TRAF2 is a Valuable Prognostic Biomarker in Patients with Prostate Cancer

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Background: TRAF2 exerts important functions in regulating the development and progression of cancer. The aim of this study is to investigate whether TRAF2 is a valuable prognostic biomarker and to determine if it regulates TRAIL-induced apoptosis in prostate cancer.

Material/Methods: Microarray gene expression data from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases were used to determine TRAF2 expression in prostate cancer. TRAF2 expression in prostate cancer was further investigated by immunohistochemistry assay. Kaplan–Meier curves and log-rank test were used to assess the recurrence-free rate. Cox regression was used to analyze prognostic factors. Effects of TRAF2 on regulating TRAIL-induced apoptosis in DU-145 cells were further investigated.

Results: We found that TRAF2 was significantly upregulated in prostate cancer compared with normal prostate samples (P<0.001). In addition, compared with primary prostate cancer, TRAF2 was upregulated in metastatic prostate cancer (P=0.006). Furthermore, our results showed that high expression of TRAF2 was significantly associated with tumor stage of prostate cancer (P=0.035). TRAF2 high expression was associated with poorer recurrence-free survival in prostate cancer patients (P=0.013). TRAF2 was found to be a valuable independent prognostic factor for predicting recurrence-free survival (P=0.026). In addition, the present results indicate that TRAF2 affects TRAIL-induced apoptosis in prostate cancer DU-145 cells via regulating cleaved Caspase-8 and c-Flip expression.

Conclusions: TRAF2 could be a novel prognostic biomarker for predicting recurrence-free survival in patients with prostate cancer, which might be associated with the effects of TRAF2 in regulating TRAIL-induced apoptosis in prostate cancer cells via c-Flip/Caspase-8 signalling.

MeSH Keywords: Biological Markers • Clinical Medicine • Disease Progression • Prostatic Neoplasms • Urologic Neoplasms

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Prostate cancer is thought to be one of the leading causes of death among older men. It was documented that about 220,800 new cases and 27,540 deaths were reported in the United States [1,2]. In Asia, the incidence rate of prostate cancer has also been increasing [3]. Patients with localized prostate cancer could undergo initial surgical, radiological therapy, or subsequent treatment of androgen deprivation therapy (ADT). However, among some patients, the disease eventually becomes metastatic and resistant to treatment, which is known as lethal castration-resistant prostate cancer (CRPC). Castration-resistant phenotype influences about 20% of patients within 5 years. Currently, the molecular mechanisms governing the development and progression of prostate cancer are not well understood.

Immune surveillance could affect tumor development and progression [4–7]. One of these functions is to lead to apoptosis of cancer cells, which could be regulated by members of the tumor necrosis factor (TNF) and TNF receptor (TNF-R) superfamily. It was reported that TNF-related apoptosis-inducing ligand (TRAIL) is an important member of the TNF superfamily, and is responsible for extrinsic induction of apoptosis. It was suggested that multiple cell types in the immune system could secrete TRAIL, including natural killer (NK) cells, dendritic cells, and macrophages [5]. It is well documented that TRAIL-mediated immune surveillance can influence tumor development and progression [5].

Apoptosis, also known as programmed cell death, has been shown to be related to changes in cell morphology, including cell shrinkage, chromatin condensation, and nuclear fragmentation. Both extrinsic and intrinsic apoptosis pathways were extensively studied in past years. The intrinsic apoptosis pathway is activated by extracellular signals, mainly depending on the release of proteins from mitochondria. The extrinsic apoptosis pathway is activated by extracellular ligand binding to death receptor, which causes the formation of the death-inducing signalling complex (DISC). Exogenous TRAIL is shown to induce cancer cell death without killing normal cells in humans [5], which makes TRAIL a potential anticancer agent [8]. TRAIL can bind to death receptor 4 (DR4) or 5 (DR5), which subsequently induces activation of Caspase-8 and initiates extrinsic apoptosis [9–11]. Sedger et al. [12] reported that endogenous TRAIL can suppress tumor growth in vivo. Endogenous TRAIL secreted by NK cells can effectively kill cancer cells in the liver after implantation of breast and renal cancer cells. Other studies also showed that TRAIL has vital roles in regulating tumor development and metastasis [13–16]. It was reported that cellular FLICE-inhibitory protein (c-FLIP), as a protease-dead Caspase-8 homologue, can suppress the activation of Caspase-8 and thereby inhibit Caspase-8-mediated apoptosis [17,18]. It has been well documented that c-FLIP/Caspase-8 signalling is important in regulating activation of extrinsic apoptosis pathways [19]. The difference in the extent of Caspase-8 activation might be associated with the cell-to-cell variation in TRAIL responsiveness [20,21]. In addition, various molecules have been found to regulate TRAIL-induced apoptosis in tumor cells [22].

The tumor necrosis factor receptor (TNF-R)-associated factor 2 (TRAF2), also known as a signalling adaptor, belongs to the TRAF superfamily, which consists of TRAF1 to 7 in mammals. One of these functions is to serve as an adaptor protein in the assembly of receptor-associated signalling complexes and to link upstream receptors to downstream molecules [23]. TRAF2 is an important member of these TRAFs. Zhang et al. [24] found that TRAF2 expression was significantly increased in gastric cancer patients, which might be an independent diagnostic and prognostic indicator in gastric cancer patients. In addition, TRAF2 expression was upregulated in malignant pleural effusion cells compared with normal breast tissues [25]. TRAF2 in malignant pleural effusion cells might be a potential prognostic factor and biomarker for predicting invasion and metastasis of breast cancer [25]. Currently, TRAF2 expression in prostate cancer and mechanisms governing the development and progression of prostate cancer are still unknown. We speculate that TRAF2 may affect growth of prostate cancer cells via regulating TRAIL-induced apoptosis.

Different androgen-insensitive prostate cancer cell lines were used for investigating the mechanisms governing tumor development and progression, including DU-145 and PC-3, which were from metastatic prostate cancer lesions of brain and bone, respectively [26]. Because they originate from distinct organs, the tumor microenvironments and characteristics of these cells were different. DU-145 cells are androgen-insensitive and also show PSA (prostate-specific antigen)-positive expression [27]. Hence, the characteristics of DU-145 cells are typical of difficult-to-treat lesions of prostate cancer [28].

In this study, our results showed that TRAF2 was significantly upregulated in prostate cancer. Upregulation of TRAF2 was also observed in metastatic prostate cancer compared with primary prostate cancer. In addition, TRAF2 was found to be an independent prognostic factor for predicting recurrence-free survival in patients with prostate cancer, which might be associated with the effects of TRAF2 on regulating TRAIL-induced apoptosis in prostate cancer cells via c-FLIP/Caspase-8 signalling.

Material and Methods

Cell culture, reagents, and antibodies

Human prostate cancer cell line DU-145 was obtained from American Type Culture Collection (ATCC). DU-145 cells were

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cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO\textsubscript{2} atmosphere.

Recombinant human TRAIL (BK0176) was purchased from Bioworld Technology (Minneapolis, USA). Polyclonal rabbit anti-GAPDH (AP0063), TRAF2 (AP0698), c-FLIP (BS2545), Caspase-3 (BS1518), cleaved Caspase-3 (BS7004), cleaved Caspase-8 (AP0358), and PARP (BS70001) antibodies were purchased from Bioworld Technology (Minneapolis, USA). Secondary antibodies goat anti-rabbit/mouse IgG (H+L) HRP and goat anti-rabbit IgG (H+L) Rhodamine (TRITC) were obtained from Bioworld Technology (Minneapolis, USA).

Microarray analysis

The Cancer Genome Atlas (TCGA) normalized level 3 IllumiaHiSeq RNASeqV2 microarray gene expression data were downloaded from TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/). For TCGA prostate cancer cohort, 52 normal prostate samples and 498 prostate cancer samples were included in our study. The normalized RNASeqV2 values were log\textsubscript{2} transformed in the R statistical environment (http://www.r-project.org) and used to determine TRAF2 expression in normal and cancerous prostate tissues. All TCGA data were processed using SPSS 16.0 software.

Prostate specimens

The study was performed after approval by the Ethics Committee of Wuxi People’s Hospital. A total of 72 samples were collected to detect TRAF2 expression in prostate cancer. Patients were diagnosed with prostate cancer and samples were collected between December 2007 and December 2015. In addition, 26 benign prostatic hyperplasia (BPH) samples were collected to determine TRAF2 expression in normal prostate tissues. All prostate cancer patients were treated with radical prostatectomy. None of the patients received immunotherapy or radiotherapy before surgical treatment. The median age (range) at time of surgical treatment was 71 (57–84). The surveillance in an outpatient department included a serum PSA measure at least every 3 months for the first year and every 6 months thereafter. Median follow-up (range) time was 2442 (148–2590) days, which was defined as the time between the surgery and the endpoint of the study or the last record. The endpoint was time to biochemical recurrence, which was defined as the time between surgery to the first of at least 2 consecutive values of 0.2 ng/mL or greater in total serum PSA level [29].

| Parameters                | Cases (%) | TRAF2 expression | P*   | P#  |
|---------------------------|-----------|------------------|------|-----|
| Age (years)               |           |                  |      |     |
| <70                       | 34 (47.22) | 23               | 11   | 0.009 | 0.469 |
| ≥70                       | 38 (52.78) | 14               | 24   |      |
| Tumor grade (Gleason score)|           |                  |      |     |
| <7                        | 13 (18.06) | 9                | 4    | 0.155 | 0.149 |
| ≥7                        | 59 (81.94) | 28               | 31   |      |
| Tumor stage               |           |                  |      |     |
| T1–T2                     | 38 (52.78) | 24               | 14   | 0.035 | 0.004 |
| T3                        | 34 (47.22) | 13               | 21   |      |
| Biochemical recurrence    |           |                  |      |     |
| Positive                  | 11 (15.30) | 3                | 8    | 0.082 | 0.013 |
| Negative                  | 61 (84.70) | 34               | 27   |      |

* Pearson Chi-Square; # Log-Rank test.
The patients were classified according to 2010 AJCC/UICC TNM classification for stage and postoperative Gleason score (2005 ISUP modified) for grade [30,31]. Clinicopathological features of all prostate cancer patients are shown in Table 1. Follow-up was completed until August 2016.

**Immunofluorescence assay**

Immunofluorescence assay was performed according to the methods described in a previous study [32]. Briefly, prostate cancer cells were incubated with polyclonal rabbit anti-TRAF2 antibody at a dilution of 1: 500, and then were subsequently exposed to goat anti-rabbit IgG Rhodamine (TRITC) (diluted 1: 200 in 2% BSA/PBS) for 1 h at 37°C. DAPI was used to stain nuclei. Immunofluorescence staining was photographed under fluorescence microscopy.

**Immunohistochemistry**

Immunohistochemical assay was performed to determine TRAF2 expression in prostate samples according to the manufacturer’s protocol, as previously described [33].

**Lentivirus transduction of TRAF2 and shRNA knockdown of TRAF2 in prostate cancer cells**

Human TRAF2 cDNA was amplified by PCR method and subcloned into lentiviral transfer plasmid pLVX-Puro (Clontech). Lentiviral particles were generated in 293T/17 cells by co-transfection of lentiviral transfer plasmid pLVX-Puro-TRAF2 or control plasmid pLVX-Puro using Lipofectamine 2000 (Invitrogen), along with the packaging plasmids psPAX and pMD2.G. DU-145 cells were infected with medium containing lentiviral particles (LV-TRAF2 and LV-Control) and selected using 2 μg/ml of puromycin (Sigma). DU-145 cells infected with lentivirus LV-TRAF2 can stably express exogenous TRAF2 and were named as DU-145-TRAF2 cells.

For TRAF2 knockdown, shRNA sequence targeting TRAF2 was designed as follows: GCAGGTACGGCTACAAGAT. The TRAF2 shRNA was synthesized and inserted into the lentiviral vector.

**RNA extraction and quantitative real-time RT-PCR**

Lentivirus-infected prostate cancer cells were normally maintained in medium containing 10% FBS and were selected with puromycin for at least 4 weeks. Then, total RNA was extracted with the RNAiso Plus reagent (TaKaRa) according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized from extracted RNA using the PrimeScript™ RT reagent kit with gDNA Eraser kit (TaKaRa). A relative quantification of TRAF2 and GAPDH mRNA expression was performed by quantitative real-time RT-PCR (qRT-PCR). The qRT-PCR was performed with the SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa). The primer sequences for qRT-PCR were as follows: for TRAF2, 5’-CACACCTGTGCCCTTCTTGTG-3’ (forward) and 5’-TTCTGGTCCAGCAGCATTAAG-3’ (reverse); for GAPDH, 5’-TGCAACACAACTGCTTACG-3’ (forward) and reverse primer 5’-GGCATGGACTGTTGTCATAG-3’ (reverse).

**WST-8 cell viability assay**

WST-8 colorimetric cell viability was performed using the Cell Counting kit-8 (CCK-8) (Dojindo, Japan) according to the manufacturer’s instructions. Prostate cancer cells were seeded at a density of 1×10^4 cells per well in 96-well plates. After overnight incubation, the medium was removed and replaced with DMEM containing 1% FBS. TRAIL in 0.1% BSA was added to each well. After 24-h treatment, WST-8 was added to each well, and plates were incubated for an additional 1 h at 37°C. Absorbance was measured at 450 nm. The experiments were repeated 4 times, and cell proliferation rate was calculated using the following formula: Proliferation rate=(OD test/OD control)×100%.

**Western blot analysis**

Lentivirus-infected prostate cancer cells were treated with 200 ng/ml TRAIL for 24 h. Western blot analysis was performed according to the methods described in a previous study [10]. Briefly, cells were incubated with RIPA lysis buffer on ice for 20 min. Different proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T (Tris-Buffered Saline solution containing 0.1% Tween-20). The membranes were immunoblotted with the primary antibodies overnight at 4°C, and then processed with secondary antibodies.

**Apoptosis using flow cytometry with Annexin V and PI staining**

Cell apoptosis was detected according to a previously described method [34]. The Annexin V-FITC Apoptosis Detection kit (Dojindo, Japan) was used to detect apoptotic cells by flow cytometry according to the manufacturer's instructions.

**Statistical analysis**

Statistical analyses in this study were performed using SPSS 16.0 software. All data were calculated based on at least 3 independent experiments and expressed as mean ± standard deviations (SD). The chi-squared test was performed to evaluate categorical variables. The recurrence-free rate of prostate cancer after surgery was analyzed by the Kaplan-Meier curves, and the significance of differences was further assessed by the log-rank test. In addition, Cox proportional hazard analysis was
Figure 1. TRAF2 expression in prostate cancer. (A) A heatmap showed TRAFs (TRAF1-7) expression in normal prostate samples and prostate cancer samples from TCGA cohort. TRAF2 expression was calculated by log2 transformation of RSEM in TCGA dataset. (B) Levels of TRAF2 expression were analyzed in primary prostate cancer samples (n=65) and metastatic prostate cancer samples (n=25) from GEO dataset GSE6919 and are shown as a heatmap. (C) Levels of TRAF2 expression were analyzed in non-CRPC (n=49) and CRPC (n=27) from GEO dataset GSE35988 and are shown as a heatmap. Asterisks indicate statistically significant differences (*** P<0.01).
performed for the univariate and multivariate analyses to investigate the effect of TRAF2 in prostate cancer on recurrence-free survival. A P value <0.05 was considered statistical significance.

Results

Upregulation of TRAF2 in prostate cancer

To obtain an overall view of TRAF2 expression in prostate cancer, the publicly available datasets were downloaded from the Cancer Genome Atlas (TCGA). For TCGA cohort, 52 normal prostate samples and 498 prostate cancer samples were included. Compared with normal prostate tissues, high expression of TRAF2 in prostate cancer was observed (P<0.001, Figure 1A).

For GSE6919 dataset, 65 primary prostate cancer samples and 25 metastatic prostate cancer samples on platform GPL8300 were downloaded and analyzed. We found that TRAF2 was significantly upregulated in metastatic prostate cancer compared with primary prostate cancer (P=0.006, Figure 1B). In addition, for GSE35988 dataset, there were 49 prostate cancer samples and 27 castrate resistant prostate cancer (CRPC) samples. Upregulation of TRAF2 was detected in CRPC samples when compared with non-CRPC prostate cancer (P<0.001, Figure 1C).

Figure 2. Immunohistochemical staining of TRAF2 in human prostate cancer. (A) Representative images of TRAF2 low expression in normal prostate samples, and (B) prostate cancer samples. (C) Representative images of TRAF2 high expression in normal prostate samples, and (D) prostate cancer samples. Scale bar indicates 50 μm.
Table 2. Prognostic factors for recurrence-free survival in univariate and multivariate analyses.

| Parameters                        | Univariate analysis | Multivariate analysis |
|-----------------------------------|---------------------|-----------------------|
|                                   | HR (95% CI)         | P         | HR (95% CI)         | P         |
| Age                               | 1.570 (0.458–5.376) | 0.473     | NA                  | NA       |
| Tumor stage                       | 7.037 (1.508–32.841)| 0.013     | 5.506 (1.134–26.733)| 0.034    |
| Tumor grade (Gleason score)       | 4.148 (0.516–33.318)| 0.181     | 3.285 (0.339–31.819)| 0.305    |
| TRAF2 expression                  | 4.798 (1.239–18.583)| 0.023     | 5.600 (1.228–25.529)| 0.026    |

NA – not available.
To determine TRAF2 expression in prostate cancer in a Chinese population, 26 normal prostate samples and 72 prostate cancer samples were collected from the WXPH cohort. Using immunohistochemical staining, we observed that TRAF2 was upregulated in 19.2% (5/26) of normal prostate cancer samples and in 48.6% (35/72) of prostate cancer samples (Figure 2, Table 1). Furthermore, we found that high expression of TRAF2 was significantly associated with tumor stage (P=0.035, Table 1).

**Figure 4.** Overexpression of TRAF2 in human prostate cancer cells. (A) Representative images of overexpression of TRAF2 in DU-145 cells by immunofluorescence staining. Cells were stained using 6-diamidino-2-phenylindole (DAPI) to visualize nuclei. (B) TRAF2 overexpression in DU-145 cells was confirmed by quantitative real-time RT-PCR and (C) Western blot analysis. Quantification of TRAF2 expression was normalized by GAPDH. Data are presented as mean ±SD. Asterisks indicate statistically significant differences (** P<0.01).

**TRAF2 expression and clinicopathological features in patients with prostate cancer**

To determine TRAF2 expression in prostate cancer in a Chinese population, 26 normal prostate samples and 72 prostate cancer samples were collected from the WXPH cohort. Using immunohistochemical staining, we observed that TRAF2 was upregulated in 19.2% (5/26) of normal prostate cancer samples and in 48.6% (35/72) of prostate cancer samples (Figure 2, Table 1). Furthermore, we found that high expression of TRAF2 was significantly associated with tumor stage (P=0.035, Table 1).

**Effect of TRAF2 expression on recurrence-free rate of prostate cancer**

The recurrence-free rate was investigated using Kaplan–Meier curves. The last follow-up time was August 2016. Our results showed that the 5-year overall recurrence-free rate of prostate cancer patients was about 74.8% (Figure 3). The effects of TRAF2 expression and tumor stage on the recurrence-free rate were investigated. A log-rank test showed that TRAF2 expression and tumor stage were significantly associated with recurrence of prostate cancer (Figure 3).
Analyses of prognostic factors in prostate cancer patients

To investigate the effects of TRAF2 expression on other clinical pathological features in prostate cancer patients, Cox regression was performed for univariate and multivariate analyses. Both TRAF2 expression and tumor stage were found to be significant prognostic factors according to univariate analysis (Table 2). Multivariate analyses further showed that TRAF2 expression and tumor stage were independent prognostic factors. TRAF2 may be a valuable prognostic biomarker for predicting recurrence-free survival of prostate cancer patients (P=0.013, Table 2).

Exogenous expression of TRAF2 in prostate cancer cells

In this study, TRAF2 expression was found to be upregulated in prostate cancer. This led us to further explore the effects of TRAF2 on regulating TRAIL-induced apoptosis in prostate cancer cells, which could influence the growth of prostate cancer. To investigate the roles of TRAF2, DU-145-TRAF2 cells were constructed, which could stably express TRAF2. Overexpression of TRAF2 in DU-145 cells was confirmed by immunofluorescence assay (Figure 4). Using quantitative real-time RT-PCR and Western blot analysis, we found that expression levels of TRAF2 mRNA and protein were significantly increased in DU-145 cells (Figure 4).

Figure 5. Overexpression of TRAF2 attenuated TRAIL-induced cell death in human prostate cancer cells. (A) Cells were treated with indicated concentrations of TRAIL for 24 h. Representative images under a light microscope in human prostate cancer DU-145 cells. (B) TRAF2 overexpression enhanced cell viabilities when treated with indicated concentrations of TRAIL in human prostate cancer DU-145 cells. Experiments were performed 4 times. Data are presented as mean ±SD for 4 independent experiments. Asterisks indicate statistically significant differences (* P<0.05, ** P<0.01). Scale bar indicates 100 μm. (C) We found downregulation of cleaved Caspase-8, cleaved Caspase-3, and cleaved PARP expression, and upregulation of c-Flip expression in TRAF2-overexpressed prostate cancer DU-145 cells.
We further investigated the effects of TRAF2 on regulating TRAIL-induced apoptosis in human prostate cancer DU-145 cells. After TRAIL treatment, cell viabilities of TRAF2-overexpressed DU-145 cells were significantly higher when compared with control groups (Figure 5). This finding suggested that TRAF2 overexpression could significantly attenuate TRAIL-induced cell death in DU-145 cells (Figure 5). Using Western blot analysis, our results indicated that TRAF2 overexpression could upregulate c-Flip expression (Figure 5), and TRAF2 overexpression could downregulate cleaved Caspase-8, cleaved Caspase-3, and cleaved PARP expression (Figure 5).

TRAF2 regulated TRAIL-induced apoptosis in human prostate cancer cells

Lentiviral particles that expressed shRNA targeting TRAF2 were constructed to downregulate TRAF2 expression in prostate cancer cells. We confirmed that TRAF2 knockdown in DU-145 cells enhanced TRAIL-induced cell death according to cell viability analysis (Figure 6). Using Annexin V/PI staining by FACS analysis, we found that TRAF2 knockdown significantly enhanced TRAIL-induced apoptosis in DU-145 cells (Figure 7). Western blot analysis showed that TRAF2 knockdown upregulated cleaved Caspase-8, cleaved Caspase-3, and cleaved PARP expression in DU-145 cells (Figure 6). However, TRAF2 knockdown downregulated c-Flip expression in DU-145 cells (Figure 6).

Figure 6. TRAF2 knockdown enhanced TRAIL-induced cell death in human prostate cancer cells. (A) TRAF2 knockdown inhibited cell viabilities in TRAIL-treated human prostate cancer DU-145 cells. Experiments were performed 4 times. Data are presented as mean ±SD for 4 independent experiments. Asterisks indicate statistically significant differences (** P<0.01). (B) TRAIL-induced cell death was blocked by treatment of pan Caspase inhibitor Z-VAD-FMK in DU-145-shTRAF2 cells. Experiments were performed 4 times. Data are presented as mean ±SD for 4 independent experiments. No significant difference was found in DU-145-shTRAF2 cells. (C) TRAF2 knockdown upregulated cleaved Caspase-8, cleaved Caspase-3, cleaved PARP expression, and downregulated c-Flip expression in human prostate cancer DU-145 cells.
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TRAF2 attenuated TRAIL-induced apoptosis in DU-145 cells via c-Flip/Caspase-8 signalling

It was reported that c-Flip/Caspase-8 signalling played a vital role in regulating the extrinsic apoptosis pathway [19]. Western blot analysis showed that TRAF2 knockdown could upregulate cleaved Caspase-8, cleaved Caspase-3, and cleaved PARP expression in TRAIL-treated DU-145 cells, and TRAF2 knockdown could downregulate c-Flip expression in TRAIL-treated DU-145 cells. Importantly, it was observed that TRAIL-induced apoptosis could be blocked by treatment of pan Caspase inhibitor Z-VAD-FMK (Figure 6). Similar results were also found when cells treated with Caspase-8 inhibitor Z-IETD-FMK (data not shown). These findings suggest that TRAF2 might affect TRAIL-induced apoptosis in prostate cancer cells via regulating c-Flip/Caspase-8 signalling.

Discussion

The tumor necrosis factor receptor (TNF-R)-associated factor 2 was originally identified as a signalling adaptor that bound to the cytoplasmic regions of receptors in the TNF-R superfamily. It was documented that TRAF2 plays a crucial role in regulating immunity response, embryonic development, and stress response. TRAF2 expression has been reported to be upregulated in breast cancer [25]. Zhao et al. [25] found that TRAF2 expression was upregulated in primary tumor and metastatic lymph nodes compared with normal breast tissues. Different biomarkers of prostate cancer have recently been identified [35–38]. In the present study, we found that TRAF2 expression was significantly upregulated in prostate cancer. TRAF2 high expression was significantly associated with tumor stage and recurrence-free survival of prostate cancer patients. In addition, using GEO datasets, we also detected that TRAF2 expression was significantly increased in castration-resistant prostate cancer. Overall, the results of our study suggest that TRAF2 is a novel prognostic biomarker for predicting recurrence-free survival of prostate cancer patients, and might affect development and progression of prostate cancer. Considering the important roles of TRAF2 in cancers, our findings are reasonable and convincing.

TRAIL is an important member of the TNF superfamily and plays vital roles in cancer immunosurveillance. We confirmed that TRAF2, as a valuable prognostic biomarker in prostate cancer patients, can regulate TRAIL-induced apoptosis in human prostate cancer cells. It has been documented that TRAIL affects the development and progression of cancer [5]. The present results show that TRAF2 affects the development and progression of prostate cancer.

Figure 7. TRAF2 knockdown enhanced TRAIL-induced apoptosis in human prostate cancer cells. Cell apoptosis was detected using Annexin V/PI staining by FACS analysis. TRAF2 knockdown enhanced apoptosis when treated with 200 ng/ml of TRAIL in human prostate cancer DU-145 cells. Graphs represent apoptosis rates of DU-145 cells, which were quantified by Annexin V/PI staining method. Experiments were performed 3 times. Data are presented as mean ±SD for 3 independent experiments. Asterisks indicate statistically significant differences (** P<0.01).

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prostate cancer though regulating TRAIL-induced apoptosis in prostate cancer cells.

It was reported that c-Flip/Caspase-8 signalling plays a vital role in regulating the extrinsic apoptosis pathway [19,39]. Our results indicate that TRAF2 overexpression inhibits Caspase-8 activation in TRAIL-treated DU-145 cells, and TRAF2 overexpression upregulates c-Flip expression in TRAIL-treated DU-145 cells. Furthermore, TRAF2 knockdown in DU-145 cells shows consistent results. Hence, it is further confirmed that TRAF2 regulates TRAIL-induced apoptosis in TRAIL-treated human prostate cancer cells via c-Flip/Caspase-8 signalling (Figure 8).

**Conclusions**

The present study firstly shows that TRAF2 may be a valuable prognostic biomarker for predicting recurrence-free survival of prostate cancer patients, which might be associated with the effects of TRAF2 on regulating TRAIL-induced apoptosis in prostate cancer cells. In addition, we found that TRAF2 can affect TRAIL-induced apoptosis via c-Flip/Caspase-8 signalling in prostate cancer cells.

**Conflicts of interest**

None declared.

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