PRDX1 and PRDX6 are repressed in papillary thyroid carcinomas via BRAF V600E-dependent and -independent mechanisms

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Received September 18, 2013; Accepted November 6, 2013

DOI: 10.3892/ijo.2013.2208

Abstract. Many clinical studies highlight the dichotomous role of PRDXs in human cancers, where they can exhibit strong tumor-suppressive or tumor-promoting functions. Recent evidence suggests that lower expression of PRDXs correlates with cancer progression in colorectal cancer (CRC) or in esophageal squamous carcinoma. In the thyroid, increased levels of PRDX1 has been described in follicular adenomas and carcinomas, as well as in thyroiditis, while reduced levels of PRDX6 has been found in follicular adenoma. We studied the expression of PRDX1 and PRDX6, in a series of thyroid tissue samples, covering different thyroid diseases, including 13 papillary thyroid carcinomas (PTCs). Our results show that PRDX1 and PRDX6 are significantly reduced in all PTCs compared to normal tissues, to non-neoplastic tissue (MNG) or follicular neoplasms. This reduction is strongly evident in PTCs harboring BRAF V600E (31% of our cases). Using TPC-1 and BCPAP and FRTL-5 cell lines, we demonstrate for the first time that the presence of BRAF V600E is responsible of the hypoxia-expression of PRDX1 and PRDX6 both at mRNA and protein levels. Finally, independently of BRAF status, we observe an interesting correlation between the tumor size, the presence of lymph node metastasis and the lowest PRDX1 and PRDX6 levels. Therefore, these data indicate that PRDX1 and PRDX6 expression not only may play a key role in papillary thyroid carcinogenesis via a BRAF V600E-dependent mechanism, but their determination could be considered as potential tumor marker for indicating tumor progression in PTCs, independently of BRAF status.

Introduction

In recent years, several studies have linked oxidative stress (OS) to thyroid cancer (1-3). The thyroid gland itself generates reactive radical molecules, through the process of iodine metabolism and thyroid hormone synthesis. During this process, TSH stimulates H₂O₂ production, which is the substrate of thyroperoxidase (TPO) on the apical membrane of the thyroid follicular cells (4). Therefore, thyrocytes need protective mechanisms that limit the oxidative damage of H₂O₂ production by catalase, glutathione peroxidases and peroxiredoxins (PRDXs) (4,5). PRDX proteins belong to a highly conserved family of six proteins. All of the PRDXs contain a conserved cysteine residue in the N-terminal region, that is the active site that catalyzes peroxide reduction of H₂O₂ (6). These proteins can be classified in 2-cysteine (2-Cys) PRDXs including PRDX1-5, and 1-Cys PRDX including PRDX6. Together with their cytoprotective antioxidant function, PRDXs play a role in cell proliferation, differentiation, immune response, protection of oxidation-sensitive proteins, regulation of cellular H₂O₂, control of apoptosis and redox signaling (7). PRDX1 is the most abundant and ubiquitously distributed member of the mammalian PRX family, identified in a large variety of organisms, while PRDX6 is the only 1-Cys PRDX that has glutathione peroxidase and phospholipase A2 (PLA2) activities (8). Their function has been mainly related to regulating cell proliferation, differentiation, and apoptosis (9,10). Data obtained in cells and in mice lacking PRDX1 indicate that it exerts a strong tumor suppressor function (11), interacting with c-Myc and selectively inhibiting its transcriptional activity (12). On the other hand, because of their antioxidant function, PRDXs may support tumor survival and maintenance, by protecting tumor cells from oxidative stress-induced apoptosis (13). Many clinical studies highlight this dichotomous role of PRDXs in various kinds of human cancers. Increased or reduced level of PRDX1 was described in lung and breast cancer (14-17), as well as in aggressive esophageal squamous carcinomas (18). Aberrant expression of PRDX6 has been reported in a variety of tumors such as malignant mesothelioma (19), esophageal carcinoma (20), oligodendroglioma (21), breast cancer (22) and squamous cell carcinomas (23).
Studies on the role of PRDXs in thyroid diseases demonstrated modification in the expression of these proteins mainly in follicular neoplasms. Increased level of PRDX1 has been described in follicular adenomas and carcinomas, as well as in thyroiditis (24). Recently, thyroid proteomic approach highlighted a reduction of PRDX6 in follicular adenomas (25), suggesting a possible role for this protein as complementary marker to distinguish between different follicular neoplasms. Based upon this knowledge, we studied the expression of two representative members of PRDXs family, PRDX1 and PRDX6, in a group of 32 thyroid tissue samples covering different thyroid diseases, including 13 papillary thyroid carcinomas (PTCs). PTC accounts for 80-90% of all thyroid cancers (26,27). Although the majority of these cancers, treated with surgical thyroidectomy, followed by radiodiablation in many patients, have an excellent prognosis, there are 20-30% of cases that show recurrence at 15-20 years (28,29). At least 45% of PTCs with worst prognosis harbor the oncogenic BRAF V600E mutation, which leads to a constitutive activation of the RAF/MEK/ERK pathway. PTCs with this BRAF mutation behave more aggressively than wild-type BRAF (WT) tumors, and their clinical outcome correlates to extrathyroid extension, positive nodal status, disease recurrences, and mortality (30-32).

Our data show that PRDX1 and PRDX6 levels are strongly reduced in all PTCs compared not only to normal tissue (NT), but also to non-neoplastic tissue or follicular neoplasms (FA and FC). We demonstrate for the first time that PRDXs reduction correlates with the presence of BRAF V600E mutation both in human samples and in papillary thyroid carcinoma cellular model. The significant reduction of PRDX1 and PRDX6 obtained in FRTL-5 cells, transiently transfected with of BRAF V600E, allowed us to state that these molecules are a new target of BRAF V600E. Finally, independently of BRAF status, we observed an interesting correlation between tumor size, the presence of lymph node metastasis and the lowest PRDX1 and PRDX6 levels. Therefore, these data indicate that PRDX1 and PRDX6 reduction may play a key role in pathogenesis of papillary thyroid cancer and they could be considered as potential tumor markers for indicating tumor progression in PTCs.

**Materials and methods**

**Tumor samples.** Thirty-two tissue samples of different histological subtype, collected between 2005 and 2011 in the Department of Experimental and Clinical Sciences University ‘G. d’Annunzio’ Chieti-Pescara, frozen immediately in liquid nitrogen and stored at -80°C, were analyzed by qRT-PCR to evaluate the expression levels of PRDX1 and PRDX6 genes. In all cases, the contralateral normal thyroid tissue was simultaneously processed in a similar manner. Sample collection includes: 13 cases of PTC (10 females, 3 males; median age 52.85±15.10 years; median ± SD), 13 follicular adenomas (FA) (10 females, 3 males; median age 44.92±17.33 years), 3 multinodular goiter (MNG) (3 females; median age 44±8.19 years), and 3 follicular carcinomas (FC) (2 females, 1 male; median age 40.33±22.90 years). The clinical and histological features, including the TNM staging, are shown in Table I. Samples of normal thyroid tissue were obtained from healthy contralateral lobe of each of patient.

### Table I. Clinical and histopathological features of the study population.

<table>
<thead>
<tr>
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<th>Benign nodule (n=16)</th>
<th>Malignant nodule (n=16)</th>
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<tr>
<td></td>
<td>Multinodular goiter (MNG)</td>
<td>Follicular adenoma (FA)</td>
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<tr>
<td>No. of patients</td>
<td>3</td>
<td>13</td>
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<tr>
<td>Gender</td>
<td>Female/male</td>
<td>Female/male</td>
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<tr>
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<td>3/0</td>
<td>10/3</td>
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<tr>
<td>Median age</td>
<td>44±8.19</td>
<td>44.92±17.33</td>
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|                      | Papillary thyroid carcinoma (PTC) | Follicular carcinoma (FC) |
| No. of patients      | 13                      | 3                        |
| Gender               | Female/male             | Female/male              |
|                      | 10/3                    | 2/1                      |
| Median age           | 52.85±15.10             | 40.33±22.90              |
| TNM no. (%)          | T₁N₁M₀                  | T₂N₁M₀                   |
|                      | 4 (30.8)                | 3 (100)                  |
|                      | T₂N₂M₀                  | T₃N₂M₀                   |
|                      | 3 (23.1)                | 2 (15.4)                 |
|                      | T₃N₃M₀                  | T₄N₄M₀                   |
|                      | 2 (15.4)                | 1 (10)                   |

For the IHC study, the same formalin-fixed, paraffin-embedded cases were analyzed to evaluate the expression levels of PRDX1 and PRDX6 proteins. Informed consent from individual patients was not necessary because all data were made anonymous. This investigation was performed according to the guidelines of the Ethics Committee of the University Hospital Essen and in accordance with the precepts established by the Helsinki declaration. Histopathology evaluation and TNM classification were performed by two expert thyroid pathologists (R.C. and C.D.G.).

**Immunohistochemistry.** Immunohistochemistry was performed, as described in Lazzareschi et al (33), on formalin-fixed, paraffin-embedded tissue sections of thyroid tumors from 13 patients. Consecutive histological sections (2 μm), mounted on slides coated with a suitable tissue adhesive, were deparafinized in xylene and rehydrated through graded alcohol series. Endogenous peroxidase was blocked by 3% hydrogen peroxide/methanol for 10 min. Sections were placed in blocking serum (UCS Diagnostic, Morlupo, Rome, Italy) for 10 min and then incubated, at room temperature for 1 h, with primary antibody: rabbit polyclonal anti-human PRDX1 (1:400) or mouse monoclonal anti-human PRDX6 (1:400) (Abcam, Cambridge, MA, USA). After incubation, tissue sections were washed with PBS buffer and incubated with the appropriate biotinylated secondary antibody for 15 min at room temperature and then with streptavidin/peroxidase complex (UltraTek HRP Ready-
to-use, UCS Diagnostic) for 15 min at room temperature. Tissues were then developed in 3',3'-diaminobenzidine (DAB, Dako, CA, USA), resulting in a brown reaction product. The slides were subsequently counterstained with hematoxylin.

Thyroid tissue with histological diagnosis of Graves' disease were used as positive control. Negative controls were obtained omitting the primary antibodies. Two investigators examined the slides in a blinded manner by a Leica microscope (Leitz Camera, Wetzlar, Germany) using normal light. The evaluations of the two pathologists were 100% concordant.

**RNA isolation and analysis.** Total RNA from tissue and cells was extracted using TRI Reagent (Sigma-Aldrich, Co.), following the manufacturer's instructions. Using 1 µg RNA, cDNAs were synthesized using MuLV reverse transcriptase (Applied Biosystems, Hammonton, NJ, USA) and random primers (Roche) according to the manufacturer's instructions. Real-time reverse transcription (RT)-PCR was performed three times in six replicates on ABI PRISM 7900HT (Applied Biosystems, Foster City, CA), using the SYBR Green detection system. mRNA levels were compared with reference curves and normalized to 18S ribosomal RNA. Each experiment was repeated three times using different total RNA extracts. The primers used in qRT-PCR and relative parameters are reported in Table II.

For analysis of the BRAF gene mutation, exon 15 of BRAF was amplified using the polymerase chain reaction (PCR) with the following primers: BRAF h F(1): 5'-CGA CAG ACT GCA CAG GGC A-3' and BRAF h R(1): 5'-GCC ATC CAC AAA ATG GAT CCA-3' (62˚C; amplicon of 191 bp).

Amplification products were analyzed using a 2% agarose gel and purified with ExoSap-IT (USB Corporation). Nucleotide sequence analysis was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The purified PCR products were sequenced in both directions using BRAF h F(1) and BRAF h R(1) primers. Cycle sequencing products were analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

**Cell cultures and transient transfection analysis.** For the present study, FRTL-5 cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. They were designated as ATCC CRL 8305 and described as passage 14. On arrival, they were divided into aliquots and frozen in liquid nitrogen until required.

FRTL-5 cell line, diploid between their 5th and 25th passage, maintains the functional characteristics of iodide uptake, thyroglobulin synthesis, and cyclic nucleotide metabolism over prolonged periods of culture and doubling time of 36 h (34). The cells were grown as previously described (35) at 37˚C in a humidified atmosphere of 5% CO₂ air in w/o supplemented with 5% calf serum and 6H mixture. FRTL-5 were transiently transfected with pBABE BRAF V600E plasmid (kindly provided by Professor G. Canettieri) using lipofectin technique following the manufacturer's instructions. After 48 h of incubation, transfected cells were used in qRT-PCR and western blot assays.

The human PTC cell lines, TPC-1 (BRAF WT/WT) and BCPAP (BRAF V600E/V600E), characterized according to Schweppes et al (36), were maintained in a 5% CO₂ culture humidified atmosphere, at 37°C in DMEM supplemented with 10% calf serum (37).
Protein extraction and immunoblotting. Protein extracts from subconfluent cells, transfected or not, were obtained using ice-cold TNE extraction buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with 1 mM PMSF, protease and phosphatase cocktail inhibitors. Protein lysates (50 µg) were subjected to immunoblotting as previously described (38), using primary antibodies to PRDX1 (1:1000) (Abcam), PRDX6 (1:1000) (Abcam), CREB (Cell Signaling Technology, Beverly, MA, USA) (1:1000).

Then the membranes were incubated with anti-rabbit (1:50000) or anti-mouse (1:10000) HRP-conjugated secondary antibodies (Sigma-Aldrich Co.). The western blots were revealed by chemiluminescence using the Super Signal Kit from Pierce (Rockford, IL, USA), according to the manufacturer’s instructions and visualized on CL-Xposure film (Pierce).

PRDX1 and PRDX6 bands were analyzed using Bio-Rad Laboratories software. Data, obtained from three different protein extracts, were collected in terms of average intensity of bands of each proteins per average intensity of bands of CREB.

Statistical analysis. All statistical analyses have been performed by JMP Software, purchased from Statistical Discovery SAS Institute (Cary, NC, USA). Data were analyzed by Student’s t-test (P<0.05, statistical significance; P<0.001, high statistical significance; P<0.0001, very high statistical significance).

Results

Peroxiredoxin 1 and peroxiredoxin 6 are reduced in PTCs. The expression of PRDX1 and PRDX6 was evaluated by qRT-PCR in a series of 32 snap-frozen thyroid samples (Table I), including 13 PTC, 3 FC, 13 FA, 3 MNG and, in all cases, in normal thyroid tissue samples from the contralateral lobe. All examined tissues showed detectable levels of PRDX1 and PRDX6. The comparative analyses of the groups demonstrated higher value of PRDX1 in follicular neoplasm FA and in FC as compared to normal tissue (~2- and 1.3-fold respectively) or to non-neoplastic tissue MNG (~1.7- and 1-fold respectively). The PRDX6 levels are moderately decreased in FA and in FC compared to NT (~2.5- and 4-fold respectively) and to MNG (~1.4- and 2.4-fold respectively).

The most interesting results were obtained in PTC group, where the PRDX1 expression was reduced with statistical significance (P<0.05) compared to normal tissue (14-fold) and FA (26-fold). The strong reduction was evident also comparing PTCs to non-neoplastic tissue MNG (16-fold) and to FC (18-fold) (Fig. 1A). PRDX6 expression showed similar behavior, with a statistically significant (P<0.05) reduction, compared to contralateral normal tissue, and consistent reduction with respect to MNG (12-fold) and to follicular neoplasms (9- and 5-fold respectively) (Fig. 1A).
cases of the same PTC were studied by immunohistochemistry, using specific antibodies against PRDX1 and PRDX6. As shown in Fig. 1B, a weak intensity of PRDX1 and PRDX6 staining was present in all PTCs compared to non-neoplastic thyroid, substantially confirming the PCR (qRT-PCR) data. Tissues from thyroid Graves’ disease were used as positive control (Fig. 1C).

Given the above findings, our study focused on the PTC group. In order to verify the possible correlation between PRDXs reduction and clinical behavior of PTC, we analyzed the data according to the TNM classification. As shown in Fig. 2 the lower levels of PRDXs were detected in PTC at stage T1N1 (5-fold less than in T1N0) and T2N1 (~2-fold less than in T2N0) for PRDX1. We observed a similar trend towards PRDX6, the reduction was 18-fold in T1N1 vs T1N0 and 3-fold in T2N1 compared to T2N0 groups. Finally, these analyses also suggested that PRDX reduction could correlate with the tumor size, T2N0 ~3- and 2-fold less than T1N0, respectively for PRDX1 and PRDX6 (Fig. 2).

PRDX1 and PRDX6 expression level correlates with BRAF status and with nodal metastases. According to the literature (31), the sequencing of BRAF exon 15, performed in all PTC samples and in contralateral healthy tissue, demonstrated that BRAF V600E mutation was present in 31% (4 out of 13) of tumors. In our data, the PRDX1 and PRDX6 reduction was strongly pronounced (about 4- and 3-fold, respectively) in all BRAF V600E mutated with respect to non-mutated samples (Fig. 3A). These results were also confirmed by immunohis-

![Figure 2. Correlation between PRDX1 and PRDX6 mRNA levels and TNM classification in PTCs.](image)

![Figure 3. Correlation between PRDX1 and PRDX6 levels and BRAF status in PTC. (A) Relative gene expression levels of PRDX1 and PRDX6 normalized to 18S RNA in PTCs WT or PTCs BRAF V600E mutated. Values represent mean of triplicate determination ± SD of three experiments. (B) Representative PRDX1 and PRDX6 immunohistochemistry (original magnification, x400) of PTCs WT (left) and BRAF V600E mutated (right). The evaluation of the two pathologists was 100% concordant.](image)
tochemistry, comparing the immunostaining of PRDX1 or PRDX6 of BRAF V600E mutated to non-mutated formalin-fixed, paraffin-embedded samples (Fig. 3B).

Since we observed that the presence of nodal metastases correlated to the lowest PRDX levels (Fig. 2), we investigated whether the reduction could be dependent or independent by the BRAF status. We analyzed the PTC data, considering the subgroups according to the presence of BRAF V600E mutation and TNM. Interestingly, by means of these analyses we observed that, independently of BRAF status, the presence of nodal metastases correlated to the reduction of PRDX1, about 7 times in T1N1 vs T1N0 and about 2 times in T2N1 vs T2N0, as well as of PRDX6, about 12 times in T1N1 vs T1N0, and about 5 times in T2N1 vs T2N0 (Fig. 4).

**PRDX1 and PRDX6 are targets of BRAF V600E.** To demonstrate that BRAF V600E might act as a repressor of PRDXs expression in PTCs, we used TPC-1 and BCPAP cells. These cell lines represent a model of human papillary thyroid carcinomas characterized, respectively, by the presence of the RET/PTC rearrangement and BRAF V600E mutation (36,39). TPC-1 and BCPAP were analyzed by qRT-PCR and western blot analysis. The data shown in Fig. 5A, clearly demonstrated that PRDX1 and PRDX6 transcripts were strongly reduced in BCPAP compared to TPC-1. Such a reduction was 31-fold (P<0.001) for PRDX1 and 22-fold (P<0.05) for PRDX6, respectively. Western blot analysis performed on total lysates of TPC-1 and BCPAP, further confirmed the strong and statistically significant reduction of PRDX1 and PRDX6 proteins in cells harboring BRAF V600E mutation (Fig. 5B). Thus, to test whether PRDX1 and PRDX6 were targets of BRAF V600E mutation, transient transfections of expression vector pBABE BRAF V600E were performed in FRTL-5. The expression of PRDXs, evaluated by qRT-PCR, revealed a statistically significant (P<0.05) reduction of both PRDX1 and PRDX6 in BRAF V600E transfected cells compared to untransfected (Fig. 6A). Similarly, western blot analysis performed on total lysates of FRTL-5 and FRTL-5 BRAF V600E, confirmed the statistically significant (P<0.05) decrease of both PRDXs in transfected cells (Fig. 6B).

**Discussion**

The thyroid cells are protected from reactive oxygen species (ROS), by means of catalase, glutathione peroxidase and peroxiredoxines production (40). TSH induces PRDX2 expression in FRTL-5 cells, indicating that the increase of the peroxidase-mediated protection is required during TSH-dependent hormogenesis (5). Low iodine content diets may stimulate the expression of PRDX3 and PRDX5 (41); moreover, an increase of PRDX5 was found in Basedow (42). It seems that the increase of ROS, as a consequence of imbalanced intracellular redox systems, is an important event in thyroid cancerogenesis (2,43). A recent study of oxidative status, performed on serum of patients with thyroid cancer, demonstrates an increase of oxidants and a decrease of antioxidants (44), especially evident in papillary thyroid carcinomas (3). Therefore, the alteration in
Serine/threonine kinase. This mutation occurs in approximately 45% of PTCs and it is strongly associated with a poor clinic pathological outcome of PTC, including aggressive pathological features, increased recurrence, loss of radioiodine avidity and treatment failures (32,45-48). In agreement with these reports, we found BRAF V600E mutation in 31% of PTC. All mutated samples, compared to WT, show a strong reduction of PRDX1 and PRDX6 expression levels, both at mRNA and protein level. To date, a specific mechanism governing the modulation of PRDXs in human cancer has not yet been described; instead, several molecular modifications have been observed associated to BRAF mutation, including the mutation-promoted overexpression of tumor-promoting molecules (49,50), suppression of tumor-suppressor genes (51,52), and silencing of iodide-handling genes with impaired radioiodine avidity in PTC (53,54). In our study, we demonstrate for the first time, that the presence of BRAF V600E in thyroid cells correlates with a reduction of PRDX1 and PRDX6 levels. BCPAP cell line, which presents BRAF V600E mutation, shows a statistically significant reduction of PRDXs mRNA and proteins compared to TPC-1 cell, characterized by the presence of RET/PTC rearrangement. When BRAF V600E is expressed in FRTL-5 cells, mRNA and protein expression of PRDX1 and PRDX6 is strongly reduced. These findings let us conclude that PRDXs represent another important target of BRAF V600E mutation, that could contribute to more aggressive clinical outcome of carcinoma, reducing the protection from oxidative stress.

Many clinical studies highlight the dichotomous role of PRDX members, depending on the tumor type and on the stage of tumor progression. Lower expression of PRDX2 correlates in CRC with advanced clinical stage, poorer tumor differentiation, distant metastasis and poor survival (55). PRDX1 appears to be upregulated in lung tumors (56), while it is downregulated in esophageal squamous carcinomas, where its reduction correlates with a cancer progression (18). Interestingly, we observed that the strong reduction of PRDXs correlates also with lymph node metastasis. Independently of BRAF status, lower levels of PRDX1 and PRDX6 are observable in PTC at stage T_{1N_1} and T_{2N_2}. Thus, these preliminary data suggest that the determination of PRDX1 and PRDX6 could be of relevance for monitoring PTC clinical behavior.

In conclusion, our study demonstrates that the PRDXs reduction is a feature of PTCs. Their expression is strongly modulated by BRAF V600E, as shown in human PTCs and in cellular model. PRDX1 and PRDX6 reduction correlates with the stage of the diseases, independently of BRAF status. Our findings provide strong evidence that PRDX1 and PRDX6 are targets of BRAF V600E and that their reduction in PTCs contributes to more aggressive clinical outcome of PTCs, reducing the protection from oxidative stress. Finally, although based on a small number of cases, we think PRDXs could be considered as potential tumor markers for indicating tumor progression and lymph nodal metastases in PTCs.

**Acknowledgements**

We thank Dr Graziano De Luca (Unit of Pathology and Molecular Medicine, Department of Medicine and Science of Aging, Hospital ‘SS. Annunziata’ Chieti) and Dr Chiara Tarantelli, for collection, storage and management of Biobank samples. The authors would also like to thank Mr. Daniel Kenton for the

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**Figure 6.** Effect of BRAF V600E transfection on PRDX1 and PRDX6 expression in FRTL-5 cells. (A) The expression of PRDX1 and PRDX6 was evaluated by qRT-PCR analysis on mRNA obtained from FRTL-5 untransfected or transiently transfected with pBABE BRAF V600E plasmid. Relative gene expression levels were normalized to 18S RNA. *Statistical significance (Student's t-test, P<0.05) of FRTL-5 transfected vs untransfected cells. (B) Whole protein lysates (50 µg/lane) from FRTL-5 transfected or not with pBABE BRAF V600E plasmid were analyzed by western blot analysis using an antibody against PRDX1 or PRDX6. Densitometric evaluation of the PRDX signals was performed normalizing to the levels of CREB. *Statistical significance (Student's t-test, P<0.05) of FRTL-5 transfected vs untransfected cells.
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