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Original Article

Research on the antitumor mechanism of plumbagin to treat differentiated thyroid cancer based on network pharmacology and experimental validation

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Abstract: Objective: Differentiated thyroid carcinoma (DTC) is the most common type of thyroid cancer (TC), accounting for 70% to 80% of all TC cases. However, 10% to 15% of DTC patients experience neoplasm metastasis and recurrence. Plumbagin is a bioactive molecule that inhibits the formation of neoplastic cells by inactivating signal pathways. Methods: We investigated the antitumor activity of plumbagin in DTC and its molecular mechanisms based on network pharmacology, molecular biological techniques and animal experiments. The underlying processes of plumbagin were investigated using gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG). Results: Plumbagin reduced the growth of DTC cells and increased the cells' apoptotic rate *in vitro*. Tumor volumes were significantly lessened after administration of plumbagin ($P<0.05$) as compared to tumor dimensions in the control group. In total, 1,225 cancer target genes and 89 putative plumbagin target genes were distinguished. As revealed in bioinformatics data, 12 predominant targets of plumbagin for treating DTC were obtained, and western blotting further confirmed that TP53, AKT1, CASP3, and PTEN were the most important biomolecules in the effects exerted by plumbagin. Conclusion: These findings show that plumbagin induced DTC cell apoptosis and suppressed tumor growth via TP53, AKT1, CASP3, and PTEN mediated pathways in human DTC cells. GO and KEGG enrichment analysis of differentially expressed genes (DEGs) related to plumbagin activation indicated pathways involved in cell survival, apoptosis, and metabolism, that were all significantly enriched.

Keywords: Differentiated thyroid cancer, plumbagin, molecular mechanism, network pharmacology, target

Introduction

Thyroid cancer (TC) is one of the most common malignant endocrine neoplasms [1]. TC has become a worldwide health problem, and 1,000,000 individuals have been diagnosed with TC annually, worldwide, as well as in China [2-4]. Thyroid cancers can be categorized into different histological types: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), and anaplastic thyroid cancer (ATC). Differentiated thyroid cancer (DTC) including PTC and FTC is the most common kind of TC and accounts for 70%-80% of all types of TC. Surgical operation is the usual management for many individuals with well-differentiated thyroid cancer, and individuals with more complex situations might need thyrotropin (TSH) suppression and radioiodine medical care (RAI) after surgery [5]. However, 10%-15% of individuals with DTC have recurrent disease, and about 5% will develop distant metastasis to the lung or bone, resulting in cancer-specific death which occurs in some individuals. Several scientific advances have highlighted a number of molecular pathways such as tyrosine kinases (TKs), MAPK signaling, and BRAFV600E which may be responsible for DTC. The evolving understanding of disease-specific molecular therapeutic targets has led to the development of specific medical specialties tailored to those molecular changes [6], such as sorafenib and lenvatinib for RAI-refractory DTC. While these medicines are reserved for DTC individuals who fail all other treatment choices, their ability to enhance individuals' overall survival (OS) remains hindered by their low effectuality and alternative mole-

cular factors. Therefore, the development of a new, more effective, and low-toxicity tumor inhibitors is of great significance for improving the survival rate of individuals with DTC and reducing disease recurrence.

Plant-derived herbs and medicines have been historically used as anticancer agents for several centuries and are starting to become more progressively utilized in modern medicine. Plumbagin, a plant-derived quinoid constituent, was initially isolated from the roots of the medicinal herb plumbago and exerts various pharmacological effects [7]. In recent studies, plumbagin has gained attention, and encouraging results which are reported in cases of many types of cancer, such as prostate, breast, lung, and colorectal cancer [8]. Plumbagin has been shown to halt the growth of non-small lung cancer cells through multiple mechanisms [9] and inhibits PI-5 kinase to induce its cytotoxic effects in breast cancer cells [10]. In melanoma cells, plumbagin induces ROS-mediated apoptosis to suppress cancer cell proliferation [11]. Few studies have investigated the consequences of plumbagin in DTC, or the bio targets and molecular mechanisms concerned with the effects of plumbagin. During this study, we investigated the potential impact of plumbagin in regard to DTC; this research is part of a rising and novel technique for identifying the general mechanisms of therapeutic compounds in illness [12, 13]. We aimed to use network pharmacology, molecular docking, and cell biology experiments to determine the resulting effects of plumbagin on DTC and from this we predicted the core targets and biological functions, as well as pathways, and mechanisms.

Materials and methods

Biological functions and enrichment pathway analysis of identifiable targets from the dose-response curve based on MTT assay

DTC cell lines KTC-1, TPC-1, and FTC-133 were obtained with STR profiling from the Chinese Academy of Sciences, Shanghai Institute of Biochemistry, and Cell Biology. The cells were grown in RPMI-1640 (Lonza, Verviers, Belgium) and incubated at 37°C in a humidified 95% air and 5% CO_2 atmosphere, seeded onto 96-well plates at a density of 1×10^5 cells/mL for 24 h with plumbagin (Sigma-Aldrich, St. Louis, MO, USA) 6 μM, 12.5 μM, 25 μM, 50 μM, 100 μM, and subsequently incubated with 20 μL methyl thiazolyl tetrazolium (MTT) (5 mg/ml, Sigma-Aldrich, Merck KGaA) for 4 h. Then, 150 μL dimethyl sulfoxide (DMSO) (Invitrogen, Thermo Fisher Scientific, Inc.) was added to each well and shaken for 10 min after the medium was removed, and the continuous-wavelength multi-function microplate reader was used to measure the optical density (OD) value at 490 nm. Inhibition rate (%) = (control-plumbagin/control) ×100%.

Mouse xenograft

All animal studies complied with the management rules of the National Health and Birth Control Commission of China and were approved by the Ethics Committee of the Peking University Shenzhen Hospital. In brief, we implanted 1×10^5 FTC 133 cells and cells which were injected subcutaneously into six nude mice (6-8 weeks old) in the thyroid glands, and three nude mice treated with plumbagin (6 mg/kg) intra-gastrically starting approximately 7 days following cell inoculation. More than two weeks (17 days) after treatment, subcutaneous tumors were harvested and weighed following animal sacrifice by cervical dislocation, and the three nude mice in the control group were left untreated (mock). The tumor volumes were estimated using the formula (length × width^2)/2.

Identification of candidate targets of plumbagin and DTC

We obtained all candidate targets of plumbagin from the Traditional Chinese Medicine System Pharmacology Technology Platform (TCMSP, http://tcmspw.com/tcmsp.php) database [14], SwissTargetPrediction (a tool for target prediction according on 2-dimensional and 3-dimensional similarity measures with known ligands) [15], PharmMapper server (http://59.78.98.102/pharmmapper/), HERB data base (http://herb.ac.cn/) [16], Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) [17], and Drug-Gene Interaction Data base (DGIdb, www.dgidb.org) [18]. The GeneCards (https://www.genecards.org/), OMIM data base (http://www.omim.org/), CTD, and DisGeNET (http://www.disgenet.org) were used to identify DTC-associated gene targets [19]. The OMIM database focuses on illustrating genetic disorders [20]. GeneCards is a comprehensive database that provides human genes for annotation and prediction [21].

Cluster analysis and the protein-protein interaction (PPI) network of plumbagin and DTC

Identification of protein-protein interaction (PPI) networks of plumbagin and DTC were analyzed using the Search Tool for the Retrieval of Interacting Genes (STRING database, V10.5, http://string-db.org/), which predicted protein functional associations and protein-protein interactions [22]. The potential targets of DTC were introduced into the string database with the species restricted to "Homo sapiens" and therefore the PPI information of them was obtained, and the results were saved in TSV format. A Cytoscape software system was utilized to construct a PPI network and analyze the interactions of the DEGs [23]. PPIs were collected if the combined score value was >0.8.

Gene function and pathway enrichment analysis

The DAVID database (https://david.ncifcrf.gov/) and the KOBAS database (http://kobas.cbi.pku.edu.cn/) were utilized to conduct GO and KEGG enrichment analysis of the DEGs. Statistical significance was set at $P<0.05$.

Western blot analysis

RIPA lysis buffer (Sangon Biotech NO.C500-005) and protease inhibitor cocktail (Sigma-Aldrich) were exploited to lyse DTC cells. Total protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Solarbio, P1200) and transferred onto nitrocellulose membranes (Roche, 03010040001). The membranes were blocked with 5% non-fat dry milk and incubated overnight with the various primary antibodies at 4°C. Specific antibodies like anti-GAPDH (Bioworld Technology, AP0063), anti-TP53 (Santa Cruz Biotechnology, sc-126), anti-AKT1 (Bioworld Technology, BS4009), anti-PTEN (Santa Cruz Biotechnology, sc-7974), and anti-CASP3 (Santa Cruz Biotechnology, sc-56053) were diluted to according to the manufacturer's protocol. The goat anti-mouse (Santa Cruz Biotechnology, sc-2005) antibody was diluted to 1:1,000 in 5% skim milk, Tris-HCl and 0.1% Tween-20. The immunoblots were subsequently washed and incubated with a goat anti-mouse antibody for 1.5 h at room temperature. The results were visualized using a chemiluminescence reagent (Santa Cruz Biotechnology, sc-2048).

Statistical analysis

All results were analyzed by GraphPad Prism (GraphPad software package, Inc., La Jolla, CA, USA) and SPSS 22.0 (IBM Corporation, Armonk, NY, USA). Two-tailed paired Student's t-tests were conducted to analyze the two groups. One-way analysis of variance (ANOVA) and Bonferroni post-hoc tests were used to evaluate differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MTT assay

As demonstrated using the MTT assay (**Figure 1A** and **1B**), cell viability was significantly reduced in cells treated with plumbagin for 24 h. As the concentration of the drug increased, cellular inhibition became more pronounced, and following intervention with 50 μM plumbagin, the proliferation inhibition exceeded approximately 50%.

Plumbagin inhibits xenograft tumor growth

All tumor volumes were monitored every three days, and also the mice were sacrificed after seventeen days of treatment for isolation of the tumor tissue. Compared with the tumor sizes in the control group (100.65±99.65 mm^3 in mock xenograft mice), the tumor volumes were considerably minimized with the administration of plumbagin (10.46±2.15 mm^3) ($P<0.001$). We discovered a significant reduction and growth delay in tumors of mice given plumbagin compared to tumors in control mice (**Figure 2A** and **2B**).

Screening and identification of DTC and plumbagin-related genes

The molecular structure of plumbagin was obtained from the PubChem database (**Figure 3**). To acquire a list of human genes associated with plumbagin, the five databases including TCMSP, STP, HerB, CTD, and DGIdb were employed. A total of 89 target genes related to plumbagin were identified. To acquire a list of related targets of DTC, the four databases including GeneCards, OMIM, CTD, and Disgenet were employed. A total of 1,225 targets related to DTC were identified (**Figure 4**). We used Venn diagram software to identify the

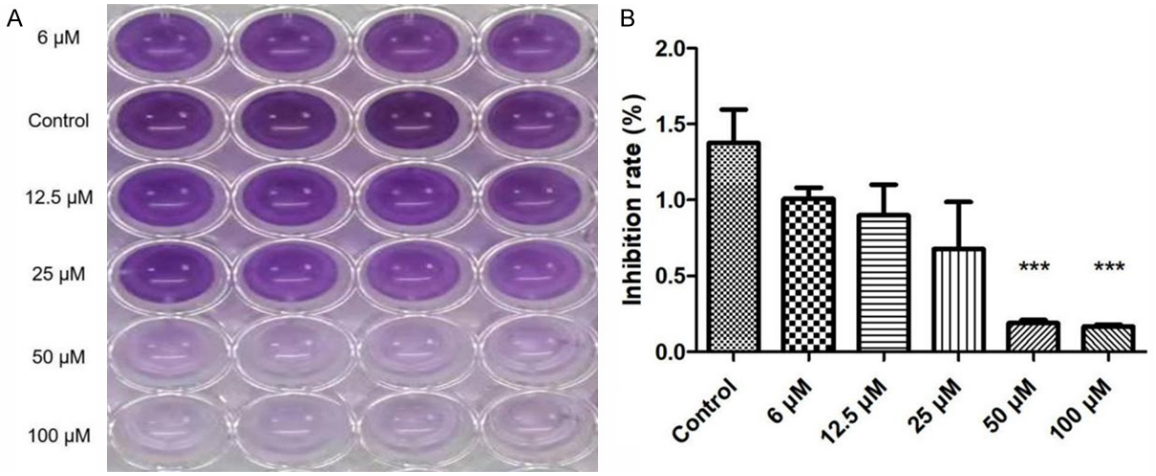

Figure 1. Inhibition of plumbagin inhibited the proliferation in a co-culture with DTC cells. A: The cells were treated with different concentration of plumbagin for 24 h. B: Inhibition rate (% value) of plumbagin, star shows the statistical significance of change between the groups. ***P<0.001.

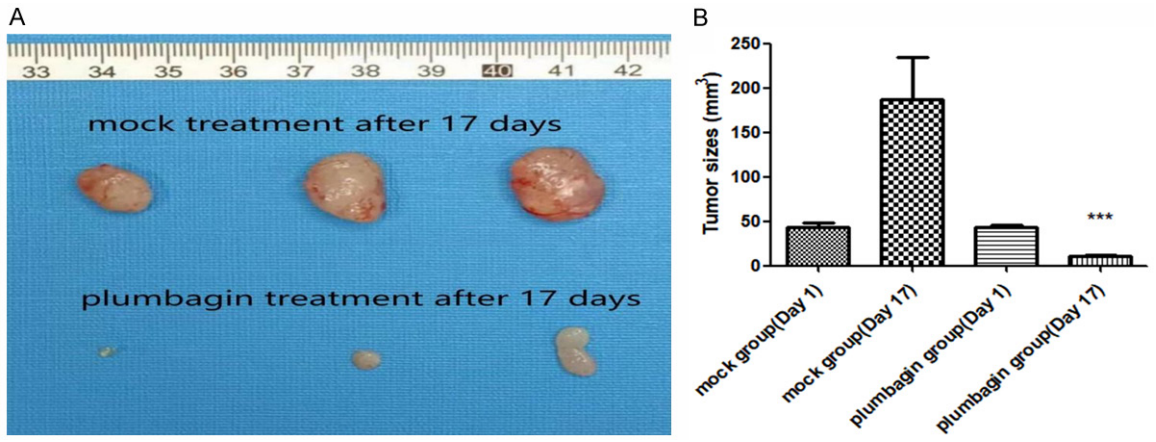

Figure 2. Inhibition of plumbagin inhibited the xenograft tumor growth. A: The tumor volumes in two groups after 17 days of treatment. B: The tumor sizes after treatment in two groups from 1 to 17 day. ***P<0.001 compare to mock xenograft mice group.

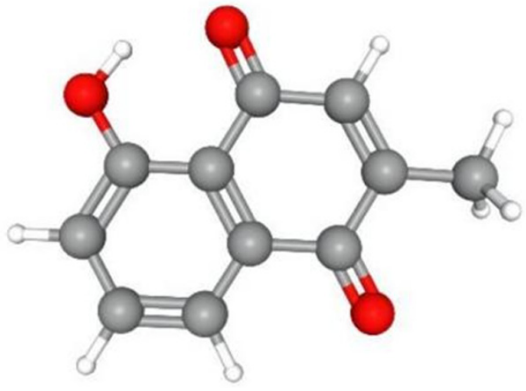

Figure 3. The molecular structure of plumbagin.

common DEGs, and a total of 37 target genes related to plumbagin and DTC were identified (**Figure 5A** and **5B**).

PPI network of DEGs and identification of key genes

The STRING online information was used to analyze the integrated DEGs and construct the PPI network. The results were downloaded and analyzed using the Cytoscape software package, the highest 12 hub genes were screened consistent with their degree values, and the identified hub genes were TP53, AKT1, CASP3, PTEN, EGFR, ESR1, MAPK3, CAT, CASP8,

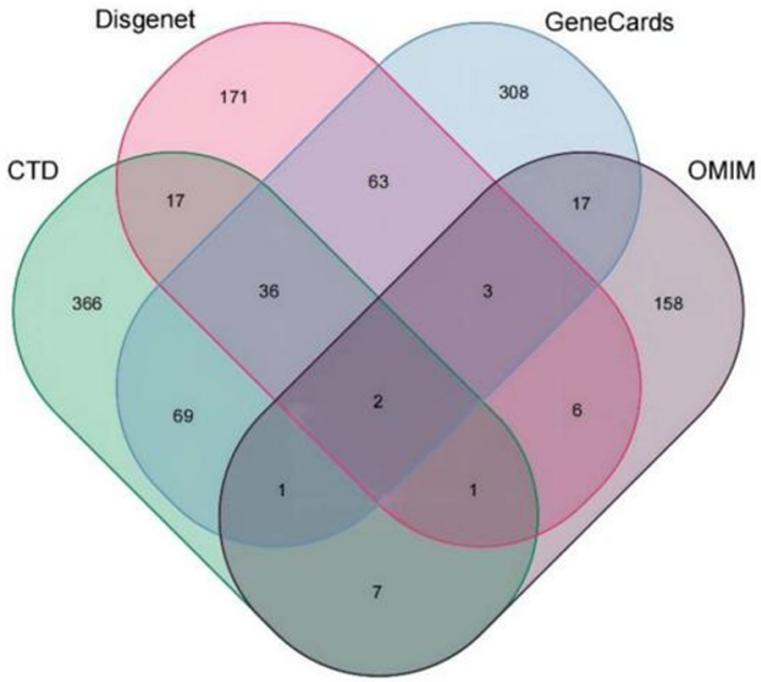

Figure 4. Information of targets related to DTC from four databases. The four databases including GeneCards, OMIM, CTD and Disgenet.

HSPA5, CASP9, NFE2L2 (**Figure 6A** and **6B**), and the high 4 prognostic bio targets for plumbagin in DTC were identified as TP53, AKT1, CASP3, PTEN as shown in **Table 1**.

Effect of plumbagin on the activities of major signaling pathways in DTC cells

Western blotting results confirmed that TP53, AKT1, CASP3, and PTEN were inhibited by plumbagin in a dose-dependent manner in FTC133 cells. However, we did not find an obviously inhibiting effect of TP53 by plumbagin in TPC-1 and KTC-1 cells, and no important distinction was found concerning the expression of TP53 in TPC-1 and KTC-1 cells compared to control groups after being affected by 12 μM and 24 μM plumbagin for 24 h ($P>0.05$). Treatment with plumbagin significantly downregulated the protein expression of AKT1, CASP3, and PTEN in FTC133, TPC-1, and KTC-1 cells compared to that in the control groups ($P<0.05$) (**Figure 7**).

GO and KEGG pathway enrichment analyses

GO functional annotation of the integrated DEGs was performed using the DAVID database and its online analysis tool. The GO functional analysis of the integrated differential genes was divided into the following three parts: biological process (BP), molecular function (MF), and cell component (CC). The main enriched GO terms showed that the DEGs were involved in the establishment of responses to nutrient levels, response to extracellular stimulus, cellular response to chemical stress, cellular response to external stimulus, cellular response to abiotic stimulus, and cellular response to environmental stimulus. KEGG pathway analyses were mainly enriched in pathways such as apoptosis, proteoglycans in cancer, chemical carcinogenesis-reactive oxygen species, thyroid hormone signaling pathway, and so on (**Figure 8A** and **8B**).

Discussion

Plumbagin is a fat-soluble molecule isolated from the roots of the medicative plant Plumbago zeylanica L. and it has been reported to possess various biological activities [24-26]. To be explicit, the antineoplastic activity and mechanism of plumbagin has become a research hotspot, and increasing attention has been paid to it, since researchers have shown that plumbagin has a repressive result on the expansion of neoplastic cells each *in vivo* and *in vitro*. It can also block the "vicious cycle" of neoplasm metastasis and inhibit bone metastasis of neoplastic cells by controlling the bone microenvironment [27, 28]. Plumbagin inhibits the expansion of cancer cells principally by modulating signals such as PI3K/Akt/mTOR, AMPK, and Ras [29, 30]. The clinical significance and biological performance of plumbagin in DTC are under-reported, and during this study we first discovered that different concentrations had an anti-proliferative impact on DTC cells *in vitro* and *in vivo*, and with the rise in drug concentration, the anti-proliferation impact became stronger; in addition there was a major reduction and growth delay of tumors treated with plumbagin compared with control-treated tumors.

To understand the possible mechanism of plumbagin in DTC cells, we have constructed

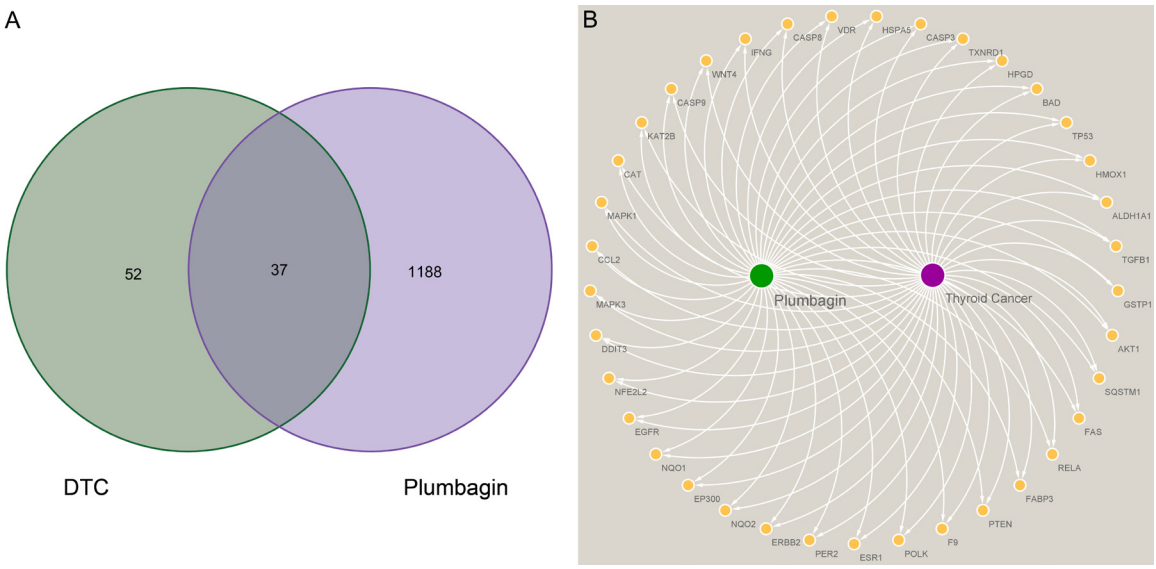

Figure 5. The commonly DEGs of 37 target genes related to plumbagin and DTC. A: Venn diagram of plumbagin and DTC. B: The 37 target genes related to plumbagin and DTC.

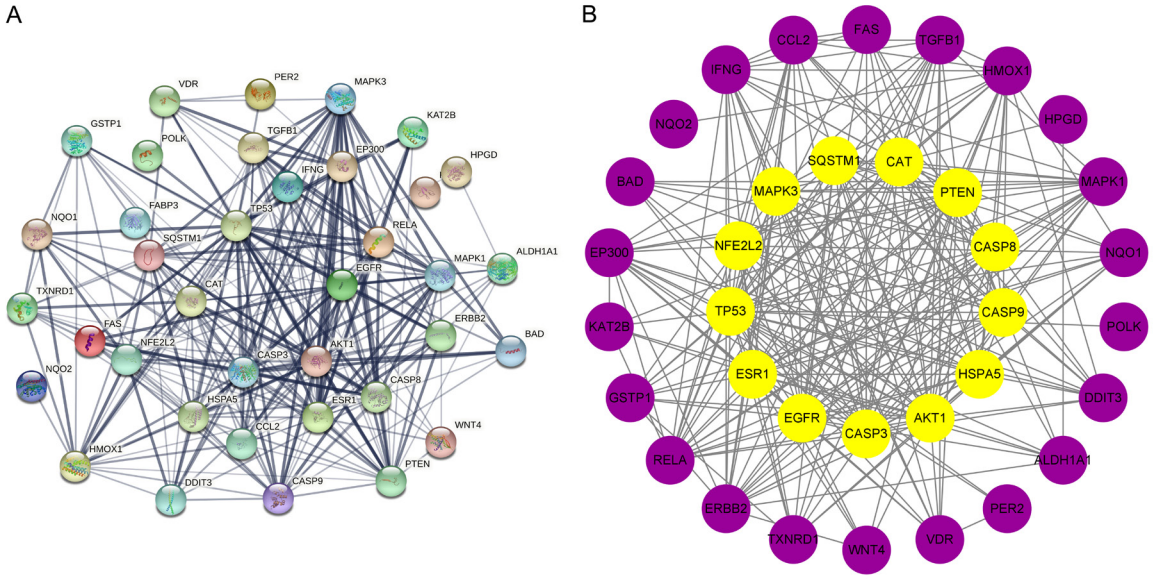

Figure 6. PPI network of plumbagin in DTC targets was constructed for visualization of interactive targets. A: PPI network from STRING online database. B: The top hub genes (yellow) were screened using the cytoscape software.

PPI networks with integrated DEGs and identified the subsequent 12 hub genes including TP53, AKT1, CASP3, PTEN, EGFR, ESR1, MAPK3, CAT, CASP8, HSPA5, CASP9, and NFE2L2. The top four predictive bio targets were identified as TP53, AKT1, CASP3, and PTEN. Mutations within the TP53 sequence are one amongst the foremost frequent alterations in human cancers, TP53 mutations are also potential prognostic markers, additionally as targets for medical intervention [31]. Previous studies showed that the expression of TP53 protein and mRNA in TC tissues was beyond that in adjacent tissues, and therefore the expression of TP53 protein in TC stages III and IV was more than that in TC stages I and II ($P<0.05$) [32]. Abnormal expression of TP53 results in resistance of TC to a variety of medicines, such as Nutlin-3, GW2580, PLX4720, ponatinib, and Dalafenib. TP53 mutation can-

Table 1. Details of corn targets of plumbagin against DTC

Targets	Degree	Eigenvector	Betweenness Centrality	Closeness centrality
TP53	32	0.2659985	183.93121	0.94444
AKT1	30	0.2639008	75.59788	0.89474
CASP3	25	0.2414419	28.51028	0.79069
PTEN	25	0.2370107	36.89059	0.79069
EGFR	24	0.2296114	28.61053	0.75556
ESR1	24	0.2253662	33.18419	0.75556
MAPK3	22	0.2237877	16.04506	0.73913
CAT	23	0.2164571	69.94124	0.75556
CASP8	20	0.2035245	13.38399	0.70833
HSPA5	20	0.2021689	13.71565	0.70833
CASP9	19	0.1955828	12.09925	0.69388
NFE2L2	19	0.1923804	10.47824	0.69388

not solely be used predict prognosis; however, it may additionally offer a target for the development of recent medicine [33]. Our research found that different concentrations of plumbagin failed to considerably inhibit the expression of TP53 protein in TPC-1 and KTC-1 cells except for FTC133 cells, suggesting that plumbagin suppressed the expansion of DTC cells not solely through the TP53 signal pathway. Another reason for this may be that FTC133 is a thyroid follicular cancer cell, and unlike papillary thyroid cancer cells TPC-1 and KTC-1, plumbagin acts differently on the three DTC cell lines. It was found that the AKT1, CASP3, and PTEN were inhibited by treating the three different DTC cell lines with increasing concentrations of plumbagin. The phosphoinositide-3-kinase-protein enzyme B/Akt (PI3K-PKB/Akt) pathway is also the main pathway of cell growth. Activated mTOR and Akt square measures are concerned with the development and progression of PTC [34]. In TC tissue samples, a rise in phosphorylated Akt is common within the invasive tumor fronts in an exceeding manner [35]. A previous study found that inhibition of the AKT-mTOR signaling pathway could directly stimulate autophagy and apoptosis in TC cell lines by downregulating the expression of p-AKT using AKT siRNA [36]. Recent studies have shown that plumbagin inhibits proliferation and promotes apoptosis of ovarian granulosa cells in PCOS by inactivating the PI3K/Akt/mTOR pathway [37], as well as inhibiting cell proliferation and promoting cell necrobiosis in bladder cancer, lung cancer, prostatic adenocarcinoma, and pancreatic cancer. The mechanism has been confirmed to be associated with repressing the PI3K/Akt/mTOR pathway [38-40]. Although there is no relevant research to prove that plumbagin treatment of TC represses the PI3K/Akt/mTOR pathway, the above research provided reference for the treatment of TC with plumbagin.

CASP3 is a crucial apoptotic element, and its abnormal performance might play a key role in the neoplasm pathological process, CASP3 promotes tumor growth by providing a pro-angiogenic microenvironment [41]. Flanagan et al reported that colon cancer patients with low levels of activated CASP3 had a longer disease-free survival time [42]. The results of a previous study additionally showed that the nucleotide polymorphism RS4647610 of CASP3 was related to a larger neoplasm size in PTC [43]. BRAFV600E mutation is one of the vital oncogenes in TC, and vemurafenib could be a sensitive drug for TC patients with BRAFV600E mutation. Run et al found that once both BRAFV600E mutated and Caspase-3 were activated in cancer cells at the same time, the sensitivity of vemurafenib would be suppressed [44]. *In vitro* and *in vivo* experiments of malignant hepatoma have shown that plumbagin might have an effect on tumor cell growth by affecting CASP3 and E-cadherin expression [45]. These results indicated that CASP3 activation is involved in tumorigenesis and may be a therapeutic target, and that plumbagin might interfere with tumor cell growth by influencing CASP3.

PTEN could be a growth suppressor, and it has been found in current studies that compared with the tumor-surrounding tissues and also in rat traditional thyroid tissues, PTEN expression in dysplastic thyroid cancer tissues is downregulated [46]. The data in the present study suggested that the miR-21/PTEN/Akt pathway might be one of the mechanisms for murine inhibition of TPC-1 thyroid cancer cells [47]. In different tumors, loss of PTEN may also be a reason for tumor development and metastasis. Previous studies have found that plumbagin inhibited prostate carcinogenesis in intact and altered PTEN knockout mice by targeting Stat3 and AKT [48], and in prostate cancer, mice treated with plumbagin with PTEN mutation

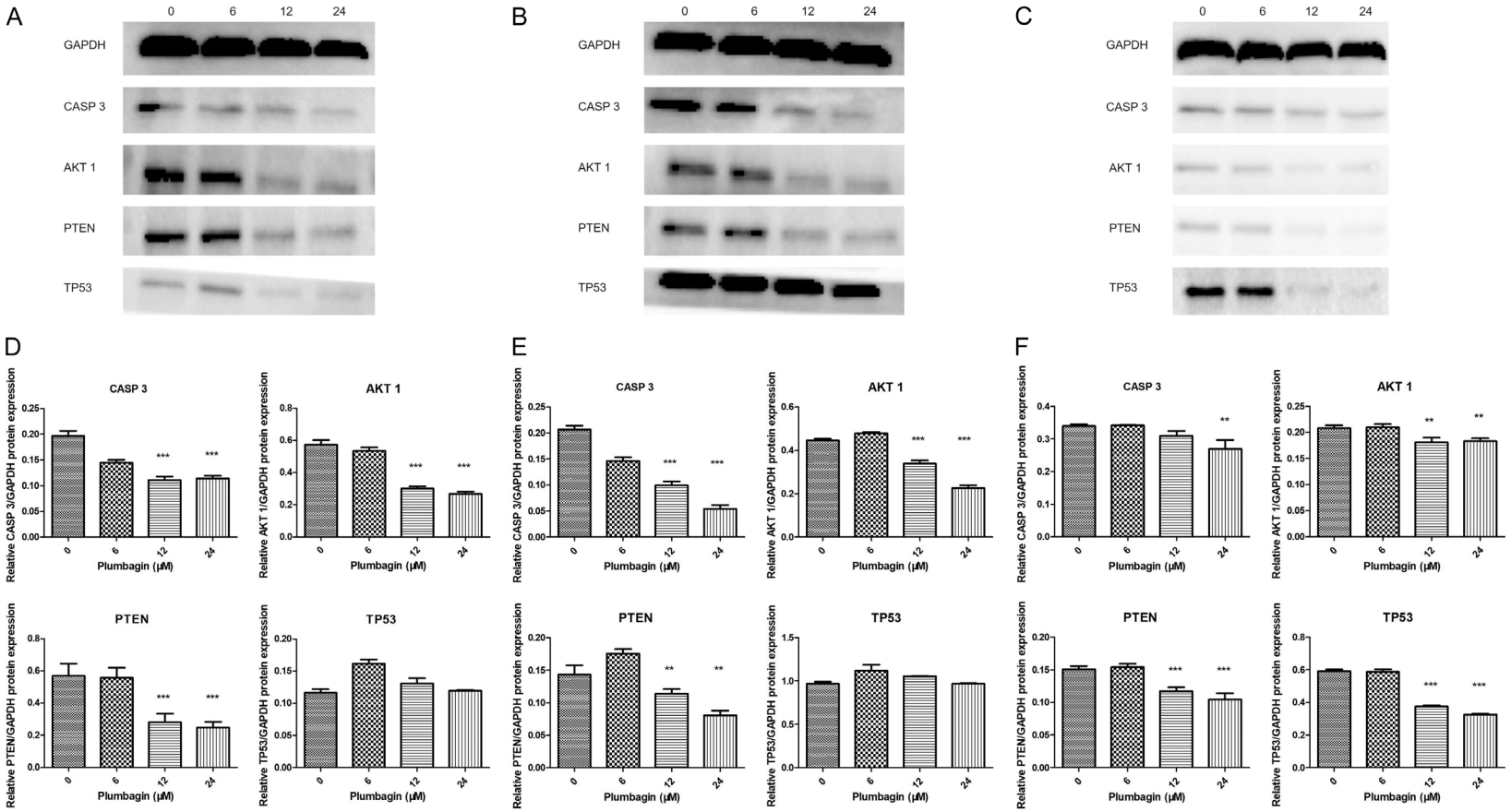

Figure 7. The Western blotting results of TP53, PTEN, CASP 3 and AKT1 after the treatment of 6 μM, 12 μM, 24 μM plumbagin for 24 h in different DTC cells. ***P<0.001, **P<0.01 vs control group, results were expressed as mean ± SEM. A, D: TPC-1 cells. B, E: KTC-1 cells. C, F: FTC 133 cells. NC: non-specific control.

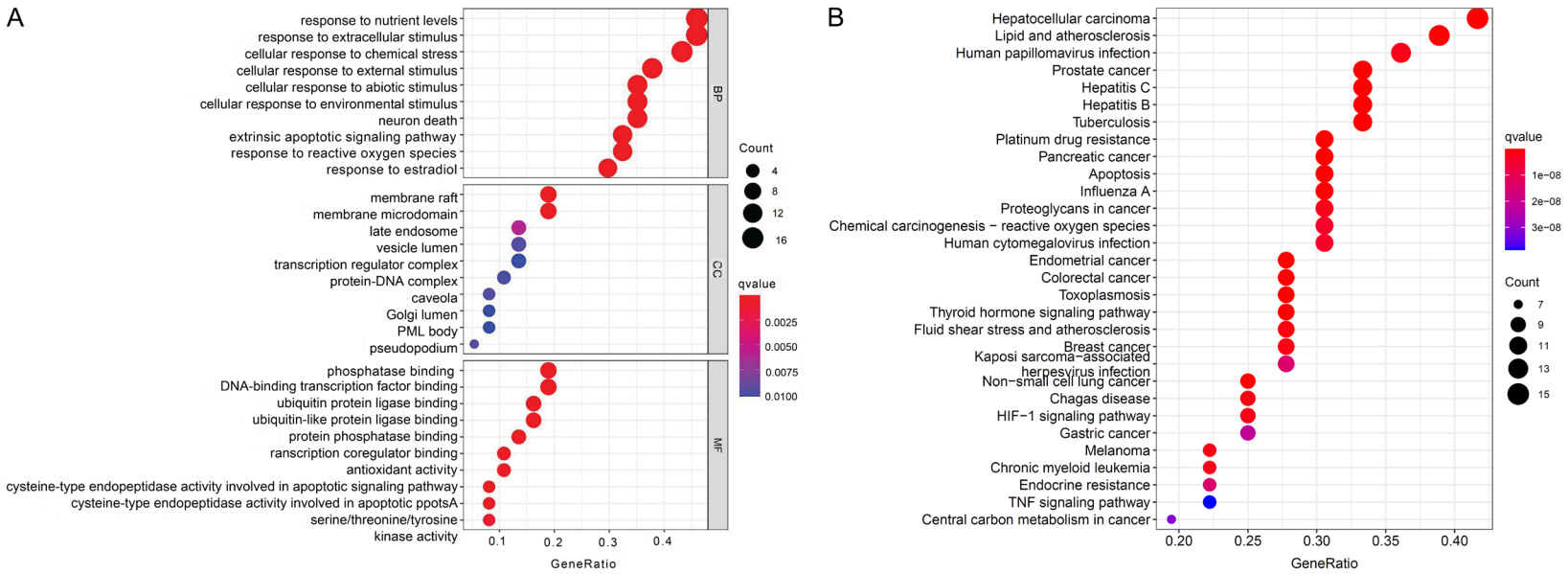

Figure 8. Biological functions and pathway enrichment assays of core targets of plumbagin through GO and KEGG pathway enrichment analyses. A: GO function annotation. B: KEGG pathway analysis.

within the prostate tumor tissue had tumor regression [49].

Conclusion

In this study, we have adopted network pharmacological strategies and western blotting to identify the active compounds and core target genes of plumbagin for the treatment of DTC. Cell and animal experiments were used to take a look at the effectuality of plumbagin within the treatment of DTC, and also the results provided new insights into the treatment mechanism of plumbagin. Plumbagin was able to considerably inhibit the proliferation and tumorigenicity of DTC cells, which may be related to the regulation of TP53, AKT1, CASP3, and PTEN. The potential biological mechanisms involved include modulation, which plays a crucial role in cell survival, apoptosis, and metabolism.

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Disclosure of conflict of interest

None.

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