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Original Article

TERT and BRAF promote proliferation of thyroid cancer cells

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Abstract: Objective: The aim of this study was to investigate the effects and mechanisms of BRAF (V600E) and TERT expression on proliferation and migration of thyroid cancer cells. Methods: BRAF (V600E) and TERT expression was detected by qRT-PCR in SW579 cell lines of thyroid cancer cells. Effects of BRAF (V600E) and TERT expression on proliferation and cell migration of thyroid cancer cells were detected. Immunoblotting was used to detect changes in levels of proteins. Results: After successful interference of BRAF siRNA and TERT siRNA, expression levels of BRAF and TERT mRNA in groups B (P=0.002) and C (P=0.009) were significantly decreased, compared to the control group. Moreover, expression levels of BRAF mRNA and TERT mRNA in group D were significantly lower than in group B (P=0.039) and group C (P=0.027). After 48 hours, BRAF siRNA and TERT siRNA significantly inhibited proliferation of SW579 thyroid cancer cells (P=0.032). In addition, cells in group D, transfected with two kinds of interference fragments at the same time, were affected more than those in groups B and C (P=0.022). Expression levels of RAS, phosphorylated MEK, and phosphorylated ERK were decreased (P=0.028) after interference with BRAF and TERT in thyroid cancer cells. Conclusion: BRAF (V600E) and TERT promote proliferation and migration of thyroid cancer cells.

Keywords: Thyroid cancer SW579 cells, BRAF (V600E), TERT, cell proliferation, cell migration

Introduction

Thyroid cancer is the most common malignancy of the endocrine system. It has developed into the fifth largest malignancy occurring in Chinese women, now at an annual growth rate of 6% and 8%. This growth rate is expected to continue rising for the next 10 years, worldwide [1].

BRAF (v-raf murine sarcoma viral oncogene homolog B1, BRAF) is a member of the tryptophan/serine kinases - RAF family [2]. Mutations in BRAF have been closely associated with papillary thyroid carcinoma (PTC) [3]. Seppala suggested that BRAF may promote proliferation and migration of thyroid cancer cells through targeted regulation of mitogen-activated protein signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways. However, specific mechanisms of action remain unclear. Telomerase reverse transcriptase (TERT) is the main limiting factor of telomerase activity [4, 5]. This mechanism may be one of the reasons why mutation of the TERT promoter contributes to occurrence of cancer. Although mutation of the TERT promoter has been proven to be closely associated with occurrence of thyroid cancer, there are no relevant studies to explain whether it enables it to further promote proliferation and migration of thyroid cells and functional mechanisms [6-8]. Since TERT promoter mutations have been reported to correlate with thyroid cancer, the roles of mutations along with TERT promoter mutations in thyroid cancer have received extensive attention [9]. The capabilities of mutations of BRAF (V600E), accompanied by TERT promoter mutations, to further promote occurrence of thyroid cancer have not been studied or reported [2].

This present study aimed to analyze the effects of BRAF (V600E) and TERT on proliferation and metastasis of thyroid cancer cells by transfecting BRAF siRNA and TERT siRNA interference fragments in SW579 thyroid cancer cells. This

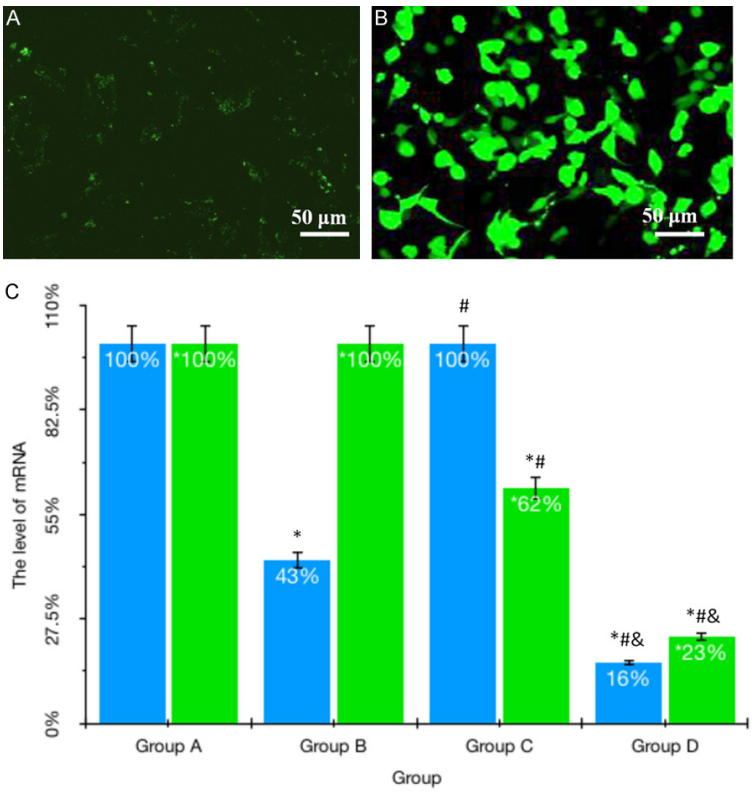

Figure 1. mRNA levels in the 4 groups after siRNA interference. A. Non-transfected cells under the fluorescence microscope. B. siRNA-transfected cells under the fluorescence microscope. C. Levels of BRAF mRNA (blue) and levels of TERT mRNA (green). *P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

Table 1. Levels of BRAF mRNA in 4 groups after siRNA interference

	Group A	Group B	Group C	Group D
BRAF mRNA	1	0.43*	1#	0.16*,#,&
TERT mRNA	1	1	0.62#,*	0.23*,#,$

*P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

study also explored individual and combined effects of BRAF (V600E) and TERT siRNA on expression of related proteins in MEK/ERK signaling pathways of thyroid cancer cells and relevant mechanisms affecting proliferation and migration, aiming to provide a theoretical basis for the novel treatment of thyroid cancer with BRAF as a bio-therapeutic target.

Materials and methods

Reagents

SW579 thyroid cancer cell line was purchased from ATCC Corporation, USA. Small interfering RNA (siRNA) sequences were synthesized by Invitrogen Corporation, USA, and labeled with cyanine 3 (cy3) fluorescence. Liposomes were purchased from the Invitrogen Corporation, USA. Dulbecco's Modified Eagle Medium (DMEM) powder, RPMI 1640 medium, and fetal bovine serum were purchased from Gibco, USA. CCK-8 cell proliferation assay kit, trypan blue, and paraformaldehyde were purchased from Sigma Corporation, USA. Matrigel was obtained from BD Bioscience, USA. Transwell chambers were obtained from Corning, USA. Anti-rat sarcoma protein (Rat Sarcoma, RAS; 1:400) monoclonal antibody was purchased from Santa Cruz, USA. Anti-BRAF monoclonal antibody (1:400) and anti-TERT monoclonal antibody (1:400) were purchased from Sigma. Anti-phospho-MEK monoclonal antibody (1:500), total MEK monoclonal antibody (1:500), anti-phospho-ERK monoclonal antibody (1:500), and total ERK monoclonal antibody (1:500) were purchased from Abcam, UK.

SiRNA transfection (preparation of thyroid cancer cells)

SW579 thyroid cancer cell lines were cultured and transfected with siRNA. Control group (group A) was transfected with the control interference segment. BRAF interference group (group B) was transfected with BRAF interference segment 1. TERT interference group (group C) was transfected with TERT interference segment 2. BRAF combined with TERT interference group (D Group), in which: Control sense siRNA: 5'-GCUAACGGCGCUGCCAGGU-3'; Control antisense siRNA: 5'-GCCCCUCAGCGCCCGCAGUC-3'; BRA F sense siRNA1: 5'-CGGGGACAUGGAGCCCGAG-3'; BRAF antisense siRNA1: 5'-CUCGGGCUCCAUGUCCCCG-3'. TERT

Table 2. Proliferation of SW579 cells after BRAF siRNA and TERT siRNA interference

	12 h	24 h	36 h	48 h
Group A	0.049	0.098	0.174	0.282
Group B	0.060	0.109	0.170	0.181*
Group C	0.032	0.102	0.160	0.182*
Group D	0.050	0.101	0.170	0.132*,#,&

*P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

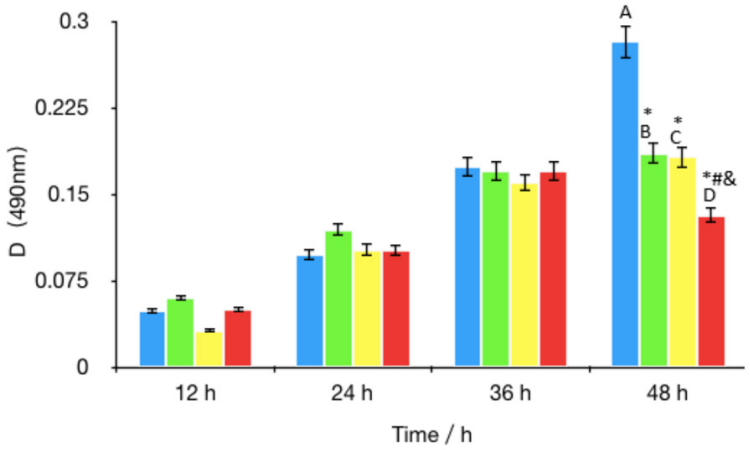

Figure 2. Proliferation of SW579 cells after siRNA interference. (A) Control group (B) Proliferation of SW579 cells after BRAF siRNA interference (C) Proliferation of SW579 cells after TERT siRNA interference (D) Proliferation of SW579 cells after BRAF siRNA and TERT siRNA interference. *P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

sense siRNA1: 5'-GAUGGCGGCGCUGAGCGGU-3'; TERT antisense siRNA1: 5'-ACCGCUCAGCGCCGCCAUC-3'; BRAF and TERT interference groups were simultaneously transfected with the above two interference fragments. The transfection procedure was as follows. Cells were inoculated and cultured overnight. SiRNA-liposome complexes were synthesized, added to each well of cells, and transfected for 48 hours. The effects of transfection were observed using fluorescence microscopy. Transfection rates were calculated and cells were collected for further experiments.

Detection of expression of BRAF mRNA and TERT mRNA in four groups by application of RT-PCR method

RT-PCR method was applied to detect expression of BRAF and TERT mRNA in 4 groups of successfully transfected cells. Total RNA was extracted, RNA concentration and purity were detected by UV spectrophotometry, and RNA integrity was detected by agarose gel electrophoresis. cDNA was synthesized by reverse transcription reaction using a kit purchased from Promega (USA). The cDNA template was obtained by reverse transcription, corresponding specific primers were designed, and PCR amplification was conducted using a 7900 fluorescence-quantitative PCR instrument. Finally, the PCR product was subjected to agarose gel electrophoresis. Primer 3.0 software was used to design target gene primers and β-actin (NM_001101.3) was applied as an internal reference. Sequence of primers was as follows:

BRAF sense strand: 5'-ACCACCCAATACCACAGGAA-3', BRAF antisense strand: 5'-CATTGGGAGCTGATGAGGAT-3'; TERT sense strand: 5'-ACCACCCAATACCACAGGAA-3', TERT antisense strand: 5'-CATTGGGAGCTGATGAGGAT-3'; beta-actin sense strand: 5'-AAACTGGAACGGTGAAGGTG-3', beta-actin antisense strand: 5'-AGAGAAGTGGGGTGGCTTTT-3'. The above sequences were sent to Shanghai Bio-engineering Company for synthesis. Expression of BRAF and TERT mRNA was detected by RT-PCR.

Detection of cell proliferation by colorimetric CCK-8 assay and trypan blue staining

Thyroid cancer cells transfected with BRAF interfering fragment 1, TERT interfering fragment 2, and both interfering fragments were trypsinized and re-inoculated into 96-well plates. After incubation for 12, 24, 36, and 48 hours at 37°C in 5% CO_2, 10 μL of CCK-8 reagent was added. After mixing, incubation was continued for 1 hour. Absorbance (A value) at 450 nm was measured using enzyme-linked immunosorbent assay and growth curves were drawn. Experiments were conducted in triplicate and live cells were directly counted after staining with trypan blue.

Table 3. Effects of siRNA interference on the migration ability of SW579 cells

	Group A	Group B	Group C	Group D
Migration cell number/field	193	84*	79*	58*,#,&

*P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

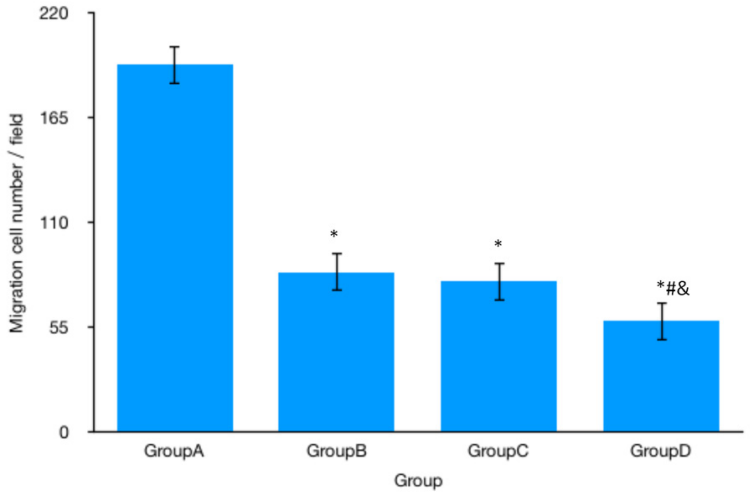

Figure 3. Proliferation of SW579 cells after BRAF siRNA and TERT siRNA interference. *P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

Detection of cell migration and invasion by Transwell assay

After 24 hours of transfection, each group of cells was trypsinized and resuspended in a serum-free medium. Cell density was adjusted to $2*10^8$/L. One hundred microliters of the cell suspension was added to the upper layer of a Transwell chamber. Also, 500 μL of culture medium containing 10% serum was added to the lower layer of the Transwell chamber for cultivation in an incubator at 37°C with 5% CO_2. After 24 hours of culturing, the chamber was washed with PBS. Next, non-migrated cells in the upper chamber were gently wiped off with a wet cotton bud and fixed for 20 minutes with 4% paraformaldehyde. After three washes with PBS, 0.1% crystal violet was used to stain cells for 10 minutes and 5 fields were randomly selected, after washing with PBS, to count the migrating cells under an optical microscope.

Detection of RAS, MEK, and ERK protein expression by Western blotting analysis

Successfully transfected cell lines were subjected to Western blotting analysis to detect changes in expression of proteins related to RAS, MEK, and ERK signaling. Successfully lysed and transfected cells were collected and centrifuged. Protein samples were extracted and subjected to electrophoresis. Membrane strips were cut to appropriate sizes, treated, and transferred to the gel. Next, the membranes were washed, treated with coating solution, washed, treated with primary antibody, washed, treated with horseradish peroxide-coupled secondary antibody, washed, and treated with a coloring solution. Color was developed by protection from light until the strips appeared. These strips were finally placed in double distilled water to stop the reaction.

Statistical analysis

SPSS 22.0 software was used for statistical analysis of data. Experimental results are expressed as $\bar{x} \pm SD$. Differences between the two groups were analyzed by t-test. Differences among multiple groups were analyzed by one-way ANOVA Values of $p<0.05$ are considered statistically significant.

Results

siRNA interferes with mRNA expression levels of BRAF and TERT

Two synthesized interfering fragments of BRAF siRNA and TERT siRNA were successfully transfected into three groups (B, C, and D groups) of SW579 thyroid cancer cells, with respective transfection rates of 90.21%, 91.11%, and 94.24%. Transfection of siRNA was observed by fluorescence microscopy (**Figure 1A** and **1B**).

After successful interference of BRAF siRNA and TERT siRNA, expression levels of BRAF and TERT mRNA in groups B (P=0.002) and C (P=0.009) were significantly decreased, compared to the control group. Moreover, expres-

Table 4. Comparison of expression of RSA, pMEK, tMEK, pERK, and tERK in 4 groups ($\bar{x}$ ± SD)

Group	RAS	pMEK	tMEK	pERK	tERK
Group A	0.236 ± 0.003	2.875 ± 0.821	5.202 ± 1.245	1.425 ± 0.418	3.123 ± 0.517
Group B	0.079 ± 0.003*	2.035 ± 0.781*	5.026 ± 0.972	0.582 ± 0.325*	3.417 ± 0.573
Group C	0.047 ± 0.002*	1.976 ± 0.627*	5.366 ± 1.025	0.671 ± 0.216*	3.252 ± 0.512
Group D	0.013 ± 0.001#,&	1.034 ± 0.871*,#,&	4.953 ± 1.016	0.316 ± 0.304*,#,&	3.240 ± 0.576

*P<0.05 vs group A, #P<0.05 vs group B, &P<0.05 vs group C.

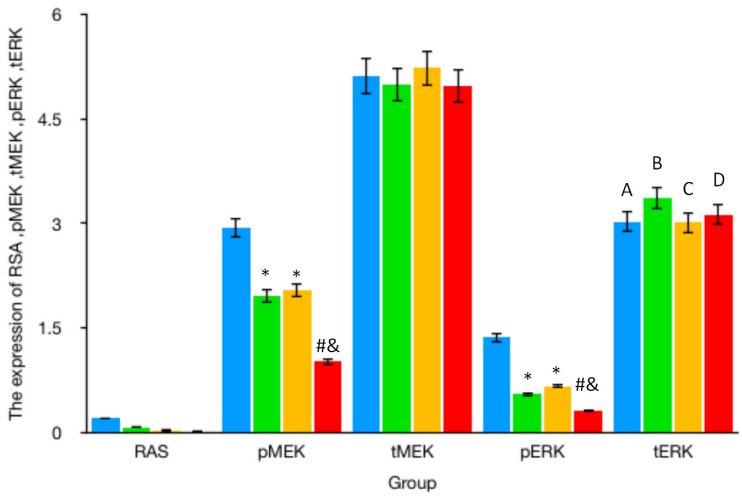

Figure 4. Comparison of expression of RSA, pMEK, tMEK, pERK, and tERK in 4 groups. (A) Control group (Blue). (B) Proliferation of SW579 cells after BRAF siRNA interference (Green). (C) Proliferation of SW579 cells after TERT siRNA interference (Yellow). (D) Proliferation of SW579 cells after BRAF siRNA and TERT siRNA interference (Red).

sion levels of BRAF mRNA and TERT mRNA in group D were significantly lower than in group B (P=0.039) and group C (P=0.027) (**Table 1**, **Figure 1C**).

Effects of silencing BRAF mRNA and TERT mRNA on proliferation of SW579 thyroid cancer cells

After transfection of SW579 thyroid cancer cells with BRAF siRNA and TERT siRNA, the effects of overexpressed BRAF siRNA and TERT siRNA2 and combination action of both on proliferation of SW579 thyroid cancer cells were detected by CCK-8 assay. Results illustrated that proliferative effects of BRAF siRNA and TERT siRNA on thyroid cancer cells had no significant differences compared to the control group (A group), suggesting that BRAF siRNA and TERT siRNA had no significant effects on proliferation of thyroid cells. After 48 hours, BRAF siRNA and TERT siRNA significantly inhibited proliferation of SW579 thyroid cancer cells (P=0.032). In addition, cells in group D, transfected with two kinds of interference fragments at the same time, were affected more than those in groups B and C (P=0.022) (**Table 2** and **Figure 2**).

Effects of low expression of BRAF mRNA and TERT mRNA on migration of SW579 thyroid cancer cells

After BRAF siRNA and TERT siRNA were transfected with SW579 cells of thyroid cancer, the Transwell method was used to detect effects of the combination of BRAF siRNA and TERT siRNA2 on migration of thyroid cancer SW579 cells. Results showed that transfection of BRAF siRNA (P=0.012) and TERT siRNA (P=0.023) could significantly inhibit migration of SW579 cells in thyroid cancer (**Table 3**, **Figure 3**).

Detection of expression of RAS, phosphorylated MEK, and phosphorylated ERK in 4 groups by Western blotting analysis

Expression of RAS, phosphorylated MEK, and phosphorylated ERK in the four groups was detected by Western blotting analysis. Results showed that expression levels of RAS, phosphorylated MEK, and phosphorylated ERK were decreased (P=0.028) after interference with BRAF and TERT in thyroid cancer cells. However, there were no significant differences in total MEK and ERK (**Table 4** and **Figure 4**).

Discussion

BRAF is a specific kinase of encoding silk/threonine, an important transduction factor in MEK/ERK pathways closely related to cell

growth, differentiation, and apoptosis [3]. Berres found, in a study of thyroid cancer with high BRAF (V600E) expression was closely correlated with incidence of thyroid cancer. In thyroid papillary carcinoma, BRAF (V600E) mutation rates are as high as 57% [10-13] Multitarget kinase inhibitor bay43-9006 can significantly inhibit increase in values of BRAF (V600E) gene mutation thyroid cancer [14]. Sanchez-Torres and Steinberg reported that high expression of BRAF (V600E) may promote proliferation and migration of cancer cells and that its mechanism may be related to inactivation of MEK/ERK signaling pathways caused by BRAF (V600E) overexpression [2, 14, 15]. The present study demonstrated that when BRAF protein expression was downregulated, after BRAF interference, expression of certain proteins such as RAS, phosphorylated MEK, and phosphorylated ERK in MEK/ERK signaling pathways was also downregulated. More importantly, downregulation of BRAF proteins may inhibit proliferation and migration of thyroid cancer cells. This suggests that BRAF interference may inhibit growth, proliferation, and migration of SW579 cell lines by inactivating MEK/ERK signaling pathways, consistent with the findings of Sanchez-Torres. Taken together, these findings confirm that high expression of BRAF (V600E) works to inhibit MEK/ERK signaling pathways, promoting proliferation and migration of thyroid cancer cells.

Liu and Landa I et al. proposed a TERT promoter mutation in thyroid carcinoma. [16, 17]. Masutomi et al. discovered that when they used mutant TERT with functional deficiency (DN-hTERT) and shRNA specific to hTERT for hTERT inhibition of the human fibroblasts BJ and WI-38 that express TERT, TERT gene expression in mouse tumor cells caused by RNA interference was inhibited. This resulted in decreased telomerase activity, telomere DNA synthesis disorders, and reduced tumor cell proliferation [18-20]. The present study interfered with expression of TERT in a SW579 thyroid cancer cell line to detect differences in cell proliferation, migration, and related protein expression. Results of this study are consistent with Masutomi's results. After TERT interference, TERT protein expression was downregulated to inhibit proliferation and cell migration of thyroid cancer cells.

With further study on the correlation between TERT promoter mutation and thyroid cancer, the roles of BRAF V600E mutation with TERT promoter mutation in thyroid cancer have gradually attracted the attention of clinicians. Qasem believed that TERT promoter mutation was more common in tumors with BRAF V600E mutations [21, 22]. Gandolfi et al. believed that BRAF V600E and TERT promoter mutations are only related to PTC with distal metastasis [23]. Lee's investigation into the common Korean thyroid cancer population with BRAF V600E mutation found that incidence of BRAF V600E and TERT promoter mutation in 242 patients was 13% [24, 25]. It can be seen that BRAF V600E and TERT promoter joint mutations have certain synergistic effects. However, Landa et al. found that BRAF V600E was negatively correlated with TERT promoter mutations [17].

In summary, the mechanisms may be that TERT promoter mutations (C228T, C250T) form a new binding site for an ETS transcription factor, BRAF (V600E) mutations activate MAPK signaling pathways and upregulate expression of ETS, and ETS transcription factors can be combined with the new site generated due to the TERT promoter mutation for upregulation of TERT expression. Consequently, high expression of TERT promotes the progression of thyroid cancer.

Disclosure of conflict of interest

None.

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