An investigation into the mechanism of nobiletin’s inhibition of papillary thyroid cancer using network pharmacology analysis and experimental pharmacology

Q.-J. DU¹, Q. LI², R.-H. ZHOU³, H. WANG³, Q. YAN³, W.-J. DANG¹, J.-J. GUO¹

¹Third Hospital of Shanxi Medical University, Shanxi Bethune Hospital, Shanxi Academy of Medical Sciences, Tongji Shanxi Hospital, Taiyuan, China
²Shanxi Medical University, Jinchong, China
³Second Clinical Medical College, Shanxi Medical University, Taiyuan, China

Qiujing Du and Qian Li have contributed equally to this work and share the first authorship

Abstract. – OBJECTIVE: Surgery and radioactive iodine therapy are the main treatments for papillary thyroid carcinoma (PTC), and effective drugs are lacking. As a promising natural product, nobiletin (NOB) has a wealth of pharmacological activities like anti-tumor, antivirus, and other effects. In this research, bioinformatics methods and cellular assays were combined to explore how NOB inhibited PTC.

MATERIALS AND METHODS: Our NOB targets were derived from three databases, including the SwissTargetPrediction database, Traditional Chinese Medicine System Pharmacology Database, and the TargetNet server. Four databases were used to identify disease-related targets: GeneCards, PharmGkb, Online Mendelian Inheritance in Man, and DisGeNET. Finally, cross-targets of disease and drug were deemed as pharmacological targets, and they were used for GO and KEGG enrichment analysis. STRING and Cytoscape were applied for PPI Network and core Targets Ranking. Molecular docking analysis validated binding affinity values for NOB and core targets. By using cell proliferation and migration assays, NOB was assessed for its effects on PTC proliferation and migration phenotype. Western blot validated the downregulation of the PI3K/Akt pathway.

RESULTS: (1) Preliminarily, 85 NOB targets were predicted for NOB intervention in PTC. (2) Our core target screening identified TNF, TP53, and EGFR, and our molecular docking results confirmed good binding between NOB and protein receptors. (3) NOB inhibited proliferation and migration of PTC cells. PI3K/AKT pathway target proteins were downregulated.

CONCLUSIONS: (1) Bioinformatics analyses revealed that NOB may inhibit PTC by regulating TNF, TP53, EGFR and PI3K/AKT signalling pathway. (2) As evidenced by cell experiments, there was an inhibition of proliferating and migrating PTCs by NOB via the PI3K/AKT signalling pathway.

Key Words: Nobiletin, Papillary thyroid carcinoma, Network pharmacology, Molecular docking.

Introduction

The incidence of thyroid carcinoma is rising worldwide. Differentiated thyroid cancer (DTC) accounts for 90% of thyroid cancers and most of them are papillary thyroid carcinomas (PTCs). There has been an increase in the diagnosis of DTCs and PTCs, which are the main causes of the rise in incidence rates. While thyroid cancer incidence rates have remained relatively stable over the past 30 years for follicular, anaplastic, and medullary types. The thyroid cancer mortality rate is relatively inert, increasing by 1.1% per year, but the poor prognosis that the rising mortality represents cannot be ignored. Thyroid cancer mortality increased mainly due to advanced-stage PTC. Approximately five to ten per cent of DTC patients will develop metastatic disease, usually affecting the lungs and bones, and two-thirds of these patients will become RAI-refractory DTC, who lose the ability to absorb iodine little by little as a result of de-differentiation and have a poor prognosis with an average lifespan of only three to five years. Therefore, PTC requires a deeper understanding of its mechanisms and novel therapeutic approaches.
A total of 49% of FDA new drug approvals from 1981 to 2014 were based on natural products or derivatives of a natural product pharmacophore. In addition to their structural diversity, natural products have diverse biologic activities, low toxicity, and they are available from a variety of sources. The flavonoid nobiletin (NOB) can be obtained in citrus peel and is an active ingredient in traditional Chinese medicine such as Centipedae Herba, Citrus Reticulata, Tripterygii Radix, which has a wealth of pharmacological activities like antitumor, antivirus, anti-inflammatory, antioxidant, and antidiabetic effects. We were pleasantly surprised by its anticancer activity in various tumors, like lung cancer, renal carcinoma, and ovarian cancer. Furthermore, in normal tissues and cancer cells, nobiletin may have a dual role, which means that it may increase the sensitivity of cancer cells to anticancer drugs in addition to protecting normal tissues, just like the characteristics of other herbal-derived agents. Research has shown that multi-drug resistance can be reversed by nobiletin safely and efficiently. In brief, considerable reports have indicated that this is a promising natural product.

Network pharmacology integrates systems biology, molecular biology, pharmacology, and other emerging macro-to-micro disciplines. Computer-aided drug design relies heavily on molecular docking to predict protein-ligand binding affinity. For the first time, we have discovered and reported that tangeretin, an antioxidant derived from citrus peel, can enhance insulin sensitivity in the liver by suppressing the MEK-ERK1/2 pathway. Based on interest in flavonoids derived from citrus and their demonstrated therapeutic potential, we predicted the core targets and key signaling pathways of NOB intervention in PTC through network pharmacology, molecular docking, and other bioinformatics methods. In cell experiments, we demonstrated that NOB inhibited the proliferation and migration of PTC cells through PI3K/AKT pathway. The design idea of our project was shown in Figure 1. When current standard treatments fail to achieve good results, bringing more treatment options to patients, including more therapeutic targets and more economical and convenient drugs, has become the direction of our research.

**Materials and Methods**

**Data Collection and Target Prediction**

To clarify the effective targets of NOB, we obtained NOB targets from three databases, in-
Mechanism of NOB inhibiting PTC

including the SwissTargetPrediction database\(^{21}\), Traditional Chinese Medicine System Pharmacology Database (TCMSP), and the TargetNet web server\(^{22}\). Disease-related targets were associated with 4 databases, which were comprised of GeneCards\(^{23}\), PharmGkb\(^{24}\), Online Mendelian Inheritance in Man (OMIM)\(^{25}\), and DisGeNET\(^{26}\). Finally, cross-targets of disease and drug were deemed as pharmacological targets.

Further Exploration of the Screened Targets

With the application of DAVID Bioinformatics Resources\(^ {27}\), we further performed further enrichment analysis on the screened targets, which were targets of NOB intervention in PTC. The objective of Gene Ontology (GO) enrichment analysis was to explore the function of pharmacological targets, containing biological process (BP), molecular function (MF), and cellular compartment (CC). Meanwhile, we could be acquainted with the signalling pathways involved in the targets, under the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. We mapped the possible transduction of the PI3K-AKT pathway in combination with predicted targets.

Establishment of Protein-Protein Interaction (PPI) Network and Pharmacological Targets Ranking

The screened targets were imported into STRING (version 11.5; https://string-db.org/) to establish a PPI network, which gave a summary of the network about anticipated interconnections for screened proteins. With the application of CytoHubba from Cytoscape (version 3.9.0)\(^{28}\), we could predict and seek crucial nodes and weak parts in an interactome network by our chosen topological algorithms, which consisted of Degree, Maximum Neighborhood Component (MNC), Maximal Clique Centrality (MCC), Closeness, Edge Percolated Component (EPC) and Betweenness.

Molecular Docking

We downloaded crystal structures of proteins from the RCSB Protein Data Bank (PDB) database\(^ {29-31}\), and we obtained the small molecule ligand structure from PubChem. DeepSite\(^ {32}\) is based on deep neural networks and can predict protein binding pockets. With the upload of PDB files, the application of DeepSite helps us predict the position of druggable binding sites very well. AutoDock Vina 1.2.3\(^ {33}\) was used for molecular docking studies. PLIP\(^ {34}\) and PyMOL\(^ {35}\) were used to analyze and visualize non-covalent interactions between biomacromolecules and their ligands.

Cultivation of the PTC Cell Line

Chinese Academy of Sciences Cell Bank provided the B-CPAP cell line (PTC cell line). The mycoplasma test result of the cell line was negative, and the STR test was correct. B-CPAP Cells were nurtured in RPMI-1640 medium mixed with 10% fetal bovine serum, in 5% carbon dioxide, at 37°C.

Nobiletin IC50 and Cell Proliferation

CCK-8 assay (Boster, China) was used to assess the proliferative impact of NOB (HY-N0155, MCE) at different concentrations. For 24 hours, the cells were cultivated on 96-well plates (3,000/well) under appropriate culture conditions. Then different concentrations of NOB (40 μM, 80 μM, 120 μM) and DMSO (0.8%, 1.6%, 2.4%) were added respectively. After 24 hours, the medium was removed, and the CCK-8 reaction solution (10% culture system) was put on plates according to the manufacturer’s instructions. We measured the absorbance at 450 nm after incubation for 1-4 hours and then calculated the NOB IC50 using GraphPad Prism software (La Jolla, CA, USA).

Wound Healing

Cells were sowed in six-well plates (2 * 10^5 cells/well). When cells came up to 90% confluence, we made an equal-width scratch in the plate with a 200 μL pipette tip and washed it three times with PBS intending to remove scraped cells. Then wells were grouped into drug (30 μL and 60 μL NOB) and control group (0 μL NOB), and incubated for 24 hours, maintaining with serum-free medium. Scratch healing was observed at 0 h and 24 h, while we chose 5 fields of view to take pictures through the microscope in each well. With the percentage of relative wound closure assessed, we contemplated differences in cell migration that were shown in drug groups by contrast with the control group.

Western Blotting

With the usage of RIPA lysis buffer (Boster, China) supplemented with protease inhibitors and phosphatase inhibitors (Keygen, China), we lysed different groups of cells on ice. Cell lysates were picked up and centrifuged at 10,000 g for 10
minutes. Quantifying protein concentrations was done using a BCA protein assay kit (Boster, China). Electrophoresis was used to separate samples with equal amounts of protein. Proteins were transferred to nitrocellulose membranes (Boster, China), and 5% nonfat milk was used to block nitrocellulose membranes for 1 hour at room temperature before blocking with primary antibodies overnight at 4°C. The primary antibodies were as follows: PI3KCA (A0265, 1:1000, Abclonal, China), P-PI3K p85 (Tyr458)/p55 (Tyr199) (#4228, 1:1000, CST, USA), AKT1 (A17909, 1:1000, Abclonal, China), P-AKT (AP0140, 1:1000, Abclonal, China), GAPDH (ab181602, 1:3000, Abcam, UK). Incubation with secondary antibodies was performed after washing the membranes three times with Tris-buffered saline containing Tween-20 (0.1%). Finally, membranes were treated with a chemiluminescent reagent and exposed to X-rays.

Statistical Analysis

The following software were utilized for data extraction and analysis: R software (version 4.1.0), Strawberry Perl software (version 5.30.1-64bit), GraphPad Prism software (version 8.0.2; La Jolla, CA, USA) and SPSS software v.26.0 (IBM, Armonk, NY, USA). In *in vitro* studies, paired t-test was used for variance analysis of two-sample means; One-way ANOVA was used for overall variance analysis of multi-sample means; Turkey and Dunnett-t statistical methods were used for pairwise comparison of multi-sample means. A description of the significance of the p-value: ns means $p > 0.05$ (not significant); * means $0.01 < p < 0.05$; ** means $0.001 < p < 0.01$; *** means $p < 0.001$.

Results

**Determination of NOB Targets Against PTC**

Referring to drug-related targets, 15 targets, 35 targets, and 153 targets were derived from SwissTargetPrediction, TCMSP and TargetNet, respectively (Figure 2A). With the filter criteria of relevance score greater than 1, we screened 2,723 targets from the 2,773 PTC disease-related targets in GeneCards. The final collection of disease-related targets was finished after we obtained 202 targets from PharmGkb, 287 targets from OMIM, and 1,348 targets from DisGeNET (Figure 2B). With duplicate targets among databases removed, 85 NOB targets for intervention in PTC were preliminarily predicted (Figure 3).

Further Exploration of Initially Predicted Targets

In GO enrichment analysis, biological processes mainly referred to signal transduction, negative regulation of cell proliferation, inflammatory response and so on (Figure 4). With the consequences of the KEGG enrichment analysis, the signalling pathways of NOB intervention in PTC were mainly focused on the pathways in cancer, PI3K/AKT signalling

![Figure 2. A, The distribution of NOB-related targets. B, The distribution of PTC-related targets.](image-url)
Mechanism of NOB inhibiting PTC pathway, chemical carcinogenesis-receptor activation, prostate cancer, pancreatic cancer (Figure 4). Combined with predicted targets, the PI3K/AKT pathway was depicted in Figure 5 with the application of ScienceSlides.

The PPI of NOB Targets against PTC and Pharmacological Targets Ranking

With the implementation of STRING and Cytoscape, a processed network based on the PPI network not only demonstrated the interaction
among pharmacological targets, but also indicated the connection among NOB, PTC, and pharmacological targets (Figure 6). Via our chosen topological algorithms in CytoHubba, we got the top fifteen rankings of targets in different algorithms (Table I). Based on the top three most consistent results shown by various algorithms, we selected TNF, TP53, and EGFR for molecular docking with NOB to assess their binding activities.

**Molecular Docking Analysis**

It is recognized that binding affinity values lower than -5.0 kcal/mol indicate good interactions, and binding is stronger at a lower number. The good binding of NOB and protein receptors verified the calculation results obtained by the CytoHubba algorithm. This suggested that NOB may inhibit PTC by interfering with the core targets of TNF, TP53, and EGFR. Docking results for the NOB ligand and core target protein receptors were depicted in Table II and Figure 7.

**NOB Reduced PTC Proliferation and Migration**

The 24h IC50 value of NOB was 57.85 μM for the B-CPAP cell line (Figure 8A). By further
Mechanism of NOB inhibiting PTC

analysis, we found that the viability of cells treated with 40 μM and 60 μM NOB was inhibited significantly compared with the respective experimental controls (DMSO controls), in a concentration-dependent manner (Figure 8B). Contrasted with the blank control (no drug added), the viability of cells treated with 40 μM, 60 μM, and 80 μM NOB was inhibited significantly, in a concentration-depen-

Table I. Pharmacological Targets Ranking based on different algorithms.

<table>
<thead>
<tr>
<th>Category</th>
<th>Degree</th>
<th>MCC</th>
<th>MNC</th>
<th>EPC</th>
<th>Closeness</th>
<th>Betweenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TNF</td>
<td>TP53</td>
<td>TNF</td>
<td>TP53</td>
<td>TNF</td>
<td>TNF</td>
</tr>
<tr>
<td>2</td>
<td>TP53</td>
<td>TNF</td>
<td>TP53</td>
<td>TNF</td>
<td>TP53</td>
<td>TP53</td>
</tr>
<tr>
<td>3</td>
<td>EGFR</td>
<td>CREB1</td>
<td>EGFR</td>
<td>ESR1</td>
<td>EGFR</td>
<td>ESR1</td>
</tr>
<tr>
<td>4</td>
<td>ESR1</td>
<td>PTGS2</td>
<td>ESR1</td>
<td>EGFR</td>
<td>ESR1</td>
<td>ESR1</td>
</tr>
<tr>
<td>5</td>
<td>CREB1</td>
<td>EGFR</td>
<td>CREB1</td>
<td>MMP9</td>
<td>PTGS2</td>
<td>CREB1</td>
</tr>
<tr>
<td>6</td>
<td>PTGS2</td>
<td>ESR1</td>
<td>PTGS2</td>
<td>MMP9</td>
<td>PTGS2</td>
<td>MMP9</td>
</tr>
<tr>
<td>7</td>
<td>MMP9</td>
<td>MMP9</td>
<td>MMP9</td>
<td>MAPK8</td>
<td>CREB1</td>
<td>MMP9</td>
</tr>
<tr>
<td>8</td>
<td>PPARG</td>
<td>RELA</td>
<td>PPARG</td>
<td>CREB1</td>
<td>PPARG</td>
<td>PTGS2</td>
</tr>
<tr>
<td>9</td>
<td>MAPK8</td>
<td>CASP9</td>
<td>MAPK8</td>
<td>PPARG</td>
<td>MAPK8</td>
<td>MMP9</td>
</tr>
<tr>
<td>10</td>
<td>RELA</td>
<td>MAPK8</td>
<td>RELA</td>
<td>RELA</td>
<td>RELA</td>
<td>KCNH2</td>
</tr>
<tr>
<td>11</td>
<td>AR</td>
<td>MCL1</td>
<td>AR</td>
<td>MCL1</td>
<td>AR</td>
<td>CA9</td>
</tr>
<tr>
<td>12</td>
<td>MCL1</td>
<td>AR</td>
<td>MCL1</td>
<td>AR</td>
<td>MCL1</td>
<td>AR</td>
</tr>
<tr>
<td>13</td>
<td>JAK2</td>
<td>PGR</td>
<td>JAK2</td>
<td>JAK2</td>
<td>JAK2</td>
<td>KIT</td>
</tr>
<tr>
<td>14</td>
<td>KIT</td>
<td>JAK2</td>
<td>KIT</td>
<td>KIT</td>
<td>PGR</td>
<td>MAPK8</td>
</tr>
<tr>
<td>15</td>
<td>PGR</td>
<td>PPARG</td>
<td>PGR</td>
<td>GSK3B</td>
<td>JAK2</td>
<td>RELA</td>
</tr>
</tbody>
</table>

Table II. Binding affinity values (kcal/mol) of nobiletin and the core targets based on molecular docking.

<table>
<thead>
<tr>
<th>Receptor ligand</th>
<th>TNF</th>
<th>TP53</th>
<th>EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nobiletin</td>
<td>-7.68</td>
<td>-8.36</td>
<td>-7.01</td>
</tr>
</tbody>
</table>

Figure 7. Docking results for the NOB ligand and core target protein receptors. The global molecular docking results of TNF, TP53, and EGFR were depicted in Figures A-C, respectively, while the local interactions were depicted in Figures D-F. The line colors that represent interactions are configured as follows: Hotpink lines represent π-stacking (parallel); Limon lines represent hydrophobic interactions; Blue lines represent hydrogen bond. The ligand NOB is highlighted with white sticks in all pictures.
dent manner. B-CPAP cells were exposed to NOB (30 µM and 60 µM) for 24 hours, and the relative wound closure rates were compared to those of blank control cells (Figure 9A). Contrasted with the blank control group, NOB significantly inhibited the migration of B-CPAP cells, but not concentration-dependently (Figure 9B). In sum, NOB curbed the proliferation and migration phenotypes of PTC cells significantly, compared with the control group. Cell proliferation was inhibited by NOB at a concentration-dependent level, but cell migration was not.

The PI3K/AKT Pathway Was Involved in the Inhibition of PTC by NOB

According to KEGG enrichment analysis, NOB suppressed tumor growth, most potentially via interfering with PI3K/AKT signalling in PTC. To verify the predicted results of network pharmacology, we determined the protein expression levels of PI3K, P-PI3K, AKT and P-AKT in PTC cells treated with different concentrations of NOB by western blotting. As shown in Figure 9C, target proteins associated with PI3K/AKT signalling were downregulated.

Discussion

The incidence of papillary thyroid carcinoma continues to rise with the upgrading of detection technology1. Although the prognosis is good, 40-90% of patients are prone to central lymph node metastasis37, and 3-15% of patients will develop distant metastasis38.
Furthermore, relapse occurs in up to 10% of patients after standard treatment. Data from a large case-control study suggest that lymph node metastases and incomplete surgical resection are the two main attributions for higher mortality. Therefore, at a time when surgery is the main treatment for thyroid cancer, it is essential to improve the clinical medical treatment methods and relieve the suffering of patients. With the effective validation of in vitro and in vivo models, the anticancer potential of natural products, especially those derived from traditional Chinese medicine, is gradually being recognized by researchers. As a promising avenue of contemporary drug discovery, network pharmacology highlights the better utility of multi-targeted drugs and the network characteristics of intervening diseases.

In this research, we explored how the natural product NOB inhibits PTC with the application of network pharmacology, molecular docking simulation techniques and papillary thyroid cancer cell models. Under the retrieval of existing reports, this is the first time to explore the mechanism of NOB inhibition of PTC through bioinformatics technology and cell experiments. Experiments performed in vitro revealed that NOB curbed the proliferation and migration phenotypes of the PTC cell line compared with the control group. It was concentration-dependent that NOB inhibited proliferation but not migration.

Founded on further analysis of the initial predicted targets, we identified three core targets via different algorithms, which were TNF, TP53, and EGFR. And this was verified in molecular docking. Tumor necrosis factor (TNF), also known as TNF-alpha or TNFSF2, is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor superfamily and plays a dual role in the intervention of cancers which relies upon the involvement of specific cell types and local concentration of TNF. A meta-analysis collecting 29 writings demonstrated that the level of TNF-alpha in serum and the ratio of TNF-alpha immunoreactivity in tissues for thyroid carcinoma were significantly higher in contrast with those in control. NIS’s downregulation affects iodine intake and the treatment of radioactive iodine, resulting in a poor prognosis for thyroid cancer patients. And NIS expression can be negatively modulated by TNF-alpha. As a tumor suppressor and a potent cancer barrier, tumor protein p53 (TP53) suppresses tumor growth and induces apoptosis in many tumors. There is a link between codon 72 polymorphism in the TP53 gene and thyroid cancer susceptibility. Mutations in TP53 are involved in thyroid tumor dedifferentiation and progression, causing poorly differentiated thyroid carcinoma (PDTC) and ATC. Dysregulated protein kinase activity contributes to many diseases, including cancer, making the protein kinase family one of the most important drug targets of the 21st century. Epidermal growth factor receptor (EGFR), a transmembrane glycoprotein that is a member of the protein kinase superfamily, is one of the most common drug targets for FDA-approved drugs. A significant increase in EGFR expression was associated with aggressiveness in PTC, and proliferation and migration of PTC cells were inhibited by EGFR inhibition. A dose-expansion study found that lifirafenib (a novel RAF family kinase inhibitor and EGFR inhibitor) had an acceptable risk-benefit profile and multitumor effects on patients with B-RAF V600E PTC.

As we all know, BRAF and RAS mutations are mainly responsible for PTC. And RAS activated PI3K/AKT signal pathway in thyroid cancer and had significant associations with AKT phosphorylation. Studies have shown that the activation of PI3K/AKT signalling was linked to proliferation and migration in PTC cells. NOB was found to exert a tumor-suppressing effect on PTC via the PI3K/AKT pathway according to our KEGG enrichment analysis, and this was demonstrated experimentally. Thus, NOB may curb B-CPAP cell proliferation and migration via PI3K/AKT signalling.

Conclusions

This research predicted the core targets and key signalling pathways of NOB intervention in PTC, utilizing network pharmacology, molecular docking, and other bioinformatics tools. In cell experiments, it was verified that NOB inhibited PTC proliferation and migration through PI3K/AKT signalling. In summary, for the first time, our study shows an inhibitory effect of NOB on PTC and explores its mechanism.

Conflict of Interest

The Authors declare that they have no conflict of interests.
Acknowledgments
The team should thank a computer professional friend who helped the team fix the difficult scripting problem during the run of the R software.

Authors’ Contribution
G.J. and D.Q. contributed to the design of the study. D.Q., W.H., L.Q., and D.W. helped collect data and debug the program. The statistical analysis was carried out by D.Q., L.Q. and Y.Q.

Funding
A total of three projects funded the research, including Senmei China Diabetes Research Fund of China International Medical Foundation (Z-2017-26-1902), Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province (2020-188), Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province (20200041).

ORCID ID
Qiujing Du: 0000-0002-2855-6209.
Ruhao Zhou: 0000-0001-5094-7997.
Jianjin Guo: 0000-0003-4160-4364.

References


Mechanism of NOB inhibiting PTC


41) Zhu XY, Guo DW, Lao QC, Xu YQ, Meng ZK, Xia B, Yang H, Li Q, Li P. Sensitization and syner-


