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$BRAF^{V600E}$ Promotes Invasiveness of Thyroid Cancer Cells through Nuclear Factor κB Activation

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The *$BRAF^{V600E}$* mutation is closely linked to tumorigenesis and malignant phenotype of papillary thyroid cancer. Signaling pathways activated by $BRAF^{V600E}$ are still unclear except a common activation pathway, MAPK cascade. To investigate the possible target of $BRAF^{V600E}$, we developed two different cell culture models: 1) doxycycline-inducible *$BRAF^{V600E}$*-expressing clonal line derived from human thyroid cancer WRO cells originally harboring wild-type *BRAF*; 2) WRO, KTC-3, and NPA cells infected with an adenovirus vector carrying *$BRAF^{V600E}$*. $BRAF^{V600E}$ expression induced ERK phosphorylation and cyclin D1 expression in these cells. The *$BRAF^{V600E}$*-overexpressing cells also showed an increase of nuclear factor κB (NF-κB) DNA-binding activity, resulting in up-regulation of antiapoptotic c-IAP-1, c-IAP-2, and X-linked inhibitor of apoptosis. Furthermore, $BRAF^{V600E}$ expression also induced the expression of matrix metalloproteinase and cell invasion into matrigel through NF-κB pathway. Increased invasive ability by $BRAF^{V600E}$ expression was significantly inhibited by a specific NF-κB inhibitor, racemic dehydroxymethylepoxyquinomicin. These data indicate that $BRAF^{V600E}$ activates not only MAPK but also NF-κB signaling pathway in human thyroid cancer cells, leading to an acquisition of apoptotic resistance and promotion of invasion. Inactivation of NF-κB may provide a new therapeutic modality for thyroid cancers with *$BRAF^{V600E}$*. (*Endocrinology* 147: 5699–5707, 2006)

THE *BRAF* GENE encodes a serine/threonine kinase that is a member of RAF family and transmits a signal from RAS to MAPK pathway. The MAPK signaling pathway regulates diverse physiological processes including cell growth, differentiation and apoptosis. *BRAF* somatic mutations have been identified in various types of human cancers including melanomas and colorectal and ovarian cancers (1). The *BRAF* mutations are the most prevalent genetic alteration (36–69%) in papillary thyroid cancers (PTCs), the most common type of endocrine malignancy (2–9). Activating *BRAF* mutations mainly occur in two regions of the kinase domain, the glycine-rich loop and the activation segment. The mutation in PTC is almost exclusively a thymine-to-adenine transversion at nucleotide 1799 (T1799A) in exon 15, resulting in a valine-to-glutamic acid substitution at amino acid residue 600 (V600E). This mutation produces a constitutively active kinase by disrupting hydrophobic interactions between residues in the activation loop and residues in the ATP binding site that maintain the inactive conformation, allowing development of new interactions that fold the kinase into a catalytically competent structure (1, 10).

Several studies have reported that the *BRAF* mutation is related to poor prognostic factors in patients with PTC (6, 8), whereas others have not found association between the mutation and any clinicopathological characteristics (11–14). The apparent controversy between these reports is likely due in part to histological diversity of PTC sample sets analyzed by different groups. A recent multicenter study of a large series of PTCs has shown that the *BRAF* mutation is associated with extrathyroidal invasion, lymph node metastasis, and tumor recurrence, even in patients with stage I/II initial disease (15). Thus, the *BRAF* mutation seems to be a predictor of poorer prognosis of PTCs.

Transgenic mice overexpressing $BRAF^{V600E}$ in their thyroids developed PTCs with high penetrance after 12 wk of age (16). The histological examination showed tall-cell features, areas of invasion, and foci of poorly differentiated carcinoma. These findings suggest that $BRAF^{V600E}$ confers on cancer cells malignant properties such as invasion. However, the underlying molecular mechanism still remains unknown.

CRAF (Raf-1), another member of RAF kinase family, has been shown to activate nuclear factor κB (NF-κB) (17–20). NF-κB is a transcription factor consisting of a heterodimeric or homodimeric complex. When inactive, this complex is sequestered in the cytoplasm by IκB, which is thought to mask the nuclear localization signal of NF-κB. Once IκB is phosphorylated by upstream kinases, it is subjected to ubiquitination followed by proteosomal degradation, allowing

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Abbreviations: DHMEQ, Racemic dehydroxymethylepoxyquinomicin; Dox, doxycycline; ECM, extracellular matrix; GFP, green fluorescent protein; HRP, horseradish peroxidase; MEK, MAPK kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; PTC, papillary thyroid cancer; TetR, Tet repressor; XIAP, X-linked inhibitor of apoptosis.

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NF-κB to translocate into the nucleus. NF-κB is a key regulator of genes involved in cellular proliferation and apoptosis (21). In tumor tissues, it is generally believed that the NF-κB-induced genes promote apoptotic resistance, transformation, cell growth, metastasis, and angiogenesis (22).

To investigate the possible mechanisms of BRAF^{V600E}-dependent oncogenesis, we established a thyroid cancer cell line with doxycycline (Dox)-inducible BRAF^{V600E} and also constructed an adenovirus vector carrying *BRAF^{V600E}*. Here we report that BRAF^{V600E}-induced NF-κB activation upregulates its downstream target genes that are responsible for antiapoptotic behavior and invasiveness of thyroid cancer cells, consistent with the clinicopathological features of human PTCs with this mutation.

Materials and Methods

Reagents

Antibodies used in this work were as follows: anti-p50 polyclonal, anti-p65 polyclonal, anti-BRAF monoclonal, anti-β-actin polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA); anti-IκB-α polyclonal, anti-X-linked inhibitor of apoptosis (XIAP) polyclonal, anti-phospho-ERK1/2 monoclonal, anti-His-tag polyclonal, horseradish peroxidase (HRP)-conjugated antirabbit IgG, and antimouse IgG (Cell Signaling Technology, Beverly, MA); anti-c-IAP-1 polyclonal and anti-c-IAP-2 polyclonal (R&D Systems, Minneapolis, MN); anti-TetR polyclonal (MoBiTec, Göttingen, Germany); anti-proliferating cell nuclear antigen (PCNA) monoclonal (BD Transduction Laboratories, San Jose, CA).

Stock solutions of racemic dehydroxymethylepoxyquinomicin (DHMEQ) (10 mg/ml) were prepared in dimethyl sulfoxide and stored at −20 C until use (23). U0126 was purchased from Calbiochem (La Jolla, CA).

Cell culture

Human thyroid cancer cell lines WRO and NPA were kindly provided by Dr. G. Juillard (University of California-Los Angeles, Los Angeles, CA) and KTC-3 was by Dr. J. Kurebayashi (Kawasaki Medical School, Okayama, Japan). All cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% (wt/vol) penicillin/streptomycin at 37 C in 5% CO_2-95% air environment.

Establishment of Dox-inducible BRAF^{V600E} cells

A T-REx expression system (Invitrogen, Carlsbad, CA) was used to generate Dox-inducible BRAF^{V600E} cells as directed by the manufacturer. Briefly, WRO cells were initially trasfected with the regulatory vector (pcDNA6/TR), which encodes Tet repressor (TetR) and blasticidine-resistant gene, using Lipofectamine (Invitrogen). Blasticidine-resistant clones were selected by limiting dilution in 96-well plates in the medium containing 3 μg/ml blasticidine (Promega, Madison, WI). The best clone with the highest expression of TetR (WRO-TetR cells) was determined by Western blot and further transfected with pcDNA5/TO carrying 6-Histidine-tagged BRAF^{V600E} using Lipofectamine. The double transfectants were selected in the medium with 200 μg/ml hygromycin and screened for Dox-inducible expression of BRAF^{V600E} by immunoprecipitation with anti-BRAF antibody followed by Western blotting for 6-histidine tag.

Adenovirus constructs

The *BRAF^{V600E}* and green fluorescent protein (*GFP*) cDNAs were subcloned into pAdHM4CMV (24). The plasmids were linearized with *Pac*I and transfected into human embryonal kidney 293 cells (HEK293; American Type Culture Collection, Manassas, VA) with SuperFect (QIAGEN, Valencia, CA). BRAF^{V600E} and GFP-expressing adenoviruses (Ad-BRAF^{V600E} and Ad-GFP) were propagated in HEK293 cells and purified by CsCl density-gradient centrifugation. The concentration of viral particle was determined by measuring the absorbance at 260 nm (25).

Western blotting

Cells were lysed in a buffer containing 20 mM HEPES (pH 7.5), 0.35 M NaCl, 20% glycerol, 1% Nonidet P40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Equal amount of protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) by semidry blotting. After incubation with appropriate primary antibody, the antigen-antibody complexes were visualized using HRP-conjugated secondary antibody and enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

DNA-binding assay

The multiwell colorimetric assay for active NF-κB was performed as described previously (26, 27), using a TransAM NF-κB p65 and p50 transcription factor assay kit (Active Motif North America, Carlsbad, CA). Briefly, nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotides containing NF-κB consensus binding site. NF-κB binding to the target oligonucleotides was detected with primary antibody specific to p65 or p50 subunit and HRP-conjugated secondary antibody. For quantification, OD was read at 450 nm using a microplate reader ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan).

In vitro invasion assay

The Chemotaxicell Invasion Chamber (Kurabo, Osaka, Japan) was used according to the manufacturer's instructions. In brief, 2.5×10^3 cells in serum-free medium were seeded onto matrigel-coated filters, and 5% fetal bovine serum was added to the lower wells as a chemoattractant. After incubation, the cells on the interior of the inserts were removed by swabbing, and the exterior of the inserts were stained with Diff-Quik staining kit (BD Biosciences, San Jose, CA). The cells that had penetrated through the filter were counted under bright-field microscopy.

Statistical analysis

Differences between groups were examined for statistical significance using one-way ANOVA followed by Fisher's protected least significant difference or unpaired *t* test as appropriate. A *P* value not exceeding 0.05 was considered statistically significant.

Results

Activation of MAPK pathway in BRAF^{V600E}-expressing cells

To explore the role of BRAF^{V600E} in thyroid cancer cells, Dox-inducible BRAF^{V600E}-expressing cells were generated using a thyroid carcinoma cell line WRO harboring wild-type *BRAF* (*BRAF^{wt}*). The whole cell lysates of WRO cells transfected with Dox-inducible BRAF^{V600E} (WRO-BRAF^{V600E} cells) were immunoprecipitated with anti-BRAF antibody, and the precipitates containing both endogenous and exogenous BRAF (BRAF^{wt} and BRAF^{V600E}) were immunoblotted with anti-6-Histidine (His) antibody to detect only exogenous BRAF^{V600E}. As shown in Fig. 1A, WRO-BRAF^{V600E} cells showed a marked induction of BRAF^{V600E} in a dose- and time-dependent manner.

Because BRAF is an upstream activator of MAPK kinase (MEK)/ERK pathway, we assessed the activation of MEK and ERK in the transfectants. In WRO-BRAF^{V600E} cells, the induction of MEK and ERK phosphorylation was apparent 12 h after the addition of Dox (Fig. 1B), whereas no changes were observed in control WRO-TetR cells (Fig. 1B).

To further confirm the BRAF^{V600E} effect, WRO cells were infected with recombinant adenovirus encoding *BRAF^{V600E}*. The enhanced ERK phosphorylation was observed in the

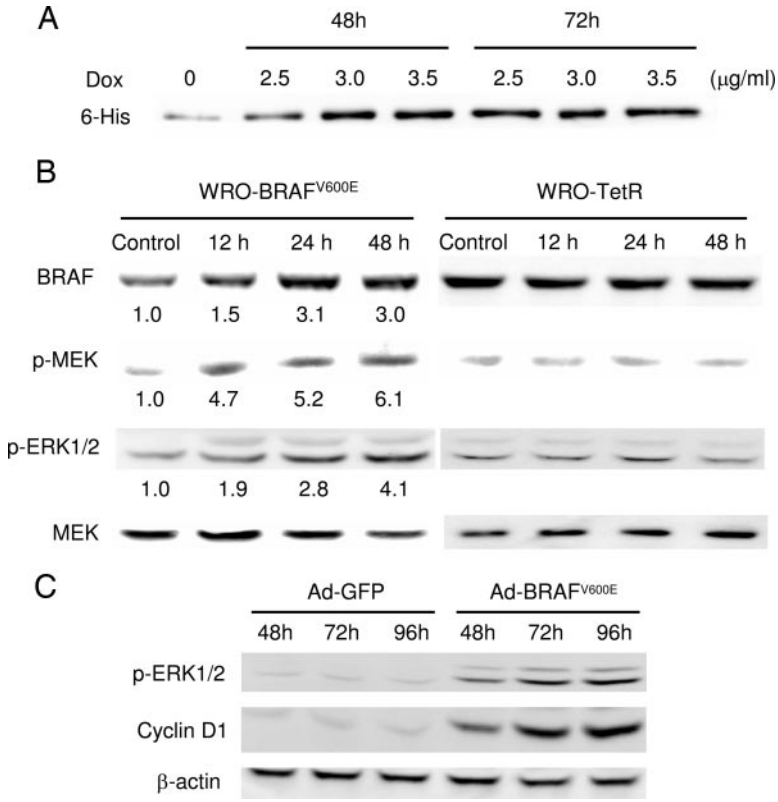

FIG. 1. Activation of MAPK pathway in BRAF^{V600E}-expressing cells. A, WRO-BRAF^{V600E} cells were incubated with the indicated concentration of Dox for the indicated times. Total cell lysates were subjected to immunoprecipitation with anti-BRAF antibody and Western blot was done using anti-6-histidine (His) antibody. B, Cells were treated with 3.0 μg/ml Dox for the indicated times. Western blot was done using the indicated primary antibodies. Relative expressions determined by densitometry are shown below the bands. C, WRO cells were infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI (multiplicity of infection) and incubated for the indicated times. Western blot was done using the indicated antibodies. β-Actin level was used as a loading control. Similar results were obtained in three independent experiments. p, Phosphorylated; Ad, adenovirus.

cells infected with Ad-BRAF^{V600E} but not with Ad-GFP (Fig. 1C).

The MEK/ERK signaling pathway controls cell proliferation in part by modulating the transcription of genes involved in cell cycle regulation such as cyclin D1. Consistently, Western blotting analysis showed that cyclin D1 was up-regulated in a time-dependent manner in cells infected with Ad-BRAF^{V600E} (Fig. 1C).

BRAF^{V600E} induces IκB-α degradation

To determine whether BRAF^{V600E} induces NF-κB activation through IκB-α degradation, we examined IκB-α expression by Western blot analysis in the both models. In Dox-inducible model, BRAF^{V600E} expression attenuated IκB-α expression in WRO-BRAF^{V600E} cells treated with Dox (Fig. 2A). In contrast, no degradation of IκB-α protein was seen in WRO-TetR cells (Fig. 2A). In the adenovirus model, IκB-α expression was similarly decreased by Ad-BRAF^{V600E} (Fig. 2B). Note that IκB-α expression was slightly restored at 96 h after infection. This is perhaps due in part to NF-κB-dependent transcription of *NFKBIA* gene that encodes IκB-α (negative feedback loop) but not adenovirus degradation because ERK phosphorylation and cyclin D1 expression at this time point were the strongest (Fig. 1C).

Induction of NF-κB DNA-binding activity in BRAF^{V600E}-expressing cells

To confirm the BRAF^{V600E}-induced NF-κB activation in thyroid cells, we performed DNA-binding assay. In Dox-inducible WRO-BRAF^{V600E} cells, NF-κB binding activity was

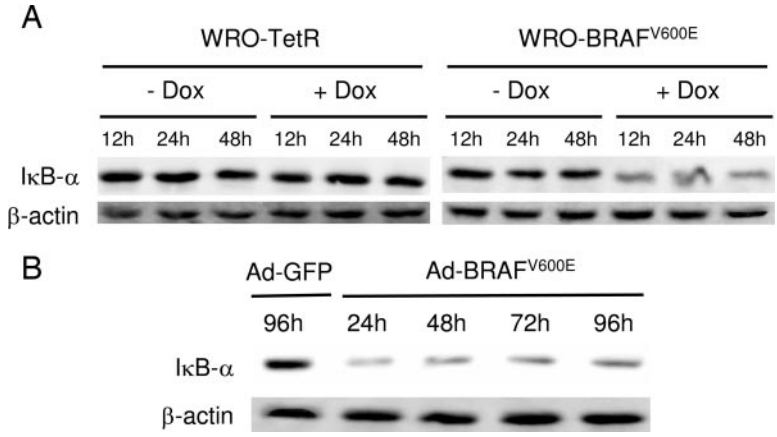

FIG. 2. Cytoplasmic degradation of IκB-α protein in BRAF^{V600E}-expressing cells. A, Cells were incubated with or without 3.0 μg/ml Dox for the indicated times. Western blot was performed using indicated antibody. B, WRO cells were infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI (multiplicity of infection) and harvested at the indicated time points. Western blot was performed using anti-IκB-α antibody. β-Actin level was used as a loading control. Similar results were obtained in three independent experiments.

significantly elevated at 24 and 48 h after the addition of Dox (Fig. 3A). There was no significant difference in the ratio of p65:p50 subunit, indicating that the active DNA-binding complex was mostly p65/p50 heterodimer. NF-κB binding activity did not change in control WRO-TetR cells after the addition of Dox (data not shown).

NF-κB binding activity was also up-regulated in WRO cells infected with Ad-BRAF^{V600E} (Fig. 3B). Treatment with DHMEQ, a specific NF-κB inhibitor (28), blocked the binding activity completely. On the other hand, treatment with U0126, a specific MEK inhibitor, had no effect on BRAF^{V600E}-induced NF-κB activation. Similar results were obtained in another thyroid cancer cell line KTC-3 also harboring wild-type *BRAF*. These results indicate that BRAF^{V600E} is a potent activator of p65/p50 NF-κB transcription factor and this activating pathway is MEK independent in these cells.

BRAF^{V600E} increases IAPs expression levels

To further confirm the functional downstream of BRAF^{V600E}-induced NF-κB activation, the expression of c-IAP-1, c-IAP-2, and XIAP proteins, well-known NF-κB-dependent antiapoptotic factors (29–31), was analyzed in BRAF^{V600E}-expressing cells. In WRO-BRAF^{V600E} cells, the expression of all the IAPs was increased after the addition of Dox in a time-dependent manner (Fig. 4A). In WRO-TetR cells, on the other hand, there was no change in IAPs protein levels (Fig. 4A). In adenovirus-infected cells, c-IAP-1 and

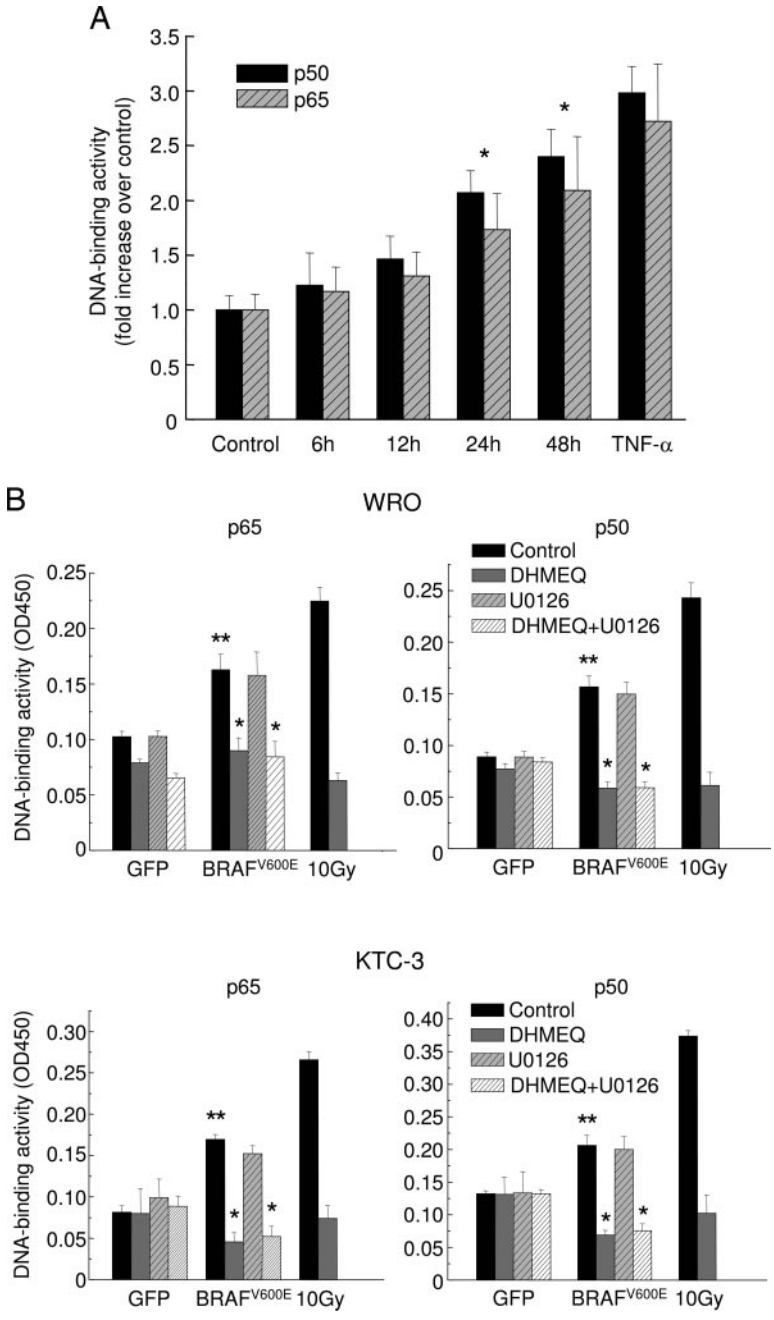

FIG. 3. Effect of BRAF^{V600E} expression on NF-κB DNA-binding activity. A, WRO-BRAF^{V600E} cells were incubated with 3.0 μg/ml Dox for the indicated times. Nuclear extracts were subjected to the DNA-binding assay as described in *Materials and Methods*. Cells treated with TNF-α were used as a positive control. *, $P < 0.05$ *vs.* control. B, Indicated cell lines were infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI (multiplicity of infection) and incubated for 72 h in the presence of indicated inhibitors (5 μg/ml DHMEQ and/or 10 μM U0126). Cells exposed to 10 Gy of γ-rays were used as a positive control. **, $P < 0.05$ *vs.* Ad-GFP control; *, $P < 0.05$ *vs.* Ad-BRAF^{V600E} control. A and B, Each *bar* indicates the mean and SD of the data collected in triplicate. Data are representative of three separate experiments.

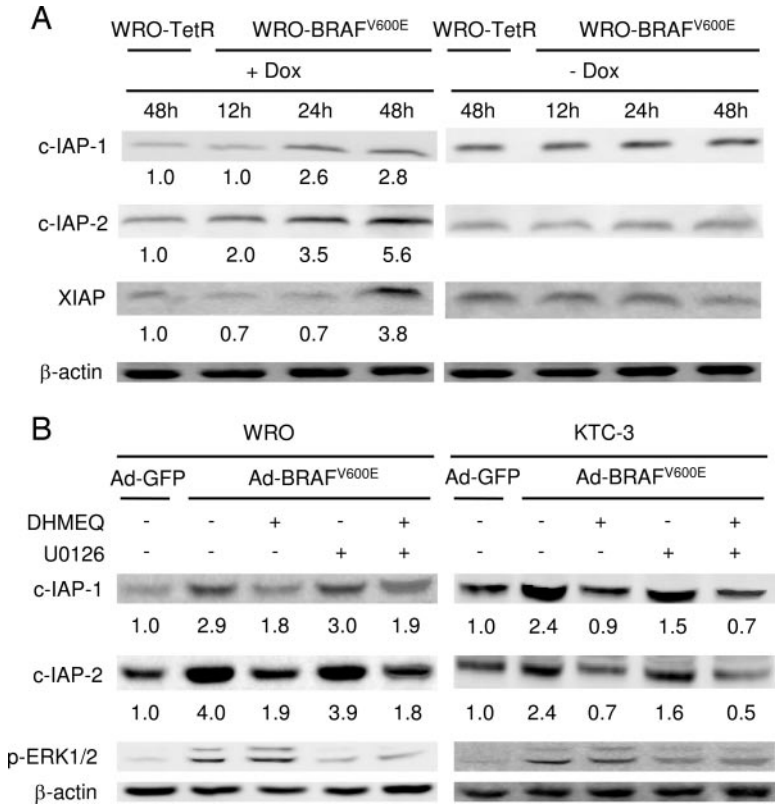

FIG. 4. Expression levels of IAP family proteins in BRAF^{V600E}-expressing cells. A, Indicated cell lines were incubated with or without 3.0 μg/ml Dox for the indicated times. B, Indicated cell lines were infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI (multiplicity of infection) with or without inhibitors (5 μg/ml DHMEQ and/or 10 μM U0126) and harvested at 96 h. A and B, Western blot was performed using the indicated antibodies. Relative expressions determined by densitometry are shown below the bands. β-Actin level was used as a loading control. Similar results were obtained in three independent experiments.

c-IAP-2 were similarly up-regulated in cells infected with Ad-BRAF^{V600E}, and this induction was blocked in the presence of DHMEQ but not U0126 (Fig. 4B).

BRAF^{V600E} enhances invasiveness of thyroid cancer cells

We next evaluated the functional consequence of BRAF^{V600E}-induced NF-κB activation. Because NF-κB induces a variety of genes that play an important role in invasion and metastasis of cancer cells, we explored the involvement of BRAF^{V600E}/NF-κB activation in cell invasiveness.

It has been reported that matrix metalloproteinase (MMP) family is associated with cancer invasion (32, 33). MMPs are the enzymes that degrade components of extracellular matrix (ECM) and basement membrane. As shown in Fig. 5A, MMP-1 expression was remarkably elevated after the addition of Dox in WRO-BRAF^{V600E} cells. We also evaluated the expression of MMP-1, MMP-7, and MMP-9 in adenovirus-infected WRO and KTC-3 cells. Similarly, an induction of MMP-1 was observed in Ad-BRAF^{V600E}-infected cells (Fig. 5B). MMP-9 was up-regulated in KTC-3 cells but not in WRO

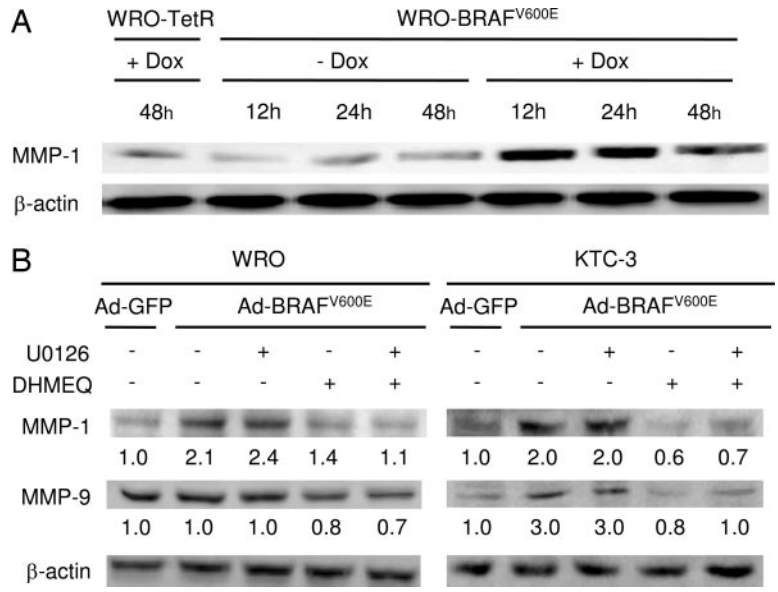

FIG. 5. Expression levels of MMPs in BRAF^{V600E}-expressing cells. A, Cells were incubated with or without 3.0 μg/ml Dox for the indicated times. B, Cells were infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI in the presence of indicated inhibitors (5 μg/ml DHMEQ and/or 10 μM U0126) and harvested at 96 h. A and B, Western blot was performed using the indicated antibodies. Relative expressions determined by densitometry are shown below the bands. β-actin level was used as a loading control. Similar results were obtained in three independent experiments.

cells (Fig. 5B). There was no change in MMP-7 expression in both lines (data not shown). These BRAF^{V600E}-induced MMP-1/MMP-9 accumulations were completely blocked in cells treated with DHMEQ but not changed with U0126 (Fig. 5B).

We next performed functional assay to investigate the consequence of BRAF^{V600E} expression on cell invasiveness. BRAF^{V600E} expression enhanced matrigel invasion by approximately 2-fold in the Dox-inducible model (Fig. 6A). In adenovirus-infected cells, BRAF^{V600E} overexpression also robustly enhanced invasiveness (Fig. 6B). Because BRAF^{V600E} did not confer a significant growth advantage on the cells (data not shown), the observed difference in cell invasiveness was not due to the increase in cell number. Interestingly, there were some differences between WRO and KTC-3 cells in the experiments with U0126 and DHMEQ. U0126 attenuated BRAF^{V600E}-induced cell invasiveness by about 60% in KTC-3 cells, but produced no effect in WRO cells (Fig. 6B). DHMEQ blunted the invasiveness by half in WRO cells but completely blocked in KTC-3 cells (Fig. 6B). In addition, to further strengthen our observation, we used PTC cell line NPA in key experiments even though this line harbors BRAF^{V600E}. As shown in Fig. 6C, BRAF^{V600E} overexpression clearly enhanced p50/p65 translocation and cell invasiveness. As a whole, these results demonstrated the involvement of NF-κB pathway in BRAF^{V600E}-induced cell migration and invasion.

Discussion

In PTCs, *RET/PTC* and *NTRK* rearrangements and point mutations of *RAS* and *BRAF*, all of which activate MAPK pathway, are cumulatively found in approximately 70% of cases and they are mutually exclusive (2, 5, 34). This provides a compelling genetic evidence that constitutive active MAPK signaling is a key component of transformation to PTC.

However, each of these oncoproteins elicits clearly distinct phenotypic features and biological behaviors. For example, *RAS* mutation or *PAX8*-peroxisome proliferator-activated re-

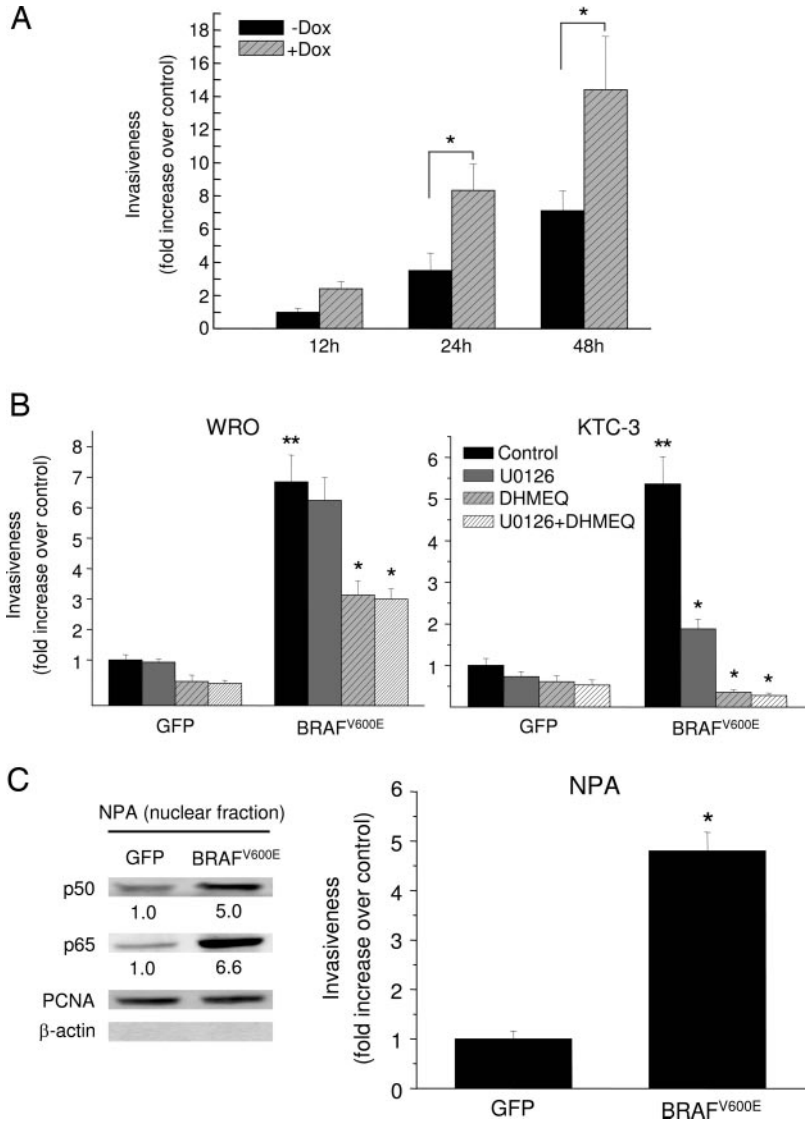

FIG. 6. BRAF^{V600E} enhances invasiveness of thyroid cancer cells. A, WRO-BRAF^{V600E} cells were seeded onto the matrigel-coated filters with or without 3 μg/ml Dox. Plates were incubated at 37 C in 5% CO_2 for the indicated times and subjected to invasion assay. *, $P < 0.05$. B, Indicated cells were seeded onto the matrigel-coated filters, infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI (multiplicity of infection). Plates were incubated at 37 C in 5% CO_2 for 72 h with or without inhibitors (5 μg/ml DHMEQ and/or 10 μM U0126) and subjected to invasion assay. **, $P < 0.05$ *vs.* GFP control, *, $P < 0.05$ *vs.* BRAF^{V600E} control. C (*Left*), NPA cells were infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI (multiplicity of infection) and incubated for 72 h. Nuclear extracts were subjected to Western blotting using indicated primary antibodies. Relative expressions determined by densitometry are shown below the bands. PCNA (proliferating cell nuclear antigen) level was used as a loading control. β-Actin was used as a negative control for nuclear extracting. *Right*, NPA cells were seeded onto the matrigel-coated filters, infected with Ad-BRAF^{V600E} or Ad-GFP at 50 MOI. Plates were incubated at 37 C in 5% CO_2 for 72 h and subjected to invasion assay. *, $P < 0.05$ *vs.* GFP control. A–C, Each *bar* indicates the mean and SD of the data collected in triplicate. Data are representative of three separate experiments.

ceptor γ recombination was more frequently found in follicular variant of PTC (35, 36), whereas PTCs with conventional growth pattern and tall-cell features often harbor mutated *BRAF*, which has been reported to associate with extrathyroidal invasion (8) and distant metastasis (6). Using microarray technique, Giordano *et al.* (37) have demonstrated that gene expression profiles, despite a profound overlapping, were discernible in PTCs harboring *RET/PTC* or mutant *RAS* and *BRAF*. Thus, besides the common propensity to activate MAPK signaling, PTC-specific oncogenes also evoke alternative pathways, and the diversity of clinicopathological manifestations is likely due to the different spectrum of downstream genes activated by each oncoprotein.

The primary purpose of this study was to explore the mechanisms by which $BRAF^{V600E}$ induces invasion and metastasis of thyroid cancer cells. To address this issue, we first established a Dox-inducible $BRAF^{V600E}$ clonal line derived from human thyroid cancer cell WRO harboring wild-type BRAF. However, Dox-inducible systems often show leaky expression even in the absence of Dox, or additional genetic changes might be acquired during sequential selection steps. In our WRO-$BRAF^{V600E}$ cells, Western blot for His-tag showed a faint expression of $BRAF^{V600E}$ in the absence of Dox, indicating small leakiness. To complement our experiments, we used another system, the adenovirus vector carrying $BRAF^{V600E}$ and another cell line, KTC-3, also harboring wild-type BRAF. In both systems, we confirmed $BRAF^{V600E}$-induced ERK phosphorylation.

CRAF, which is another isoform of RAF family proteins and similarly transmits signals from RAS to MEK, has been shown to activate NF-κB. Vale *et al.* (20) have demonstrated that CRAF-induced transformation of NIH3T3 cells requires the activation of NF-κB-IL-1 autocrine loop. We and others have reported that NF-κB plays an important role in a variety of thyroid cancer cells (27, 28, 38). In terms of the relationship between *BRAF* mutation and NF-κB activation, only one group, to our knowledge, has reported that oncogenic $BRAF^{V600E}$ up-regulates NF-κB transcriptional activity in NIH3T3 cells (39, 40). In the present work, we showed that the degradation of IκB-α, a cytoplasmic inhibitor of NF-κB, started shortly after a sufficient accumulation of $BRAF^{V600E}$ protein, resulting in the activation of NF-κB signaling. A specific NF-κB inhibitor, DHMEQ, efficiently blocked $BRAF^{V600E}$-induced NF-κB activation. Interestingly, however, this activation was independent of MEK-ERK pathway. This is consistent with a previous report showing that the constitutive active mutant of MEK failed to induce NF-κB-dependent gene expression in NIH3T3 cells, whereas RAS^{V12} or $CRAF^{BXB}$ (both are also constitutive active mutants) were capable of it (20). NF-κB is known as a potent regulator of antiapoptotic genes. Distortions of apoptotic processes are the main contributors to tumor formation and tumor cell resistance to therapeutic agents.

The family of IAPs, which are under transcriptional control of NF-κB, has been shown to play a principal role in the suppression of apoptotic cell death (41–43). In our setting, the protein expression of IAP family was induced after $BRAF^{V600E}$ expression in thyroid cells, suggesting that $BRAF^{V600E}$ contributes to inducing apoptotic resistance. This induction was also NF-κB dependent and MEK-ERK independent.

Tumor invasion and metastasis require proteolytic degradation of ECM and basement membrane by MMPs. The MMPs are zinc-dependent endopeptidases subdivided into collagenases, gelatinases, stromelysins, and matrilysins on the basis of their specificity for ECM components (32). One of the MMP family members involved in the breakdown of the most abundant ECM proteins, collagen type I and III, is interstitial collagenase, MMP-1. In our model, $BRAF^{V600E}$ induced MMP-1 expression in both WRO and KTC-3 cells and MMP-9 in KTC-3 cells. The promoter of *MMP-1* contains several AP-1 binding sites. Recent studies have shown that the AP-1 site must cooperate with a variety of cis-acting sequences found in the upstream of the promoter to up-regulate *MMP-1* transcription (44). For example, induction of MMP-1 by IL-1 in rabbit fibroblasts requires the interaction between the AP-1 site at –77 bp and a NF-κB-like element located at −3030 bp. Whereas both IL-1 and TNF-α activate NF-κB pathway, only IL-1 is capable of inducing *MMP-1* transcription in rabbit primary synovial fibroblasts (45). Presumably, this is due to the inability of TNF-α to activate MAPK pathway in these cells, whereas IL-1 activates both. On the other hand, Reunanen *et al.* (46) reported that TNF-α induced ERK phosphorylation and MMP-1 up-regulation in human skin fibroblasts, and PD98059, another MEK inhibitor, had no effect on modulating the MMP-1 mRNA and protein expression. Thus, the regulation of MMP-1 expression seems to be cell type specific. In our model using thyroid cancer cells, MMP-1 protein level appeared to be regulated by only NF-κB pathway but not by MEK-ERK signaling.

Although the promoter of MMP-9 also contains both AP-1 and NF-κB binding sites (47), MAPK pathway was not involved in MMP-9 up-regulation in KTC-3 cells. In WRO cells, $BRAF^{V600E}$ did not induce MMP-9 expression; however, the basal MMP-9 expression in WRO cells was relatively higher than in KTC-3 cells, implying the possibility that the induction was already saturated by other oncogenic signals in this type of cells.

We also observed the increased invasiveness of thyroid cancer cells after $BRAF^{V600E}$ expression. The $BRAF^{V600E}$-induced cell invasiveness was correspondingly observed in papillary cancer cell line NPA, suggesting that this mechanism is well conserved in thyroid cells. In WRO cells, U0126 did not reduce $BRAF^{V600E}$-induced cell invasiveness, which was consistent with Western blot for MMP-1. However, in KTC-3 cells, U0126 suppressed the invasiveness by about 60%. There are several possible explanations. Some degree of increased invasiveness could result from elevated activity of MAPK pathway alone. It has been reported that active MAPK may influence cell migration and metastasis through modulating the expression of integrins (48) and also the integrin distribution in cancers is associated with tumor progression (49). Active MAPK is also able to directly phosphorylate myosin light chain kinase leading to enhanced phosphorylation of myosin light chain and in turn stress fiber assembly (50). These mechanisms may participate in the induction of thyroid cell invasion after the expression of $BRAF^{V600E}$ oncoprotein. However, the treatment with DHMEQ alone completely blocked $BRAF^{V600E}$-induced cell invasiveness in KTC-3 cells and reduced by half in WRO

cells, suggesting that NF-κB is a potent downstream factor directly affecting BRAF^{V600E}-induced cell invasiveness.

In conclusion, our results demonstrate that BRAF^{V600E} activates NF-κB signaling independently of MAPK pathway in thyroid cancer cells, and enhances cell invasion, presumably via NF-κB-MMP expression. Specific NF-κB inhibitors such as DHMEQ may therefore be useful for the treatment of more aggressive thyroid cancers carrying *BRAF^{V600E}* mutation by dampening not only apoptotic resistance but also cell invasiveness.

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