Genome-wide alterations in gene methylation by the *BRAF* V600E mutation in papillary thyroid cancer cells

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Abstract

The *BRAF*V600E mutation plays an important role in the tumorigenesis of papillary thyroid cancer (PTC). To explore an epigenetic mechanism involved in this process, we performed a genome-wide DNA methylation analysis using a methylated CpG island amplification (MCA)/CpG island microarray system to examine gene methylation alterations after shRNA knockdown of *BRAF* V600E in thyroid cancer cells. Our results revealed numerous methylation targets of *BRAF* V600E mutation with a large cohort of hyper- or hypo-methylated genes in thyroid cancer cells, which are known to have important metabolic and cellular functions. As hypomethylation of numerous genes by *BRAF* V600E was particularly a striking finding, we took a further step to examine the selected 59 genes that became hypermethylated in both cell lines upon *BRAF* V600E knockdown and found them to be mostly correspondingly under-expressed (i.e. they were normally maintained hypomethylated and over-expressed by *BRAF* V600E in thyroid cancer cells). We confirmed the methylation status of selected genes revealed on MCA/CpG microarray analysis by performing methylation-specific PCR. To provide proof of concept that some of the genes uncovered here may play a direct oncogenic role, we selected six of them to perform shRNA knockdown and examined its effect on cellular functions. Our results demonstrated that the *HMGB2* gene played a role in PTC cell proliferation and the *FDG1* gene in cell invasion. Thus, this study uncovered a prominent epigenetic mechanism through which *BRAF*V600E can promote PTC tumorigenesis by altering the methylation and hence the expression of numerous important genes.

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Introduction

Papillary thyroid cancer (PTC) is the most common endocrine malignancy, accounting for 80% of all thyroid cancers (Hundahl *et al.* 1998, Altekruse *et al.* 2010). A strong oncogenic mutation that has been characterized in recent years in PTC is the *BRAF* mutation, which, by far, is the most common oncogenic genetic event found in this cancer, occurring in about 45% of cases on average (Xing 2005). There are several types of *BRAF* mutations found in PTC and the T1799A transverse point mutation accounts for vast majority of them (Nikiforov 2008). The T1799A *BRAF* mutation causes a substitution of valine with glutamic acid in codon 600 (V600E), resulting in constitutive

and oncogenic activation of the *BRAF* kinase in the Ras/Raf/MEK/ERK signaling pathway (MAPK pathway; Davies *et al.* 2002). Through activating the MAPK pathway, the *BRAF* mutation plays a fundamental role in the tumorigenesis of PTC and promotes and predicts its poor clinical outcomes (Xing 2005, 2007a). The specific molecular mechanisms underlying PTC tumorigenesis driven by the *BRAF* mutation have not been well understood. This is particularly the case in epigenetic aspects. For example, the role of aberrant gene methylation and its extent in this process have not been defined for *BRAF* mutation in PTC.

Gene methylation is an epigenetic phenomenon in which a methyl group is covalently added to the fifth

carbon of the cytosine residue in a CpG dinucleotide in CpG islands, typically located in the promoter area of a gene. Promoter methylation often silences a gene and aberration in its methylation state can thus seriously affect its function. As such, hypermethylation can silence tumor suppressor genes and hypomethylation can cause over-expression of oncogenes. Consequently, aberrant alterations in gene methylation play a fundamental role in human tumorigenesis (Jones & Baylin 2007, Hsiao *et al.* 2009, Kondo & Issa 2010), including thyroid tumorigenesis (Xing 2007b).

Given the importance of this epigenetic aberration in thyroid tumorigenesis, we hypothesize that the *BRAF* V600E mutation, through activating the MAPK pathway, may aberrantly affect gene methylation in thyroid cancer, as a molecular mechanism in *BRAF* mutation-promoted thyroid tumorigenesis. In this study, we performed a genome-wide screening of gene methylation in thyroid cancer cells using a methylated CpG island amplification (MCA)/CpG island microarray approach (Estéicio *et al.* 2007) and selectively examined the cellular functions of some genes among which many identified to be altered in the methylation state by *BRAF* V600E.

Materials and methods

Thyroid cancer cell lines

The PTC-derived cell lines BCPAP and OCUT1 were from Dr Massimo Santoro (University of Federico II, Naples, Italy) and Dr Naoyoshi Onoda (Osaka City University Graduate School of Medicine, Osaka, Japan) respectively. We chose these two thyroid cancer cell lines because both harbor the *BRAF* V600E mutation. Cells were routinely grown at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum (FBS) with standard supplements. Genomic DNA was isolated by SDS and proteinase K digestion, followed by standard phenol–chloroform extraction and ethanol precipitation.

Lentivirus-mediated RNA interference

The lentiviral pSicoR-PGK-puro vectors (Addgene, Inc., Cambridge, MA, USA) encoding seven self-complementary hairpin RNA sequences were used to knock down seven selected genes, including *BRAF*, *HLX1*, *KLHL14*, *HMGB2*, *NR4A2*, *FGD1*, and *ZBTB10*. The sequences are presented in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article. Lentiviral vector DNAs and packaging vectors were transfected into human embryonic kidney 293 cells (ATCC, Manassas, VA, USA)

to generate lentiviral particles. Cells were then exposed to lentivirus-containing supernatant for 48 h in the presence of polybrene (8 µg/ml final concentration). After 2 days, transfected cells were serum starved (0.5% FBS) and then harvested for 24 h in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blotting assays were used to detect the protein expression of *BRAF*, *HLX1*, *KLHL14*, *HMGB2*, *NR4A2*, *FGD1*, and *ZBTB10*. Cell pools with stable transfection were selected using puromycin.

Western blotting assay

Cells were lysed in RIPA buffer using standard protocol. Protein lysates were separated on 10% SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech), followed by incubation with specific primary antibodies. Anti-*BRAF* (sc-166), anti-phospho-ERK (sc-7383), anti-*ZBTB10* (sc-87402), and anti-actin (sc-1616-R) were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-*HLX1* (AV100839), anti-*KLHL14* (AV34677), anti-*HMGB2* (AV31939), anti-*NR4A2* (AV38753), and anti-*FGD1* (AV31673) were purchased from Sigma. Antigen–antibody complexes were visualized using HRP-conjugated anti-mouse (sc-2005, Santa Cruz) or anti-rabbit (sc-2004, Santa Cruz) IgG antibodies and ECL western blotting analysis system (Amersham Pharmacia).

Methylated CpG island amplification

The MCA assay was performed as described previously (Toyota *et al.* 1999). Briefly, ~5 µg of each genomic DNA from cells stably transfected with *BRAF* shRNA (termed ‘test’ hereafter) and vector (termed ‘control’ hereafter) were digested with 100 units of methylation-sensitive restriction endonuclease *Sma*I (New England Biolabs, Ipswich, MA, USA) for 16 h at 20 °C in a total volume of 100 µl, followed by digestion with 20 units of methylation-insensitive restriction endonuclease *Xma*I for 6 h at 37 °C, which leaves sticky ends (C/CCGGG). DNA fragments were then precipitated with ethanol and their sticky ends ligated with 0.5 nmol of unphosphorylated linkers RXMA–24/–12, as described (Toyota *et al.* 1999). The oligonucleotide sequences were as follows: (RXMA–24) 5'-AGC ACT CTC CAG CCT CTC ACC GAC-3' and (RXMA–12) 5'-CCG GGT CGG TGA-3'. PCR reactions were performed using the digests as templates and subjected to 20 cycles of amplification as described previously (Estéicio *et al.* 2007). Ten microliters of PCR product were resolved in 1.5% agarose gel and visualized under u.v. after ethidium bromide staining. Successful MCA reactions

resulted in amplicon smear ranging from 300 bp to 3 kb, with most amplicons at 1 kb. The PCR products were purified and reserved for fluorescence labeling.

CpG island microarray

Amino-allyl dUTP (aa-dUTP; Sigma) was incorporated into 600 ng of ‘test and control’ amplicons using the BioPrime DNA labeling system (Life Technologies, Inc., Carlsbad, CA, USA). Alexa 647 (red color) and Alexa 555 (green color) fluorescent dyes were coupled to aa-dUTP-labeled ‘test and control’ amplicons respectively. Equimolar amounts of the labeled amplicons were co-hybridized to the 12K Human CpG-island Array chip (Microarray Center, University Health Network, Toronto, ON, Canada), which contained 12 192 CpG-island clones that were originally created at the Sanger Institute (Heisler *et al.* 2005). Hybridized slides were scanned and the acquired images were analyzed with the corresponding software. Two-step global lowess normalization was done using the background-subtracted median intensity of each spot, and the resultant $\log_2$ ratios were averaged from triplicate experiments. CpG island tags (or probes) hybridized predominantly with the test amplicon, but not with the control amplicon, appear as red spots. We set an arbitrary cutoff value of 1.5 for the Alexa 647/555 ratio as previously defined (Yan *et al.* 2001, Shi *et al.* 2002); loci with ratios ≥ 1.5 (equally $\log_2$ ratio ≥ 0.6) were identified as hypermethylated in the ‘test’ samples. In contrast, CpG island tags hybridized predominantly with the ‘control’ amplicon, but not with the test amplicon, appear as green spots. We set a cutoff value of 0.5 for the Alexa 647/555 ratio; loci with ratios ≤ 0.5 (equally $\log_2$ ratio ≤ -1) were identified as hypomethylated in the ‘test’ samples (Yan *et al.* 2001, Shi *et al.* 2002). Triplicate experiments were performed for each cell line and they were averaged for data analysis.

Pathway and functional analyses of the hypermethylated genes with Ingenuity Pathways Analysis

Genes were uploaded to Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Inc., Redwood City, CA, USA; www.ingenuity.com) and pathway and functional analyses were performed. IPA is a commercial web-based interface that uses various computational algorithms to identify and establish cellular networks. This analysis uses a database of gene interactions culled from the literature and updated every quarter of the year.

RNA extraction and real-time quantitative RT-PCR analysis

RNA expression analysis was performed by real-time quantitative PCR using the ABI Prism 7900HT Sequence Detection System Instrument (Applied Biosystems, Carlsbad, CA, USA). Two micrograms of total RNA underwent RT using Oligo-dT and SuperScript II according to the instructions of the manufacturer (SuperScript First-Strand Synthesis kit, Invitrogen). PCR reactions were performed using SYBR GreenER qPCR SuperMix according to the instructions of the manufacturer (Applied Biosystems). PCR primers were designed using Primer Express (Applied Biosystems) to span a large intron whose location and sequences are presented in [Supplementary Table 2](#), see section on [supplementary data](#) given at the end of this article. C_t values were calculated by the ABI PRISM Software and the relative amount of RNA was presented for each sample according to the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). The specificity of real-time quantitative PCR of samples was confirmed by running the PCR products on 1.5% agarose gel to confirm single specific bands at the expected sizes (data not shown). Each sample was run in triplicate.

Quantitative methylation-specific PCR

Genomic DNA was subjected to bisulfite treatment as described previously (Hou *et al.* 2008). A methylation-specific and SYBR-green-based quantitative PCR (QMSP; Hattermann *et al.* 2008) was performed to validate the methylation status of selected 11 genes on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using iTaq SYBR Green Supermix (Bio-Rad) according to the instructions of the manufacturer, including *NRA4A2*, *HLX1*, *KLHL14*, *HMGB2*, *FGD1*, *ZBTB10*, *KIAA0247*, *SLC5A1*, *TBC1D7*, *SLMO1*, and *UCK2*. The methylation-specific primers were designed using Methyl Primer Express Software v1.0 (Applied Biosystems) and are presented in [Supplementary Table 3](#), see section on [supplementary data](#) given at the end of this article. Normal leukocyte DNA was methylated *in vitro* with Sss I methylase (New England Biolabs) to generate completely methylated DNA as a positive control. Each plate contained triplicate samples, multiple water blanks, and serial dilutions of positive methylated control to construct the standard curves. The relative methylation level of each DNA sample was calculated as described previously (Hu *et al.* 2006).

Cell proliferation and invasion assays

For cell proliferation assay, cells (800/well) were seeded onto 96-well plates and cultured with 2.5% FBS. MTT assay was performed daily over a 5-day time course to evaluate cell proliferation. Cell culture was added with 10 μ l of 5 mg/ml MTT agent (Sigma) and incubated for 4 h, followed by the addition of 100 μ l of 10% SDS solution and a further incubation overnight. The plates were read on a microplate reader using the test wavelength of 570 nm and the reference wavelength of 670 nm. Three triplicates were done to determine each data point.

Cell invasion assay was performed using Matrigel-coated Transwell cell culture chambers with 8 μ m pore (BD Biosciences, Sparks, MD, USA). Briefly, 2.5×10^4 cells were suspended in serum-free medium and seeded in the upper chamber of the Transwell insert. RPMI 1640 medium containing 10% FBS was added to the lower chamber. After a 22 h incubation at 37 °C with 5% CO₂, non-invasive cells in the upper chamber were removed and invasive cells that migrated to the underside of the membrane were

fixed in 100% methanol and stained with 0.5% crystal violet in 2% ethanol. The number of invasive cells was counted after photography.

Statistical analysis

Experiments were done at least twice. DNA methylation and gene expression data were analyzed using program ScanAlyze as described by Michael Eisen (<http://rana.lbl.gov/eisensoftware.htm>). Data were compared using the *t*-test. Statistical significance was defined as $P < 0.05$.

Results

Genome-wide identification of hyper- or hypo-methylated genes by the BRAF V600E mutation in thyroid cancer cells

To explore the relationship between *BRAF* V600E mutation and aberrant DNA methylation and identify the methylation targets of the *BRAF* mutation, we used the shRNA approach to specifically knock down *BRAF* in thyroid cancer cell lines, BCPAP and OCUT1,

Figure 1 Global identification of *BRAF* V600E mutation-mediated methylation targets in thyroid cancer cells using the MCA/CpG island microarray approach. (A) Effects of stable siRNA knockdown of BRAF on the MAPK pathway in thyroid cancer cells harboring the *BRAF* V600E mutation. BCPAP and OCUT1 cells were infected with lentiviruses carrying BRAF shRNA and stable populations were selected with 2 μ g/ml puromycin. The empty vector was used as the control. After a 2-week selection, cells were lysed and immunoblotted with BRAF and phosphorylated ERK (p-ERK) antibodies. The antibody against β -actin was used for quality control of western blotting. (B) Schematic illustration of MCA/CpG island microarray analysis. Methylated CpG islands were amplified by serial digestion with SmaI (methylation sensitive) and XmaI (methylation insensitive) restriction endonucleases. The methylated fraction of genomic DNA from cells stably transfected with BRAF shRNA or vector was amplified and labeled with appropriate dyes and then co-hybridized to a microarray platform as described in the Materials and methods section.

which harbored *BRAF* V600E, and examined globally the change in the methylation status of genes using the MCA/CpG island microarray approach. As shown in Fig. 1A, *BRAF* shRNA nearly completely eliminated the expression of *BRAF* protein. This effectively suppressed the signaling of the MAPK pathway, as reflected by the elimination of phosphorylation of ERK in the two cells. Hypermethylated and hypomethylated genes in ‘test’ were visualized as red and green spots, respectively, in the microarray. Normalized $\log_2$ ratios of ≥ 0.6 and ≤ -1 were used as the cutoff values for hypermethylation and hypomethylation, respectively, as conventionally used. A schematic illustration of the procedure and partial MCA/CpG island microarray image are presented in Fig. 1B.

The correlation between two different microarray experiments was statistically significant ($P < 0.0001$), with Pearson r values of 0.91 and 0.90 for cells BCPAP and OCUT1, respectively, suggesting that the MCA/CpG island microarray used to identify hypermethylated or hypomethylated genes in this study was highly reproducible and reliable (Fig. 2A). As shown in Fig. 2B, we found a similar methylation profiling in the two thyroid cancer cells after *BRAF* knockdown. Strikingly, using the corresponding gene database (University Health Network Human CpG Microarray Database, Toronto, ON, Canada; <http://data.microarrays.ca/cpg>) to identify the specifically affected genes, this approach revealed numerous hyper- or hypo-methylated genes by the *BRAF* V600E. With a cutoff value of

Figure 2 Detection of gene methylation changes by the MCA/CpG island microarray analysis. (A) Demonstration of the reproducibility of MCA/CpG island microarray performance, showing overall correlation between independent replicates for the two thyroid cancer cell lines BCPAP and OCUT1. (B) Global methylation analysis of thyroid cancer cells by the MCA/CpG island microarray analysis. Data are presented as the mean of values from three experiments for the ratios of methylation presented as $\log_2$, with red representing genes with high methylation, black representing no change, and green representing hypomethylation. The upper frame indicates 59 representative genes that became hypermethylated upon *BRAF* V600E knockdown in both BCPAP and OCUT1 cells, which were further analyzed for gene expression, which is shown in Fig. 3. The lower frame indicates ten representative genes that became hypomethylated upon *BRAF* V600E knockdown in both BCPAP and OCUT1 cells.

$\log_2 \leq -1$ as hypomethylation, we found a large cohort of genes that became hypomethylated upon BRAF knockdown in both cell lines (Fig. 2B). This result suggested that these genes were normally hypermethylated under the pressure of BRAF V600E signaling in the two cells. Similarly, with a cutoff value of $\log_2 \geq 0.6$ as hypermethylation, we identified a large cohort of genes that became hypermethylated upon BRAF knockdown in both cell lines. This result suggested that these genes were normally hypomethylated under the pressure of BRAF V600E signaling in the two cells. Compared with gene hypermethylation, gene hypomethylation has been relatively poorly investigated in human cancers. The present finding of wide gene hypomethylation by BRAF mutation in thyroid cancer cells was thus particularly interesting. We therefore selected 59 (shown in Fig. 2B) of these genes, which contain two or more SmaI/XmaI sites (CCCGGG) in their promoters, for gene expression analyses and some of them for functional studies as presented in the following sections. A summary of the known main functions of these genes is presented in

Supplementary Table 4, see section on supplementary data given at the end of this article.

Expression analysis of the genes that became hypermethylated upon BRAF V600E knockdown in thyroid cancer cells

We examined the expression status of 59 genes that became hypermethylated upon BRAF knockdown in the two cells (Fig. 2B). This gene expression analysis demonstrated the functional consequence of methylation changes in genes revealed by the MCA/CpG microarray analysis. Our results showed that most of these genes were under-expressed (indicated by green color) in the two thyroid cancer cells (Fig. 3A), consistent with their hypermethylation status upon BRAF knockdown (Fig. 2B). Many of these genes showed identical hypoexpression patterns in the two thyroid cancer cells (i.e. genes in green color in both cells in Fig. 3A). These results are consistent with the expectation that hypermethylation of genes typically leads to their silencing. It should be noted that these results from BRAF knockdown paradoxically

Figure 3 Analysis of gene expression and validation of MCA/CpG island microarray using MSQP in thyroid cancer cells. (A) Gene expression analysis of selected 59 genes from Fig. 2 in BCPAP and OCUT1 cells using RT-PCR, with red color representing over-expression and green color representing under-expression upon BRAF V600E knockdown. (B) Validation by QMSP of methylation status of selected 11 genes that were hypermethylated and under-expressed upon BRAF knockdown in cells revealed by MCA/cpG island microarray. These genes were labeled with an asterisk (*) in Fig. 3A. Details are described in the Materials and methods section. * $P < 0.05$, ** $P < 0.01$.

demonstrated that these genes were normally hypomethylated and over-expressed in thyroid cancer cells under the pressure of the *BRAF* V600E mutation.

Validation of hypermethylation of genes after BRAF knockdown in thyroid cancer cells using QMSP

We selected 11 genes from those that showed hypermethylation by MCA/CpG island microarray and hypoeexpression in both thyroid cancer cells for methylation validation using QMSP. These genes included *NRA4A2*, *HLX1*, *KLHL14*, *HMGB2*, *FGD1*, *ZBTB10*, *KIAA0247*, *SLC5A1*, *TBC1D7*, *SLMO1*, and *UCK2*. As shown in Fig. 3B, suppression of the MAPK signaling pathway through BRAF knockdown could increase methylation level of these genes in the two thyroid cancer cells. These results were consistent with

the MCA/CpG microarray and gene expression data (Figs 2B and 3A), demonstrating again the reliability of the MCA/CpG microarray approach and confirming the true methylation changes in their promoter areas.

Pathway analysis of the genes hypermethylated upon BRAF knockdown

We used the IPA Software to map the genes that became hypermethylated upon BRAF knockdown to different networks in the IPA database. We chose the top four networks in this study (Table 1). These networks describe functional relationships among gene products based on the findings presented in peer-reviewed biological pathways. Genes clustered in these networks are involved in various biological functions, including tissue development, cellular development, carbohydrate metabolism, cell death, DNA replication,

Table 1 Ingenuity networks: the genes hypermethylated upon BRAF V600E knockdown in thyroid cancer cells discovered in this study and involved in the network are presented in bold text

Top network	Molecules in network	Score	Focus molecules	Functions
1	APOL3 (includes EG: 80833), AS3MT, AUTS2 (includes EG: 26053), BCAT2 , C14ORF106, C1ORF123, CASP3, CBLL1, CD40LG, CDH1, CDK3 , CDKN1A, DBNL, ECD, ELMOD3, FGD1 , GATA3 , HLX , HNF4A, IFT122, IL12 (complex), interferon α , MAP3K14, MPP5 , NAPA , NEURL4 , NR3C1, PRR3, RPS8 , SAMHD1 , SLC5A1 , TADA3 , UNC45A , WTAP , ZC3H10	36	16	Tissue development, cellular development, and cell death
2	Amino acids, ARID5A , B3GALT2, BUD31 (includes EG: 8896), C21ORF33, CDC42EP3 , CSRP1, DOCK7 (includes EG: 85440), EEA1 , EGR2, HMGB2 , LSR, MN1, NAB1, NDST1, NFKBIA, NME6 , NXPH4 , PARP3, PDE7A, PPP5C , RABGGTA, SEC61B , SGPP2 , sphingosine-1-phosphate, ST6GALNAC3 , STK17A, TBC1D7 , TGFB1, TNFSF11, TSC1, TSC2, Tsc1-Tsc2, UCK2, YWHAA	30	14	Cellular development, nervous system development and function, and carbohydrate metabolism
3	ANKRA2, APOLD1 , APP, ATP9A , CD5L, CPEB1 , CREB1, Cu+, CYP26A1 , DUSP14, FSH, GM10681, GTPASE, hCG, hydrogen peroxide, KIAA0247 , MAP3K5 (includes EG: 293015), MCL1, NOS3, NOX5, octopamine, PAPD7, PCNA, POLD4, POLE, POLI , POLK, POLM, POLQ, PTPase, SFRS13A , SLC30A1 , UNG, WIPF1 , ZDHHC7	19	10	DNA replication, recombination, and repair, cellular compromise, and cellular assembly and organization
4	ADIPOR1, ADIPOR2, AKT3, ANP32A, APPL1 , ATP5B, CCT2, COL4A1, CSH1, DUSP4, EEF2, EEF1A1 , EIF3F, GPX1, GRB14, Insulin, KCNMA1, LEPR, MDH2, MTAP (includes EG: 4507), NR4A2 , NR4A3, PGK1, PHYHIP, PSMD11, Rab5, RPS16, SLC6A3, SP4, SYT9 , TRPV1, TUBGCP3 , Vegf, ZBTB10 , ZNF24 (includes EG: 7572)	13	7	Organismal survival, inflammatory response, and cell cycle

recombination, and repair, and other functions. Based on a score of 13 or higher in the top 1–4 networks, many genes, including the 11 genes that are specially studied as described above, that became hypermethylated upon *BRAF* V600E knockdown in the two thyroid cancer cells showed an extensive relationship and interaction among themselves in the pathways. Thus, these results strongly suggest that many of the genes aberrantly altered in methylation and hence expression by *BRAF* V600E had important functions in thyroid cancer cells.

Testing of the role in cellular functions of PTC cells of selected genes that were normally hypomethylated and over-expressed under the pressure of BRAF V600E

We speculated that some of the genes that were hypomethylated and over-expressed by *BRAF* V600E might play a direct role in cellular functions of thyroid cancer cells. From the 11 genes shown in the pathway/networks revealed by IPA, we chose six genes for such studies in BCPAP cells. These included

Figure 4 Effects of silencing *BRAF* V600E-related genes on the proliferation and invasion of thyroid cancer cells. (A) Knock down of the indicated genes coupled to the *BRAF* V600E signaling through aberrant gene methylation revealed in this study using vector-based RNA interference. BCPAP cells were infected with lentivirus expressing shRNA targeting FGD1, KLX1, HMGB2, KLHL14, NR4A2, and ZBTB10 respectively. Stable cell pools obtained after selection with puromycin were lysed and immunoblotted with corresponding antibodies. The lentivirus packaged with empty vector was used as control. (B) Bar graph, corresponding to (A), shows the average protein levels of control or shRNA-transfected cells from two independent experiments. The ratio between band intensities of specific proteins and the corresponding β -actin controls was calculated to correct for differences in protein loading on the gel. For convenient comparison, the protein levels of the control vector-transfected cells were set as 1 and the protein levels of shRNA-transfected cells were set relative to the control for each indicated gene. The error bars represent two protein levels obtained in the two different experiments. (C) Cell proliferation assay of BCPAP cell pools with stable knock down of the indicated *BRAF* V600E-coupled genes. The proliferation rate was examined using MTT assay and the OD value was measured daily over a 5-day time course. Cell numbers were significantly different ($P=0.04$) at day 6 between the group transfected with vector and the group transfected with HMGB2 shRNA. (D) Cell invasion assay of BCPAP cell pools with stable knock down of the indicated *BRAF* V600E-related genes. Cells were plated and cultured in Matrigel-coated transwell for 22 h, followed by examination and quantification of the invasive cells as described in the Materials and Methods section. The upper panel shows representative results of invasive BCPAP cells with different transfections. The bar graph in the lower panel, corresponding to the upper panel, shows the average number of invasive cells of two independent experiments, with the error bars representing the invasive cell numbers obtained from the two different experiments. C, control shRNA; S, specific shRNA; KD, knockdown.

FGD1, *HLX1*, *HMGB2*, *KLHL14*, *NR4A2*, and *ZBTB10* genes. To this end, we used the shRNA approach to specifically knock down these genes (Fig. 4A and B) and subsequently examined the change in cellular behaviors of BCPAP cells. Among these six genes, shRNA knockdown of the *HMGB2* gene could significantly inhibit the proliferation of BCPAP cells in comparison with control vector transfection ($P=0.04$; Fig. 4C). shRNA knockdown of the *FGD1* gene could dramatically inhibit the invasion of BCPAP cells (Fig. 4D). These results provide direct evidence that, as representatives of the genes coupled to *BRAF* mutation through epigenetic alterations, the *HMGB2* and *FGD1* genes may have an important role in *BRAF* mutation-promoted thyroid tumorigenesis.

Discussion

Numerous recent studies have established the important role of *BRAF* V600E mutation in the tumorigenesis and pathogenesis of PTC (Xing 2007a, Nikiforov 2008). However, information on the molecular mechanisms and derangements coupled to this mutation in this process is limited. As methylation aberrations of genes and consequent alterations in their expression are a fundamental molecular mechanism in the tumorigenesis of human cancers (Jones & Baylin 2007, Hsiao *et al.* 2009, Kondo & Issa 2010), including thyroid cancer (Xing 2007b), we explored such a mechanism in the tumorigenesis of PTC promoted by the *BRAF* mutation in this study.

We took the strategy of using a high-throughput MCA/CpG island microarray system to explore genome-wide aberrations of gene methylation coupled to the *BRAF* mutation in PTC cells. With shRNA knockdown of the *BRAF* V600E in thyroid cancer cells that naturally harbored this mutation, we examined the change in gene methylation and found, strikingly, a large number of genes to become hyper- or hypomethylated upon knock down of *BRAF* V600E in PTC cells. It should be noted that under these conditions, the hyper- or hypo-methylated genes represent, paradoxically, hypo- or hyper-methylated genes naturally existing under the pressure of *BRAF* V600E in these PTC cells. Therefore, a striking finding of this study is that numerous genes are normally hypermethylated or hypomethylated by the MAPK signaling driven by *BRAF* V600E in PTC cells. Since methylation usually silences a gene, the expression of these genes must be significantly affected by alterations in their methylation states promoted by the *BRAF* mutation. Many of these genes are already known to play various critical metabolic, molecular, and cellular functions, as also

suggested by our IPA pathway analysis for some of these genes. Changes in the expression of these genes driven by the *BRAF* mutation must have a significant impact on the functions and behaviors of the cell. Thus, this wide aberration of gene methylation in the genome coupled to the *BRAF* mutation represents a major mechanism that has not been previously revealed in the PTC tumorigenesis promoted by the *BRAF* mutation.

The gene expression profiling coupled to *BRAF* V600E was shown to be largely similar between the two thyroid cancer cell lines, BCPAP and OCUT1, in this study, but there are also many genes that are expressed differently between the two cells. This may be due to the difference in other molecular environments of the two cells, such as their different genetic backgrounds; for example, in addition to *BRAF* mutation, OCUT1 cells also harbor the *PIK3CA* mutation, which activates the PI3K/Akt pathway.

BRAF mutation was found to be associated with methylation of certain genes in colon cancer (Weisenberger *et al.* 2006, Ang *et al.* 2009). A number of genes were previously found to be hypermethylated in thyroid cancer (Xing 2007b) and some tumor suppressor genes were found to be also associated with *BRAF* mutation in PTC (Hu *et al.* 2006). However, these association studies did not establish a functional and causal link between the *BRAF* mutation and the occurrence of methylation of these genes. This study is the first to demonstrate a direct functional link between *BRAF* mutation and aberrant gene methylation in thyroid cancer cells. Strikingly, this role of *BRAF* V600E is genome-wide and affects numerous genes, suggesting that epigenetic impact of the *BRAF* mutation on PTC cells is of paramount importance. By taking a genome-wide analysis approach, the finding in this study extended well beyond the scope of previous work on the association of methylation of a few individual genes with *BRAF* mutation. A few genes previously found to be hypermethylated in association with *BRAF* mutation in PTC were important tumor suppressor genes (Hu *et al.* 2006). It is plausible to speculate that many of the new genes implicated to be normally hypermethylated by *BRAF* V600E signaling in thyroid cancer in this study may have important tumor-suppressing functions, which need to be defined in further studies.

Another novel and striking finding in this study is the numerous genes that are normally hypomethylated under the pressure of *BRAF* V600E in thyroid cancer cells (i.e. the genes that became hypermethylated upon *BRAF* V600E knockdown). In line with this finding is a recent example that aberrant MAPK signaling pathway driven by the *BRAF* mutation promoted the expression of *MAP2* gene, a neuron-specific

microtubule-associated protein, in melanoma cells by promoter demethylation (Maddodi *et al.* 2010). Compared with gene hypermethylation, gene hypomethylation has been relatively under-investigated in cancers in general. We therefore took a further step to investigate this phenomenon in thyroid cancer cells in this study. In particular, we selected 59 genes for expression analysis that contained two or more SmaI/XmaI sites in their promoters and were hypermethylated upon BRAF knockdown in the two thyroid cancer cell lines. These selected genes contained two or more SmaI/XmaI sites in their promoters, ensuring that, if identified by the MCA approach used in the present study, they can have methylation alterations in their promoter areas. As expected, most of these genes were correspondingly under-expressed upon BRAF knockdown in the two thyroid cancer cells, consistent with the classically known gene-silencing role of hypermethylation. This result paradoxically suggests that, driven by the *BRAF* V600E, these genes are normally over-expressed as well as hypomethylated in PTC cells. We also used QMSP to confirm the methylation states of selected genes revealed by the MCA/CpG island microarray with corresponding under-expression in both thyroid cancer cells and were able to validate the MCA/CpG island microarray approach in evaluating gene methylation changes.

Similar to the significance of hypermethylation and presumably under-expression of the large cohort of genes coupled to the *BRAF* V600E as discussed above, we speculated that hypomethylation and over-expression of the large cohort of genes coupled to this mutant should also have profound effect on cellular function and tumor behavior of PTC. It is tempting to propose that many of these genes coupled to the *BRAF* V600E through hypomethylation may play an important tumor-promoting role in the PTC tumorigenesis. In fact, our IPA analyses showed that many of the genes coupled to *BRAF* mutation through hypomethylation are important components of several major pathways involved in various metabolic and cellular functions. It is possible that some of these genes may prove to be novel pro-oncogenes or oncogenes in the future. To functionally and principally test this concept, we selected six genes to study their role in cellular functions of PTC cells. Even among only six genes chosen to study, we were able to demonstrate that two of them played a functional role in PTC cells. Specifically, the *FDG1* gene played a significant role in PTC cell invasion and the *HMGB2* gene played a role in cell proliferation. These results imply a potential oncogenic function of the *FDG1* and *HMGB2* genes. The *FDG1* gene is the

susceptible gene for the Aarskog–Scott’s faciogenital dysplasia syndrome (Shalev *et al.* 2006) and the *HMGB2* gene is a DNA-binding protein with pleiotropic biological functions (Yamada & Maruyama 2007). A previous study in fact showed that expression of the *FDG1* gene could cause cell transformation (Whitehead *et al.* 1998). It should be pointed out that the methylation status and consequent biological effects of many genes are not affected by the *BRAF* V600E signaling as shown in this study, although many are. This is not surprising as other more dominant regulatory mechanisms may be involved in the regulation of these genes. Also, epigenetic alterations could be irreversible under certain molecular and cellular circumstances.

In summary, using a high-throughput MCA/CpG island microarray approach, we performed a genome-wide gene methylation analysis with respect to *BRAF* mutation in PTC cells. We uncovered widespread alterations in DNA methylation with a large cohort of hyper- or hypo-methylated genes under the pressure of *BRAF* V600E signaling. Given the known important metabolic and cellular functions of many of these genes, alterations in their expression through methylation aberrations as a consequence of *BRAF* mutation may have a profound impact on cell functions and behaviors of PTC. Many of these genes, as suggested by the *FDG1* and *HMGB2* genes, may prove to play an important role in thyroid tumorigenesis. This study has thus uncovered a prominent epigenetic mechanism through which the *BRAF* mutation drives the tumorigenesis of PTC.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-11-0212>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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