstream signaling through mitogen-activated protein kinase (MAPK) pathways, including the mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway, which subsequently upregulates cell migration and proliferation (7).

Good clinical outcomes have been obtained with melanoma treatments that target BRAF mutants and with MEK/ERK inhibitors. As a first-line clinical treatment for melanoma, vemurafenib (PLX4032) is a potent inhibitor of mutated BRAF and a specific therapy for advanced melanoma (8). However, targeted inhibitors typically only maintain their efficacy for 8-9 months before the tumor develops resistance to the inhibitor, allowing rapid growth to continue (9). Thus, controlling drug resistance is a key issue in melanoma treatment.

A number of studies have attempted to elucidate the mechanisms of drug resistance in melanoma patients. Hepatocyte growth factor (HGF) expression has been observed in the stromal cells of patients carrying BRAF mutations, and an association has been demonstrated between HGF-secreting stromal...
cells and the resistance to Raf inhibitors (10). Another potential cause for the development of resistance is mitogen-activated protein kinase kinase kinase 8 overexpression by cells (11). Mutations of the asparaginyl-tRNA synthetase (NARS) gene were identified in vemurafenib-resistant cells in vitro, and in the lymph node cells of vemurafenib-resistant patients. NARS mutations may be active in the MAPK pathway, leading to the resistance of melanoma cells to targeted inhibitors (12).

The *BRAF* mutation itself may lead to resistance development. In previous reports, patients with the BRAF V600E mutation exhibited a poor prognosis due to acquired resistance to vemurafenib and trametinib (13,14). Other studies have demonstrated that the BRAF V600E mutation or MEK inhibitor resistance may be associated with epidermal growth factor receptor (EGFR) activation in tumor cells (15,16). For example, Prahallad et al identified that in a subset of patients, BRAF V600E inhibitors may lead to EGFR activation, which, in turn, may enhance the resistance of cancer cells to BRAF inhibitors (15). Sun et al demonstrated that EGFR expression enhances the proliferation of melanoma cells in the presence of inhibitors against BRAF or MEK (16).

There remains no effective clinical treatment for patients with EGFR-activating feedback. Furthermore, it has yet to be established whether patients with EGFR-activating BRAF V600E mutations experience alterations to gene expression prior to and following disease or treatment. Therefore, the aim of the present study was to analyze changes in the expression of genes by melanoma tumors in patients with EGFR-activating *BRAF* mutations, including during the development of drug resistance. The overall goal was to identify potential drug targets for melanoma treatment-resistant patients.

**Materials and methods**

Identification of differentially expressed genes (DEGs) from a public database. All cases were pathologically diagnosed to be skin melanoma while the controls were well identified as have drug resistance with EGFR-activating BRAF mutations. Sample numbers SRR961663, SRR961664, SRR961665, SRR961666, SRR961667 and SRR961668 were downloaded from dataset GSE50535 of the Gene Expression Omnibus (GEO: https://www.ncbi.nlm.nih.gov/geo/). TopHat 2.1.1 and Cufflinks 2.2.1 software packages were utilized to analyze assembly data and differences in the gene expression profiles, respectively (17). RNA-seq data of 130 samples from tumor and non-tumor sites of patients with BRAF V600E mutations of unknown EGFR activation status were downloaded from the Cancer Genome Atlas (TCGA) cutaneous melanoma database (https://cancergenome.nih.gov/). Using the edgeR3.5.5 package in Bioconductor, DEGs between pre- and post-treatment data from the GEO dataset and between pre-treatment data from the GEO dataset vs. tumor or non-tumor sample data from the TCGA site were analyzed (18). P<0.05 was considered to indicate a statistically significant difference.

Functional enrichment analysis of DEGs. A functional enrichment analysis of DEGs, including gene ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, was performed using the Database for Annotation Visualization and Integrated

**Results**

Gene expression changes in tumor samples prior to and following treatment in drug-resistant patients with EGFR-activating *BRAF* mutations. A total of 6 samples of melanoma from 3 patients prior to and following treatment from the GSE50535 dataset were analyzed. The analysis resulted in the identification of 94 significant DEGs (62 upregulated and 32 downregulated genes), which were categorized using GO analysis in DAVID (Fig. 1). EGFR and EGFR-associated mutations were also analyzed (Fig. 2).

In the CC ontology analysis (Fig. 1A), the majority of the significantly enriched genes were associated with the terms plasma membrane (PM; 30 genes, 33.25% of all DEGs; P=0.002), PM part (21 genes, 22.58%; P=0.002) and sarcolemma [4 genes, including biglycan (*BGN*), collagen type VI α-3 chain (*COL6A3*), *COL6A2* and calcium voltage-gated channel subunit α-1C (*CACNA1C*); 4.30% of all DEGs; P=0.003].

In the BP ontology analysis (Fig. 1B), 9 genes [including protein tyrosine phosphatase, receptor type C (*PTPRC*), coronin 1A (*CORO1A*), Ikaros family zinc finger 1 (*IKZF1*), clusterin (*CLU*), cluster of differentiation 4 (*CD4*), inositol polyphosphate-5-phosphatase D (*INPP5D*), complement clq C chain (*CIQC*), *CD74*, and major histocompatibility complex, class II, DR-α (*HLA-DRA*)] were enriched in the positive regulation of immune system process (P=1.08x10^-7). Other categories with significant enrichment included immune response (13; P=0.003), positive regulation of lymphocyte activation (6; P=7.29x10^-5), and lymphocyte differentiation (6; P=9.84x10^-7). A total of 8 genes were associated with the positive regulation of cell differentiation (P=7.79x10^-7).

In the MF ontology (Fig. 1C), categories with significant enrichment of DEGs included extracellular matrix structural constituent (5 genes: *BGN*, elastin, *COL6A2*, *CD4*, and *COL5A1*; P=5.43x10^-4), glycoprotein binding (4 genes: P=5.61x10^-4), kinase binding [six genes: *PTPRC*, *CORO1A*, integrin subunit β-2 (*ITGB2*), *CD4*, tropomin 13, cardiac type (*TNNI3*), and topoisomerase II-β (*TOP2B*); P=0.001], and protein kinase binding [five genes: *PTPRC*, *ITGB2*, *CD4*, *TNNI3* and *TOP2B*; P=0.003].

KEGG pathway analysis of the 94 DEGs was also performed (Table I). The most significantly enriched KEGG pathway was cell adhesion molecules (CAMs; 7 DEGs; P=1.70x10^-3). A total of 5 DEGs were enriched in the antigen processing and presentation pathway.

Gene changes in tumor compared with non-tumor melanoma samples. The present study aimed to improve the understanding of the differences between tumor samples from melanoma patients with EGFR-activating *BRAF* mutations and non-tumor samples from melanoma patients. Melanoma samples (SRR961663, SRR961665 and SRR961667) from 3 patients prior to treatment were compared with 1 non-tumor sample from the TCGA cutaneous melanoma database. A
Figure 1. Differentially expressed genes between samples prior to and following treatment, with significant enrichment in (A) CC ontology, (B) BP and (C) MF ontology terms. CC, cellular component; BP, biological process; MF, molecular function.
In the MF ontology analysis (Fig. 3C), categories with significant enrichment in DEGs included ribosome (34 genes; P=2.59x10^-3), cytosolic small ribosomal subunit (15 genes; P=3.38x10^-13), MHC protein complex (11 genes; P=1.03x10^-6) and MHC class I protein complex (6 genes; P=5.25x10^-5).

In the BP ontology analysis (Fig. 3B), the majority of categories with significant DEG enrichment were associated with the translation process, including translational elongation (33 genes; P=1.28x10^-35) and translation (37 genes; P=1.50x10^-22). DEGs were also enriched in immune-associated categories, including immune response (39 genes; P=1.08x10^-15), antigen processing and presentation (15 genes; P=5.67x10^-15), and antigen processing and presentation of peptide antigens (9 genes; P=2.59x10^-6).

In the CC ontology analysis (Fig. 3A), the most significantly enriched categories were adhesion-associated items pertaining to the ribosome, including the cytosolic ribosome (27 genes; P=3.27x10^-35), ribosome (34 genes; P=2.59x10^-24), ribosomal subunit (26 genes; P=2.59x10^-25), cytosolic small ribosomal subunit (15 genes; P=3.38x10^-13), MHC protein complex (11 genes; P=1.03x10^-6) and MHC class I protein complex (6 genes; P=5.25x10^-5).

Table I. Top 5 Kyoto Encyclopedia of Genes and Genomes pathway terms with significant enrichment of DEGs in patients with epidermal growth factor receptor-activating BRAF mutations prior to and following treatment.

<table>
<thead>
<tr>
<th>Term</th>
<th>DEGs, n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA04514: Cell adhesion molecules</td>
<td>7</td>
<td>1.70x10^-4</td>
</tr>
<tr>
<td>HSA04612: Antigen processing and presentation</td>
<td>5</td>
<td>1.88x10^-3</td>
</tr>
<tr>
<td>HSA04940: Type I diabetes mellitus</td>
<td>4</td>
<td>2.41x10^-3</td>
</tr>
<tr>
<td>HSA05414: Dilated cardiomyopathy</td>
<td>5</td>
<td>2.75x10^-3</td>
</tr>
<tr>
<td>HSA04672: Intestinal immune network for IgA production</td>
<td>4</td>
<td>3.75x10^-3</td>
</tr>
</tbody>
</table>

Table II. Top 5 Kyoto Encyclopedia of Genes and Genomes pathway terms with significant enrichment in DEGs between tumor samples from patients with EGFR-activating BRAF mutations and non-tumor samples.

<table>
<thead>
<tr>
<th>Term</th>
<th>DEGs, n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA03010: Ribosome</td>
<td>30</td>
<td>6.20x10^-27</td>
</tr>
<tr>
<td>HSA04612: Antigen processing and presentation</td>
<td>17</td>
<td>4.64x10^-11</td>
</tr>
<tr>
<td>HSA05322: Systemic lupus erythematosus</td>
<td>14</td>
<td>4.09x10^-7</td>
</tr>
<tr>
<td>HSA05330: Allograft rejection</td>
<td>9</td>
<td>1.33x10^-6</td>
</tr>
<tr>
<td>HSA05332: Graft-versus-host disease</td>
<td>9</td>
<td>2.54x10^-6</td>
</tr>
</tbody>
</table>

DEGs between tumor and non-tumor samples from melanoma patients. A total of 9 genes (CIQC, calcium-dependent secretion activator (CADPS), CD74, CLU, CORO1A, formin 1, HLA-DPA1, HLA-DRA, and lymphocyte-specific protein 1) were differentially expressed in the two DEG analyses described above (prior to vs. following treatment, and tumor vs. non-tumor). The greatest enrichments of DEGs were identified in the GO category immune process (P=1.21x10^-4) and the KEGG pathway antigen processing and presentation (17 genes; P=7.81x10^-4).

DEGs between BRAF V600E mutation patients with and without EGFR activation. Finally, DEGs between samples taken from tumor and non-tumor sites of patients with the BRAF V600E mutation of unknown EGFR activation status from the TCGA cutaneous melanoma database were analyzed. A total of 27 DGEs changed in the two groups. GO analysis with DAVID revealed that nine genes were enriched in the immune response category (P=1.22x10^-4), and eight genes were enriched in the defense response category (P=7.96x10^-6).

Discussion

Gene expression alterations in the tumors of patients with EGFR-activating BRAF V600E mutations prior to and following BRAF inhibitor treatment were analyzed. Samples taken from tumor and non-tumor sites in patients with BRAF V600E mutations of unknown EGFR activation status were
also compared. The functions of DEGs were analyzed by GO annotation and KEGG pathway enrichment analyses. The study aimed to provide information to guide the development of novel therapeutic strategies for melanoma patients with EGFR activation who are resistant to typical drugs.

RNA-sequencing (RNA-seq) technology is a powerful tool for analyzing gene expression. Using RNA-seq data for melanoma patients with EGFR activation, 94 genes were identified that were differentially expressed in samples prior to and following treatment, including 62 upregulated and 32 downregulated genes. Gene functional annotation revealed 30 genes associated with membranes in CC oncology, which provides a possible direction for future studies.

Ion channels on the membrane are involved in numerous tumor cell activities, including cell proliferation, differentiation, secretion and survival (19,20). A significant upregulation of CACNA1C (P=5.00x10^{-5}), which encodes the α1 subunit of a voltage-dependent calcium channel located on the PM (21), was observed; this gene is not detectable in normal tissues (22). Calcium channel proteins have been associated with primary tumors of the colon, lung and skin (23). Certain drugs targeting CACNA1C, including magnesium sulfate and nicardipine, have been reported; magnesium sulfate can be used to inhibit the action potential of muscle cells, thereby reducing the frequency and strength of contractions (24); nicardipine is a potent calcium channel inhibitor with important
vasodilatory and antihypertensive characteristics that can be used to enhance the efficacy of certain antitumor agents (25).

A significant downregulation of solute carrier family 4 member 10 (SLC4A10) (P=5.00x10^-5), which belongs to a small family of sodium-coupled bicarbonate transporters that regulate the intracellular pH of neurons (25), was also observed. In addition to unlimited cell proliferation, cancer is characterized by an altered cellular environment that promotes tumor cell proliferation and metastasis (26). PH homeostasis in any cell type is a complicated process. In tumor cells, these processes are even more complex owing to the internal compartment being slightly more alkaline (pH 7.4 or more) and the external compartment being more acidic than in normal cells (27). Downregulation of SLC4A10, leading to decreased Cl/HCO₃⁻ transport, may be associated with environmental alterations for melanoma growth.

A total of 274 genes were identified that were differentially expressed between untreated EGFR-activated melanoma samples and non-tumor samples from the TCGA Database. GO and pathway analyses revealed that numerous genes involved in immune-associated processes, particularly antigen processing and presentation processes, were enriched. One enriched gene was CD74, which encodes a protein associated with the class II major histocompatibility complex (MHC) and is a chaperone that regulates antigen presentation. CD74 serves as a cell-surface receptor for the cytokine macrophage migration inhibitory factor, which, when bound to the encoded protein, initiates survival pathways and cell proliferation. A previous study indicated that CD74 is only expressed in melanoma cells and not in benign melanocytes (28). Milatuzumab is a drug used for the treatment of tumors expressing the CD74 antigen (29).

Antigen presentation serves a key role in the development of melanoma vaccines (30). Broadly speaking, a tumor cell is also an antigen-presenting cell. Tumor cells form a complex with MHC class I molecules via the cytosolic processing pathway, with tumor antigens on the cell surface being recognized by CD8+ T cells. Alternatively, tumor cells can form MHC class I or II molecules by lysosomal processing of tumor antigens from dendritic cells or specialized antigen-presenting macrophages. These tumor cells are then recognized by CD8+/CD4+ T cells (30).

Significant enrichment was observed in numerous genes associated with ribosomal processes. Ribosome synthesis and translational control are essential processes for cells. Several tumor suppressor genes and proto-oncogenes can affect the formation or modification of ribosomes (31). However, the mechanisms by which these genes affect ribosomes remain unclear at present and further experiments are required.

The present study analyzed tumor and non-tumor samples from patients with BRAF V600E mutations of unknown EGFR status from the TCGA Database. These results were compared with DEGs between tumor samples from patients with EGFR-activating BRAF V600E mutations prior to and following treatment. The expression of 27 genes was altered in both comparisons. These genes were predominantly enriched in categories associated with immune response, which suggests that using immunotherapy in the early stages of melanoma may be a valid therapeutic approach. The expression of 9 genes was altered when comparing tumor and non-tumor tissues, and treated and untreated tumors. The majority of these genes exhibited different changes at different stages: For example, a gene that was downregulated in the tumor area compared with the non-tumor area might be upregulated upon acquiring resistance following treatment. DEGs that were significantly enriched in the immune process category by GO analysis included CLU, CIQC, CD74, and HLA-DRA. The CIQC protein is the target of several drugs, as it is a component of the human complement system. Studies have associated a lack of CIQA with lupus and glomerulonephritis (32). Several CIQC-targeting drugs, including tositumomab, palivizumab and cetuxima, are used clinically to treat cancer (22). CLU has been shown to be associated with a number of biological processes, including apoptosis and tumor development, as well as neurodegenerative diseases.

The present study has several deficiencies. The sample size was relatively small, with only 3 samples with EGFR activation and 1 non-tumor sample; the small sample size may lead inaccuracies when comparing the tumor and non-tumor samples. In order to improve the accuracy of the results of the present study, the authors will continue to collect samples with the relevant mutations for further research.

In conclusion, the treatment of melanoma is a complex process. Significant changes were observed in genes associated with the PM of BRAF inhibitor-resistant melanoma patients with EGFR-activated tumors prior to and following treatment. Significant changes in immune process-associated genes were also identified in melanoma patients between tumor and non-tumor samples. Although these findings may provide direction for clinical melanoma-specific therapy, follow-up studies on melanoma are required.

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References


