Abstract. Background/Aim: The BRAF\textsuperscript{V600E} mutation acts as an initiator of cancer development in papillary thyroid carcinoma (PTC). Gene expression changes caused by the BRAF\textsuperscript{V600E} mutation may have an important role in thyroid cancer development. Materials and Methods: To study genomic alterations caused by the BRAF\textsuperscript{V600E} mutation, we made human thyroid cell lines that harbor the wild-type BRAF gene (Nthy/WT) and the V600E mutant-type BRAF gene (Nthy/V600E). Results: Flow cytometry and western blotting showed stable transfection of the BRAF gene. In functional experiments, Nthy/V600E showed increased anchorage-independent growth and invasion through Matrigel, compared to Nthy/WT. Microarray analysis revealed that 2,441 genes were up-regulated in Nthy/V600E compared to Nthy/WT. Gene ontology analysis showed that the up-regulated genes were associated with cell adhesion, migration, and the ERK and MAPK cascade, and pathway analysis showed enrichment in cancer-related pathways. Conclusion: Our Nthy/WT and Nthy/V600E cell line pair could be a suitable model to study the molecular characteristics of BRAF\textsuperscript{V600E} PTC.

The BRAF\textsuperscript{V600E} mutation is a well-known driver mutation with a single nucleotide change of thymine to adenine at position 1799. This mutation results in a valine to glutamic acid substitution at amino acid 600 (c1799T\textrightarrow{}A, pV600E) and leads to carcinogenesis by activating the BRAF kinase cascade (1). The BRAF\textsuperscript{V600E} mutation accounts for 95% of BRAF gene alterations and is the most common genetic variation in papillary thyroid carcinoma (PTC) (2). The prevalence of BRAF\textsuperscript{V600E} mutation in PTC is 29-83% (3). Previous studies have reported that the BRAF\textsuperscript{V600E} mutation correlates with advanced disease such as extrathyroidal extension or lymph node metastasis, but is not clearly linked with overall survival. To explain cancer progression according to BRAF mutation, secondary gene expression alterations caused by the BRAF\textsuperscript{V600E} mutation may play important roles (4-6).

Nthy-ori 3-1 (hereafter referred to as Nthy) is an immortalized thyroid follicular epithelial cell line derived from normal adult thyroid tissue that has been transfected with a plasmid encoding the SV40 large T gene. Nthy cells are useful for studies involving the control of growth and function of the human thyroid, since it is the only human normal thyrocyte-derived cell line (7). Using a MCSV promoter-based lentivirus system, Nthy/BRAF cells expressing either wild-type or mutant BRAF were successfully developed. Functional and genomic tests were conducted to explore the biological and genomic alterations caused by BRAF\textsuperscript{V600E} in normal thyroid cells.

Materials and Methods

BRAF expression in Nthy cells by lentivirus transduction. The full-length coding sequences of wild-type BRAF and BRAF\textsuperscript{V600E} were amplified by PCR from TPC1 and 8505c cells. PCR amplification products were cloned into the pCDH-MCS-T2A-copGFP-MCSV lentiviral vector (System Biosciences, Mountain View, CA, USA) and packaged by co-transfection with psPAX2 and pMD2.G plasmids with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in HEK293FT (Invitrogen, Carlsbad, CA, USA) cells. Virus was harvested and concentrated by ultracentrifugation 48 h later. Titers

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were determined by flow cytometry as percentage of green fluorescent protein (GFP)-positive cells. For stable cell line generation, Nthy-ori 3-1 cells were treated with different titers of lentivirus for 24 h and examined for GFP expression after 3 days. Titers that generated at least 95% GFP-positive cells were chosen for further culture. Cells with low/intermediate/high GFP expression were sorted using a FACSARia flow cytometer (BD biosciences, San Jose, CA, US), and only cells with high GFP expression survived and proliferated.

Cell morphology and DNA sequencing. Transfected cells were observed using a microscope. The cells were cultured in 60-mm dishes until confluent monolayers were reached and then detached using a cell scraper. Genomic DNA was extracted using the QIaamp DNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. DNA was quantified using a Nanodrop ND-1000 spectrophotometer and used as template for PCR amplification of BRAF exon 15. PCR was performed using GeneAmp® PCR System 9700 (Applied Biosystems; Life Technologies, Carlsbad, CA, USA) as follows: initial denaturation at 95°C for 1 min was followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 15 sec, and extension at 72°C for 15 sec. The PCR primer and DNA sequencing services were provided by Cosmo Genetech (Cosmo Genetech, Seoul, Korea). The primer sequences used in this study were as follows: BRAF exon 15: F 5'-TGAAGACCTCACATGAAAATGGTG-3', BRAF exon 15: R 5'-TCCACAAAATGGATCCAGACA-3'.

Flow cytometry. Nthy cells infected with empty vector, vector encoding wild-type BRAF, or vector encoding BRAF V600E were fixed with FCM fixation buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on ice for 15 min. Fixed samples were washed in phosphate buffered saline (PBS) and permeabilized on ice for 10 min in FCM Permeabilization (Santa Cruz Biotechnology). Samples were washed and resuspended. Single cell suspensions were incubated with phycoerythrin (PE)-conjugated anti-phospho-p44/42 MAPK (Cell Signaling Technology; Beverly, MA, USA) for 1 h. The labeled cells were detected using a BD FACS Diva 8.0 System (Becton Dickinson, San Jose, CA, USA) according to the manufacturer’s protocols. Gating was implemented on the basis of negative control staining profiles.

Western blotting. Cells were cultured in 100 mm dishes until confluent monolayers were reached. Cells were cultured for 24 h with 10% fetal bovine serum (FBS) or no FBS. Cells were washed twice with PBS and detached from the culture plate using a cell scraper. Cells were lysed on ice for 15 min with radio-immuno-precipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL, USA), which contains 1% proteinase inhibitors. The samples were loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis on ice. The proteins resolved were transferred onto polyvinylidene fluoride (PVDF) membrane for 1 h and blocked for 1 h at room temperature with 5% skim milk. The membranes were incubated overnight at 4°C with the primary antibodies. Anti-alpha tubulin (diluted 1:1,000) was obtained from Santa Cruz Biotechnology. Anti-ERK1/2 (diluted 1:1,000) and anti-phospho-ERK1/2 (diluted 1:1,000) were obtained from Cell Signaling Technology. The membranes were washed in Tris-buffered saline-Tween 20 (TBST) and incubated with the secondary antibody.

Soft-agar assay. Nthy cells infected with empty vector, vector encoding wild-type BRAF, or vector encoding BRAF V600E were seeded at 3,000 cells per well in 24-well plates in a top layer of 0.4% agarose (Cell Biolabs) on a base layer of 0.6% agarose. Culture medium containing DMSO or BRAF V600E kinase inhibitors (PLX-4032, BioVision, San Francisco, CA, USA) was added to each well and cultured at 37°C in the presence of 5% CO2 for 7 days. The number of colonies containing more than 25 cells was counted using a microscope.

Invasion assay. The invasion assay was performed using the xCELLigence DP Real Time Cell Analyzer and CIM-16 plates with 8-μm pore membranes. The bottom electrodes of the CIM-16 plates were coated with 0.2% gelatin and incubated in a laminar air flow chamber for 30 min. The upper chambers of CIM-16 plates were coated with 20 μl of 0.5 mg/ml growth factor-reduced Matrigel (BD Bioscience, Bedford, MA, USA) prepared in FBS-free RPMI medium. Matrigel was allowed to equilibrate for 2 h at 37°C in 5% CO2. RPMI medium with 10% FBS was added to the bottom chambers. Empty vector control cells (Nthy/Vector), wild-type BRAF cells (Nthy/WT), or BRAF V600E cells (Nthy/V600E) were added to the top compartments (8×104 cells per well). The impedance data, reported as cell index and proportional to the area of the bottom electrodes covered by migrated/invasive cells, were collected every 15 min. The percentage of invasion was calculated as the ratio between the invasive cells and the migrated cells (8).

Gene expression microarray. Cells were cultured in 100-mm dishes until confluent monolayers were reached. Total RNA was extracted using the easy-spin (DNA free) Total RNA Extraction kit (iNIRON Biotechnology, Seoul, Korea) and quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Microarray services were provided by Macrogen (Macrogen Inc., Seoul, Korea) using the Illumina HumanHT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA, USA). Total RNA was amplified and purified using the TargetAmp-Nano Labeling kit for Illumina Expression BeadChip (EPICENTRE, Madison, WI, USA) to yield biotinylated cRNA according to the manufacturer’s instructions. Briefly, 500 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, in vitro-transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, MA, USA). Labeled cRNA samples (750 ng) were hybridized to each Human HT-12 v4.0 Expression Beadchip for 17 h at 58°C according to the manufacturer’s instructions (Illumina, Inc., San Diego, CA, USA). Detection of the array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array reader confocal scanner according to the manufacturer’s instructions.

Statistical methods for microarray data analysis. Microarray data were analyzed with two groups based on the BRAF V600E mutation status. The “Wild-type BRAF” group consisted of two Nthy/WT cultures, and the “Mutant-type BRAF” group consisted of two Nthy/V600E cultures. All statistical analyses were performed using R version 3.2.1 (9). Raw data derived from the Illumina Genome Studio version 2011.1 and Gene Expression Module version 1.9.0 were transformed.
into a "LumiBatch" object using "Lumi R package" version 1.1.0. Variance stabilization of gene expression counts was performed using the variance-stabilizing transformation (VST) method. Quantile normalization method was applied to gene expression data after VST. Packages “Annotate” and “IlluminaHumanv4.db” were used for microarray chip probe annotation, provided by bioconductor (http://www.bioconductor.org). To find differentially expressed genes (DEGs), moderated t-test using the “Limma” was applied. The Benjamini-Hochberg (BH) method was applied to correct false positive rate from multiple comparisons. p<0.05 after BH correction was considered statistically significant. A log fold change value of 2 was used as the cutoff to identify significant DEGs. Based on the DEGs identified from limma, “GO stat” package was used for gene ontology (GO) analysis (13). In the gene ontology test, false discover rate (FDR) corrected p-value under 0.01 was considered statistically significant. Pathway analysis using up-regulated DEGs in Nthy/BRAF cells was performed by Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (14).

Results

Effect of BRAF gene transduction into Nthy cells. Nthy cells were stably transfected with empty vector, wild-type BRAF, or BRAF<sup>V600E</sup> and named Nthy/Vector, Nthy/WT, and Nthy/V600E, respectively. The cell morphology of the Nthy/Vector and Nthy/WT cells was similar to that of the parental Nthy-ori 3-1 cells; however, Nthy/V600E cells had a spindle transformed shape, as shown in Figure 1A.

**BRAF gene sequences were confirmed by Sanger sequencing.** As shown in Figure 1B, the BRAF exon 15 sequences of Nthy/Vector and Nthy/WT cells were normal. Nthy/V600E cells, however, had a T>A mutation at position 1799. This result shows that the BRAF<sup>V600E</sup> recombinant plasmid was successfully constructed and the BRAF<sup>V600E</sup> expression level in Nthy-ori 3-1 cells surpassed that of the original wild-type BRAF.

By flow cytometry (Figure 2A), GFP showed a similar peak fluorescence intensity in Nthy/Vector, Nthy/WT, and Nthy/V600E cells, indicating similar BRAF protein levels. The intensity of p-ERK was increased in Nthy/V600E cells compared to Nthy/Vector or Nthy/WT cells (Figure 2A). Increased p-ERK was also confirmed by western blot (Figure 2B).
Increased anchorage-independent growth and invasion in Nthy/V600E cells. Soft-agar assay results estimating anchorage-independent growth are illustrated in Figure 3. The number of colonies observed was higher in Nthy/V600E cells than in Nthy/Vector or Nthy/WT cells. Treatment with PLX4032, a potent \(\text{BRAF}^{\text{V600E}}\) kinase inhibitor, inhibited colony growth, but the effect was small in Nthy/Vector or Nthy/WT cells; however, colony formation was decreased in a concentration-dependent manner in Nthy/V600E cells. In the invasion assay, the invasion/migration ratio was increased in Nthy/V600E cells, but not in Nthy/WT and control cells (Figure 4). This result indicates that Nthy/V600E cells are highly invasive compared to Nthy/Vector or Nthy/WT cells. Altogether, Nthy/V600E cells have increased anchorage-independent growth and a stronger invasive potential compared to Nthy/Vector or Nthy/WT cells.

Gene expression microarray. In total, 2,441 genes were differentially expressed at a higher level in Nthy/V600E cells compared to Nthy/WT cells (BH \(p<0.05\) and absolute log fold change \(>0\)). Forty-four genes were considered as significantly up-regulated DEGs in Nthy/V600E cells compared to Nthy/WT (BH \(p\)-value \(<0.05\) and absolute log fold change \(>2\). Table I. Among them, the top 20 up-regulated genes in Nthy/V600E cells and their ontology terms are listed on Table II. Their gene ontologies included carcinogenesis-related terms such as “MAPK pathway activation (\(\text{IL1B}, \text{DCLK1}, \text{TRIB1}\)”, “Wnt receptor signaling pathway (\(\text{SFRP1}\)”, “TOR signaling (\(\text{AGPAT9}\)”, “inflammation (\(\text{PLA2G7}, \text{NT5E}\)”, “cell proliferation (\(\text{IL24}\)”, migration (\(\text{SERPINE1}\)”, adhesion (\(\text{ITGA2}\)”, and “apoptosis (\(\text{G0S2}, \text{PHLDA1}\)”. 2,724 genes were differentially expressed at a lower level in Nthy/V600E cells compared to Nthy/WT cells. Forty-eight genes were considered significantly down-regulated DEGs. Information of down-regulated DEGs were also listed in Table III.

In GO analysis, 210 gene ontologies were enriched in Nthy/V600E cells (Table IV). Enriched GO terms in Nthy/V600E cells that were concordant with our functional
Figure 3. Colony formation in Nthy/Vector, Nthy/WT, and Nthy/V600E cells. Cells were grown in a 3-dimensional agar gel and treated with PLX4032, a potent inhibitor of BRAF<sup>V600E</sup> kinase. Representative images are shown.

Figure 4. Invasion/migration ratio in Nthy/Vector, Nthy/WT, and Nthy/V600E cells. The coverage of bottom electrodes, which is proportional to the number of cells that have invaded through a Matrigel-coated porous membrane or migrated through an uncoated porous membrane, was measured using a Real Time Cell Analyzer. The percentage of invasion was calculated by the ratio between the invasive cells and the migrated cells.

Analysis were as follows: morphological change to spindle shape ("cell morphogenesis"), increased cell growth in soft agar ("cell differentiation", "cell growth", "cell motility", "cell migration"), increased invasion ("cell motility", "cell adhesion"), overexpression of p-ERK ("ERK1 and ERK2 cascade", "MAPK cascade").
Significantly enriched pathways by up-regulated genes in Nthy/V600E are listed in Table V. Apoptosis, small cell lung cancer, pathways in cancer, colorectal cancer, cell cycle, and p53 signaling pathway were enriched in Nthy/V600E cells, but not in Nthy/WT cells. This result shows that the activation of cancer-related genes and pathways is increased in Nthy/BRAF cells compared to Nthy/WT cells.
Table II. Top 20 up-regulated DEGs and their gene ontology terms in Nthy/V600E mutant cells with growth factor treatment.

| Genes       | Full name                                           | Log FC | BH p-value | Gene ontology terms                                      |
|-------------|-----------------------------------------------------|--------|------------|---------------------------------------------------------|
| IL1B        | Interleukin 1, Beta                                 | 4.826  | <0.001     | Activation of MAPK activity                             |
| ANO1        | Anoctamin 1, Calcium-Activated Chloride Channel     | 4.339  | <0.001     | Ion transmembrane transport, multicellular organisational development |
| SERPINE2    | Serpin Peptidase Inhibitor, Clade E, Member 2       | 3.634  | <0.001     | Regulation of cell migration                            |
| SFRP1       | Secreted Frizzled-Related Protein 1                 | 3.553  | <0.001     | Wnt receptor signaling pathway, regulation of peptidyl-tyrosine phosphorylation |
| MPP4        | Membrane Protein, Palmitoylated 4                   | 3.26   | <0.001     | Protein localization to synapse                         |
| KHDRBS3     | Kh Domain-Containing, Rna-Binding, Signal Transduction-Associated 3 | 3.236  | <0.001     | Regulation of transcription                             |
| TMEM200A    | Transmembrane Protein 200A                          | 2.988  | <0.001     | Integral to membrane                                    |
| IL24        | Interleukin 24                                      | 2.986  | <0.001     | Regulation of cell proliferation                        |
| G0S2        | G0/G1 Switch 2                                      | 2.911  | <0.001     | Regulation of apoptotic signaling pathway               |
| ANTXR2      | Anthrax Toxin Receptor 2                            | 2.847  | <0.001     | Integral to membrane                                    |
| FOXQ1       | Forkhead Box Q1                                     | 2.82   | <0.001     | Tissue development                                      |
| DCLK1       | Doublecortin-Like Kinase 1                          | 2.705  | <0.001     | Protein kinase activity, phosphorylation                |
| AGPAT9      | 1-Acylglycerol-3-Phosphate                          | 2.601  | <0.001     | Regulation of TOR signaling                             |
| SNTB1       | Syndotrophin, Beta 1                                | 2.591  | <0.001     | Protein binding, phospholipid binding                   |
| CALB2       | Cabandin 2                                           | 2.461  | <0.001     | Calcium ion binding, cytoplasm, gap junction            |
| PHLD1A      | Pleckstrin Homology-Like Domain, Family A, Member 1 | 2.384  | <0.001     | Protein binding, phospholipid binding, apoptotic process |
| PLA2G7      | Phospholipase A2, Group Vii                         | 2.371  | <0.001     | Regulation of inflammatory response                     |
| LAMB3       | Laminin, Beta 3                                     | 2.36   | <0.001     | Structural molecule activity, extracellular matrix organization |
| ITGA2       | Integrin, Alpha 2                                    | 2.303  | <0.001     | Cell-matrix adhesion, integrin-mediated signaling pathway |
| STC1        | Stanniocalcin 1                                     | 2.229  | <0.001     | Cell surface receptor signaling pathway                  |

Discussion

The characteristics of Nthy/V600E cells were as follows: shape change to spindle type, increased anchorage-independent growth and invasion potential, increased p-ERK and overexpression of MAPK-related genes, and enrichment of cancer-related pathways.

The cell shape change in Nthy/V600E may be the result of epithelial-mesenchymal transition (EMT) induced by the BRAF mutation. A previous study reported that thyroid cancer cells differed in shape from wild-type epithelial thyroid cells and appeared spindle-shaped in BRAFV600E mice. The hallmark of EMT is the down-regulation of E-cadherin and up-regulation of vimentin expression. A significant loss of E-cadherin gene expression and an increase in vimentin gene expression were seen in BRAFV600E thyroid tumors compared to normal thyroid (15). EMT is a normal morphological event during embryonic development, tissue remodeling, and wound-healing but also occurs in neoplastic cells, especially in metastases (16). BRAFV600E expression in the rat thyroid PCCL3 cell line promotes EMT and invasion through an autocrine transforming growth factor (TGF)-β loop (1). In concordance with the previous literature, genes associated with EMT, i.e., vimentin, were highly expressed in Nthy/V600E cells compared to Nthy/WT cells in microarray experiments (data not shown). We plan to perform further functional studies to validate this observation.

The oncogenic BRAF protein is always phosphorylated to activate ERK signaling. The mechanism of phosphorylation of oncogenic BRAF has been described by Wang et al. Normal BRAF is maintained in a conformational state, where the ATP-binding domain is bound to the phosphorylation domain. However, in mutant BRAF, the combination is scattered and activates to be phosphorylated (17). PLX4032 is a selective BRAFV600E inhibitor. In cells harboring BRAFV600E, PLX4032 inhibits MAP kinase signaling effectively and suppresses phosphorylation of ERK. In tumor xenograft models of BRAFV600E melanoma, PLX4032 suppresses the proliferation of tumor cells and improves the survival of animals in a dose-dependent manner (18,19). Likewise, PLX4032 inhibited anchorage-independent growth in Nthy/V600E cells in the present study (Figure 3), suggesting that BRAFV600E may play an important role in anchorage-independent growth. In another study using PCCL3 rat thyroid cell lines with doxycycline-inducible expression of BRAFV600E, BRAFV600E protein expression and ERK phosphorylation were doxycycline dose-dependent (6, 20). In another study using PCCL3 cells to
obtain doxycycline-inducible expression of BRAFV600E, 
BRAFV600E-induced invasion through Matrigel (21). In a study 
using the human PTC-derived cell lines KAT5 and KAT10 
harboring a heterozygous BRAFV600E mutation, stable 
knockdown of BRAF using BRAF small interfering RNA (siRNA) 
suppressed anchorage-independent colony formation 
in soft agar (22). Our results are consistent with previous 
reports of thyroocyte cell lines. The clinical features of 
BRAFV600E generally thought to be associated with aggressive 
thyroid cancers, also correlate with our \textit{in vitro} data.
| GO ID     | GO Term                                         | p-Value    | Numbers of matched genes | Numbers of total genes in GO |
|-----------|-------------------------------------------------|------------|--------------------------|-----------------------------|
| GO:0008219 | Cell death                                      | <0.001     | 62                       | 1843                        |
| GO:0060560 | Developmental growth involved in morphogenesis | <0.001     | 17                       | 166                         |
| GO:0016265 | Death                                           | <0.001     | 62                       | 1847                        |
| GO:0009653 | Anatomical structure morphogenesis              | <0.001     | 74                       | 2438                        |
| GO:0007155 | Cell adhesion                                   | <0.001     | 50                       | 1344                        |
| GO:0022610 | Biological adhesion                             | <0.001     | 50                       | 1351                        |
| GO:0010941 | Regulation of cell death                        | <0.001     | 51                       | 1406                        |
| GO:0012301 | Programmed cell death                           | <0.001     | 58                       | 1755                        |
| GO:0030155 | Regulation of cell adhesion                     | <0.001     | 29                       | 561                         |
| GO:0006915 | Apoptotic process                               | <0.001     | 57                       | 1737                        |
| GO:0043067 | Regulation of programmed cell death             | <0.001     | 48                       | 1337                        |
| GO:0048585 | Negative regulation of response to stimulus    | <0.001     | 45                       | 1219                        |
| GO:0060429 | Epithelium development                          | <0.001     | 41                       | 1049                        |
| GO:0042981 | Regulation of apoptotic process                 | <0.001     | 47                       | 1328                        |
| GO:0008283 | Cell proliferation                              | <0.001     | 57                       | 1809                        |
| GO:0012427 | Regulation of cell proliferation                | <0.001     | 48                       | 1397                        |
| GO:0044011 | Locomotion                                      | <0.001     | 51                       | 1546                        |
| GO:0009888 | Tissue development                              | <0.001     | 54                       | 1687                        |
| GO:0009668 | Negative regulation of signal transduction      | <0.001     | 38                       | 986                         |
| GO:0032502 | Developmental process                           | <0.001     | 119                      | 5301                        |
| GO:0016477 | Cell migration                                  | <0.001     | 40                       | 1077                        |
| GO:0048856 | Anatomical structure development                | <0.001     | 109                      | 4706                        |
| GO:0009605 | Response to external stimulus                   | <0.001     | 60                       | 2047                        |
| GO:0048522 | Positive regulation of cellular process         | <0.001     | 98                       | 4116                        |
| GO:0048589 | Developmental growth                            | <0.001     | 22                       | 404                         |
| GO:0006928 | Movement of cell or subcellular component       | <0.001     | 52                       | 1663                        |
| GO:0048583 | Regulation of response to stimulus              | <0.001     | 83                       | 3275                        |
| GO:0043065 | Positive regulation of apoptotic process        | <0.001     | 26                       | 547                         |
| GO:0007275 | Multicellular organismal development            | <0.001     | 104                      | 4483                        |
| GO:0044767 | Single-organism developmental process           | <0.001     | 116                      | 5217                        |
| GO:0043068 | Positive regulation of programmed cell death    | <0.001     | 26                       | 552                         |
| GO:0023057 | Negative regulation of signaling                | <0.001     | 39                       | 1079                        |
| GO:0048519 | Negative regulation of biological process       | <0.001     | 98                       | 4157                        |
| GO:001648 | Negative regulation of cell communication       | <0.001     | 39                       | 1086                        |
| GO:0060602 | Branch elongation of an epithelium              | <0.001     | 6                        | 20                          |
| GO:0003401 | Axis elongation                                 | <0.001     | 7                        | 32                          |
| GO:0048468 | Cell development                                | <0.001     | 56                       | 1906                        |
| GO:0042325 | Regulation of phosphorylation                   | <0.001     | 42                       | 1240                        |
| GO:0008285 | Negative regulation of cell proliferation       | <0.001     | 27                       | 609                         |
| GO:0048513 | Organ development                               | <0.001     | 74                       | 2845                        |
| GO:0010942 | Positive regulation of cell death               | <0.001     | 26                       | 573                         |
| GO:0003269 | Cell motility                                   | <0.001     | 40                       | 1159                        |
| GO:00000574 | Localization of cell                           | <0.001     | 40                       | 1159                        |
| GO:00000668 | Protein phosphorylation                        | <0.001     | 46                       | 1439                        |
| GO:0001763 | Morphogenesis of a branching structure          | <0.001     | 15                       | 210                         |
| GO:0048729 | Tissue morphogenesis                            | <0.001     | 26                       | 584                         |
| GO:0019220 | Regulation of phosphate metabolic process       | <0.001     | 46                       | 1447                        |
| GO:0040007 | Growth                                         | <0.001     | 33                       | 866                         |
| GO:0022407 | Regulation of cell-cell adhesion                | <0.001     | 19                       | 336                         |
| GO:0035295 | Tube development                                | <0.001     | 26                       | 587                         |
| GO:0051174 | Regulation of phosphorus metabolic process      | <0.001     | 46                       | 1460                        |
| GO:0009790 | Embryo development                              | <0.001     | 35                       | 969                         |
| GO:1902532 | Negative regulation of intracellular signal transduction | <0.001 | 20 | 379 |
| GO:0048731 | System development                              | <0.001     | 92                       | 3928                        |
| GO:0030154 | Cell differentiation                            | <0.001     | 83                       | 3424                        |
| GO:0001932 | Regulation of protein phosphorylation          | <0.001     | 36                       | 1023                        |
| GO:0009666 | Regulation of signal transduction               | <0.001     | 67                       | 2549                        |
Table IV. Continued

| GO ID     | GO Term                                                      | p-Value  | Numbers of matched genes | Numbers of total genes in GO |
|-----------|--------------------------------------------------------------|----------|--------------------------|-----------------------------|
| GO:0002009 | Morphogenesis of an epithelium                              | <0.001   | 22                       | 458                         |
| GO:0044763 | Single-organism cellular process                            | <0.001   | 203                      | 11513                       |
| GO:0048518 | Positive regulation of biological process                   | <0.001   | 107                      | 4851                        |
| GO:0061338 | Morphogenesis of a branching epithelium                     | <0.001   | 14                       | 199                         |
| GO:0006469 | Negative regulation of protein kinase activity              | <0.001   | 14                       | 201                         |
| GO:0048869 | Cellular developmental process                              | <0.001   | 86                       | 3640                        |
| GO:0048523 | Negative regulation of cellular process                     | <0.001   | 89                       | 3818                        |
| GO:0051246 | Regulation of protein metabolic process                     | <0.001   | 58                       | 2123                        |
| GO:0006935 | Chemotaxis                                                  | <0.001   | 27                       | 674                         |
| GO:0042330 | Taxis                                                       | <0.001   | 27                       | 674                         |
| GO:0072001 | Renal system development                                    | <0.001   | 16                       | 271                         |
| GO:0009887 | Organ morphogenesis                                         | <0.001   | 32                       | 888                         |
| GO:0016049 | Cell growth                                                 | <0.001   | 20                       | 409                         |
| GO:0040082 | Regulation of locomotion                                    | <0.001   | 26                       | 644                         |
| GO:0001655 | Urogenital system development                               | <0.001   | 17                       | 310                         |
| GO:0033673 | Negative regulation of kinase activity                      | <0.001   | 14                       | 215                         |
| GO:0048598 | Embryonic morphogenesis                                     | <0.001   | 24                       | 569                         |
| GO:0001933 | Negative regulation of protein phosphorylation              | <0.001   | 16                       | 284                         |
| GO:0042326 | Negative regulation of phosphorylation                     | <0.001   | 18                       | 353                         |
| GO:0042221 | Response to chemical                                        | <0.001   | 86                       | 3731                        |
| GO:0060562 | Epithelial tube morphogenesis                               | <0.001   | 17                       | 322                         |
| GO:0035556 | Intracellular signal transduction                           | <0.001   | 59                       | 2243                        |
| GO:0098602 | Single organism cell adhesion                               | <0.001   | 27                       | 706                         |
| GO:0016337 | Single organism cell-cell adhesion                          | <0.001   | 26                       | 665                         |
| GO:2000145 | Regulation of cell motility                                 | <0.001   | 24                       | 585                         |
| GO:0001704 | Formation of primary germ layer                             | <0.001   | 10                       | 114                         |
| GO:0048754 | Branching morphogenesis of an epithelial tube               | <0.001   | 12                       | 167                         |
| GO:0023051 | Regulation of signaling                                     | <0.001   | 70                       | 2843                        |
| GO:0035239 | Tube morphogenesis                                          | <0.001   | 18                       | 361                         |
| GO:0007369 | Gastrulation                                                | <0.001   | 12                       | 169                         |
| GO:0010646 | Regulation of cell communication                            | <0.001   | 70                       | 2856                        |
| GO:0016310 | Phosphorylation                                             | <0.001   | 52                       | 1898                        |
| GO:0048646 | Anatomical structure formation involved in morphogenesis    | <0.001   | 34                       | 1024                        |
| GO:0033334 | Regulation of cell migration                                | <0.001   | 23                       | 554                         |
| GO:0044267 | Cellular protein metabolic process                          | <0.001   | 92                       | 4125                        |
| GO:0048588 | Developmental cell growth                                   | <0.001   | 10                       | 119                         |
| GO:0080134 | Regulation of response to stress                            | <0.001   | 34                       | 1034                        |
| GO:0022408 | Negative regulation of cell-cell adhesion                   | <0.001   | 10                       | 120                         |
| GO:0070887 | Cellular response to chemical stimulus                      | <0.001   | 60                       | 2336                        |
| GO:0060548 | Negative regulation of cell death                           | <0.001   | 30                       | 861                         |
| GO:0006793 | Phosphorus metabolic process                                | <0.001   | 68                       | 2781                        |
| GO:0032268 | Regulation of cellular protein metabolic process            | <0.001   | 52                       | 1926                        |
| GO:0048584 | Positive regulation of response to stimulus                 | <0.001   | 48                       | 1726                        |
| GO:0051270 | Regulation of cellular component movement                   | <0.001   | 25                       | 654                         |
| GO:0006796 | Phosphate-containing compound metabolic process             | <0.001   | 67                       | 2737                        |
| GO:0006904 | Cell morphogenesis involved in differentiation              | <0.001   | 29                       | 832                         |
| GO:0048699 | Generation of neurons                                      | <0.001   | 39                       | 1298                        |
| GO:0019538 | Protein metabolic process                                   | <0.001   | 101                      | 4736                        |
| GO:0010563 | Negative regulation of phosphorus metabolic process         | <0.001   | 20                       | 464                         |
| GO:0045936 | Negative regulation of phosphate metabolic process          | <0.001   | 20                       | 464                         |
| GO:0042493 | Response to drug                                            | <0.001   | 18                       | 394                         |
| GO:0043594 | Regulation of kinase activity                               | <0.001   | 27                       | 764                         |
| GO:0001822 | Kidney development                                         | <0.001   | 14                       | 256                         |
| GO:0043409 | Negative regulation of MAPK cascade                         | <0.001   | 10                       | 134                         |
| GO:0043069 | Negative regulation of programmed cell death                | <0.001   | 82                       | 812                         |
| GO:0007162 | Negative regulation of cell adhesion                        | <0.001   | 12                       | 193                         |
| GO:0044707 | Single-multicellular organism process                       | <0.001   | 124                      | 6230                        |

Table IV. Continued
Table IV. Continued

| GO ID | GO Term                                             | p-Value | Numbers of matched genes | Numbers of total genes in GO |
|-------|-----------------------------------------------------|---------|--------------------------|------------------------------|
| GO:0044092 | Negative regulation of molecular function   | <0.001  | 31                       | 952                          |
| GO:0030182 | Neuron differentiation                           | <0.001  | 36                       | 1193                         |
| GO:0051247 | Positive regulation of protein metabolic process | <0.001  | 37                       | 1243                         |
| GO:0051239 | Regulation of multicellular organismal process    | <0.001  | 56                       | 2221                         |
| GO:0072073 | Kidney epithelium development                    | <0.001  | 10                       | 140                          |
| GO:0031399 | Regulation of protein modification process       | <0.001  | 38                       | 1298                         |
| GO:0007417 | Central nervous system development                | <0.001  | 28                       | 831                          |
| GO:005680 | Negative regulation of epithelial cell proliferation | <0.001 | 9                       | 114                          |
| GO:2000026 | Regulation of multicellular organismal development | <0.001 | 40                       | 1403                         |
| GO:0022603 | Regulation of anatomical structure morphogenesis | <0.001  | 27                       | 792                          |
| GO:1902531 | Regulation of intracellular signal transduction  | <0.001  | 44                       | 1608                         |
| GO:0007165 | Signal transduction                              | <0.001  | 106                      | 5162                         |
| GO:0033993 | Response to lipid                                 | <0.001  | 25                       | 711                          |
| GO:0040166 | Negative regulation of apoptotic process         | <0.001  | 27                       | 803                          |
| GO:0014070 | Response to organic cyclic compound               | <0.001  | 25                       | 716                          |
| GO:0022008 | Neurogenesis                                      | <0.001  | 39                       | 1374                         |
| GO:0056739 | Regulation of protein kinase activity             | <0.001  | 25                       | 719                          |
| GO:0061564 | Axon development                                  | <0.001  | 22                       | 592                          |
| GO:0044700 | Single organism signaling                        | <0.001  | 113                      | 5638                         |
| GO:0023052 | Signaling                                         | <0.001  | 113                      | 5640                         |
| GO:0032270 | Positive regulation of cellular protein metabolic process | <0.001 | 34                       | 1141                         |
| GO:0014812 | Muscle cell migration                             | <0.001  | 6                        | 50                           |
| GO:0048732 | Gland development                                 | <0.001  | 17                       | 395                          |
| GO:0050673 | Epithelial cell proliferation                     | <0.001  | 15                       | 320                          |
| GO:0023014 | Signal transduction by phosphorylation           | <0.001  | 24                       | 685                          |
| GO:0022612 | Gland morphogenesis                               | <0.001  | 9                        | 124                          |
| GO:2000736 | Regulation of stem cell differentiation           | <0.001  | 8                        | 98                           |
| GO:0007154 | Cell communication                                | <0.001  | 114                      | 5728                         |
| GO:0043407 | Negative regulation of MAP kinase activity       | <0.001  | 7                        | 74                           |
| GO:0070371 | ERK1 and ERK2 cascade                             | <0.001  | 11                       | 187                          |
| GO:0032989 | Cellular component morphogenesis                  | <0.001  | 36                       | 1255                         |
| GO:0071901 | Negative regulation of protein serine/threonine kinase activity | <0.001 | 9                       | 127                          |
| GO:0001525 | Angiogenesis                                      | <0.001  | 17                       | 404                          |
| GO:0000165 | MAPK cascade                                      | <0.001  | 23                       | 653                          |
| GO:0031175 | Neuron projection development                     | <0.001  | 27                       | 832                          |
| GO:0007409 | Axonogenesis                                       | <0.001  | 21                       | 569                          |
| GO:0032501 | Multicellular organismal process                  | <0.001  | 125                      | 6465                         |
| GO:0007492 | Endoderm development                              | <0.001  | 7                        | 77                           |
| GO:0051248 | Negative regulation of protein metabolic process  | <0.001  | 27                       | 839                          |
| GO:0048608 | Reproductive structure development                | <0.001  | 17                       | 410                          |
| GO:0050186 | Response to stimulus                              | <0.001  | 141                      | 7532                         |
| GO:0000902 | Cell morphogenesis                                | <0.001  | 34                       | 1173                         |
| GO:0071310 | Cellular response to organic substance            | <0.001  | 48                       | 1892                         |
| GO:0051903 | Negative regulation of developmental process      | <0.001  | 26                       | 798                          |
| GO:0051716 | Cellular response to stimulus                     | <0.001  | 121                      | 6227                         |
| GO:0043086 | Negative regulation of catalytic activity        | <0.001  | 25                       | 753                          |
| GO:0061458 | Reproductive system development                  | <0.001  | 17                       | 413                          |
| GO:0051904 | Positive regulation of developmental process      | <0.001  | 30                       | 986                          |
| GO:0048863 | Stem cell differentiation                        | <0.001  | 15                       | 337                          |
| GO:0017066 | Endoderm formation                                | <0.001  | 6                        | 56                           |
| GO:0045785 | Positive regulation of cell adhesion              | <0.001  | 15                       | 338                          |
| GO:0010033 | Response to organic substance                     | <0.001  | 58                       | 2443                         |
| GO:0014910 | Regulation of smooth muscle cell migration        | <0.001  | 5                        | 36                           |
| GO:0007399 | Nervous system development                        | <0.001  | 50                       | 2008                         |
| GO:0030198 | Extracellular matrix organization                 | <0.001  | 16                       | 378                          |
| GO:0001934 | Positive regulation of protein phosphorylation    | <0.001  | 24                       | 715                          |
| GO:0043062 | Extracellular structure organization              | <0.001  | 16                       | 379                          |

Table IV. Continued
GO analysis of microarrays supported our functional results. DEGs up-regulated in Nthy/V600E cells are associated with cancer-related gene ontologies and pathways, showing that Nthy/V600E cells, but not Nthy/Vector or Nthy/WT cells, have carcinogenic potential.

We searched about the top four up-regulated genes in Nthy/V600E cells analyzed in light of previous research. IL-1 is a principal component of the interleukin-1 family (23). High-dose IL-1β administration causes broad inflammation and is accompanied by tissue damage and tumor invasiveness (24). In vitro analysis of melanocytes and melanoma cell lines showed that \( \text{BRAF}^{\text{V600E}} \) increases, while \( \text{BRAF}^{\text{V600E}} \) inhibition reduces the transcription of IL-1α and IL-1β (25, 26). In human thyrocytes, IL-1β alters the expression and localization of junction proteins (27). IL-1β induces the activation of cAMP responsive element-binding protein (CREB) through ERK1/2 signaling, and this mechanism was associated with poor prognosis in gastric carcinoma, non-small cell lung cancer, and breast cancer patients in previous reports (28-30).

The \( \text{ANO1} \) gene encodes the protein \( \text{ANO1} \) [transmembrane member 16A (TMEM16A)], a voltage-sensitive calcium-activated chloride channel (31). In a study in head and neck squamous cell carcinoma, \( \text{ANO1} \) overexpression significantly promoted anchorage-independent growth in vitro, whereas loss of \( \text{ANO1} \) resulted in inhibition of tumor growth. \( \text{ANO1} \)-induced cell proliferation and tumor growth were accompanied by an
increase in ERK1/2 activation and cyclin D1 induction (32). In lung cancer and colorectal cancer, ANO1 overexpression was related to tumor growth and invasion (33, 34).

The SERPINE2 gene encodes a member of the serpine protein family that inhibits serine proteases. In a study using human colorectal cell lines, BRAFV600E increased SERPINE2 mRNA and protein levels and subsequent MEK/ERK activity (35). In a pancreatic cancer study using nude mouse xenografts, SERPINE2 overexpression increased invasion through the extracellular matrix. In addition, cancer cells in SERPINE2-expressing tumors showed a spindle-shaped morphology and expressed the mesenchymal intermediate filament marker vimentin, which is consistent with our experimental results (36).

SFRP1 is the most extensively characterized gene in the SFRP family. This well-established tumor-suppressor gene generally acts as a Wnt inhibitor (37, 38). In expression and functional analysis using glioma stem cells, SFRP1 regulated the cell cycle and p53 pathways to inhibit Wnt (39). SFRP1 also increased ERK activity in lung epithelial cell lines (40).

Although Nthy is an immortalized cell line and may incompletely represent characteristics of normal human thyroid cells in vivo, our Nthy/V600E cells showed distinctive BRAFV600E mutation-associated features compared with Nthy/Vector cells. Nthy/V600E cells show a spindle-shaped morphology, anchorage-independent growth, increased invasive potential, and increased ERK phosphorylation. This cellular behavior was supported by GO analysis (cell adhesion, migration, and proliferation) of microarrays. Genes overexpressed in Nthy/V600E cells were also associated with ERK1/2, the MAPK cascade, and cancer-related pathways. Even if the results of this study cannot fully explain the pathogenesis of thyroid cancer, these Nthy/BRAF cells may be useful for basic research to evaluate the effect of the BRAFV600E mutation in normal human thyroid cells.

In conclusion, we generated a new cell line model aiming to study the carcinogenic mechanism of the BRAFV600E mutation. Functional experiments and microarrays revealed that Nthy/V600E cells have increased growth and invasion potential and increased expression of MAPK pathway components. Our Nthy/WT and Nthy/BRAF cell lines model human BRAFV600E PTC and may be useful in revealing the molecular characteristics of BRAF-mutant thyroid cancer.

Conflicts of Interest

None.

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