3,3'-Diindolylmethane inhibits the proliferation of esophageal squamous cell carcinoma cells via downregulation of STIM1

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Abstract. 3,3'-Diindolylmethane (DIM) is a natural phytochemical derived from cruciferous plants that has inhibitory effects on a wide range of tumor cells; however, its relevant effects on esophageal cancer cells have been poorly studied. Therefore, in the present study, a pharmacology network approach was used to predict the possible core targets of DIM acting on esophageal cancer. Subsequently, using *in vitro* experiments, TE-1 human esophageal cancer cells were treated with different concentrations of DIM (0, 40, 60 and 80 μM) for 24 h. Changes in cell activity were detected by Cell Counting Kit-8 assay, and changes in the expression levels of stromal interaction molecule 1 (STIM1) and apoptosis-related proteins, B-cell lymphoma-2 (Bcl-2) and Bax, were assessed by western blotting, followed by the upregulation of STIM1 by thapsigargin (Tg). Network pharmacology analysis showed that there were 39 potential core targets of DIM in esophageal cancer. The results of the *in vitro* experiments showed that DIM could inhibit the viability of esophageal cancer cells, downregulate the expression of STIM1 and Bcl-2 proteins and upregulate the expression of Bax protein, all in a concentration-dependent manner. The results also demonstrated that toxic carotenoids were agonist against STIM1 protein and upregulated STIM1 and Bax protein expression. After agonizing STIM1 protein expression using Tg, DIM was able to counteract the expression trend of STIM1, Bcl-2 and Bax protein in TE-1 cells. In summary, DIM induced apoptosis and inhibited the viability of esophageal cancer cells by downregulating the expression of STIM1 protein; therefore, the natural phytochemical, DIM, may be a potential substance for the early prevention and treatment of esophageal cancer cells.

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Introduction

Esophageal cancer is a common malignant tumor of the gastrointestinal tract, the eighth most common malignant tumor and the sixth leading cause of cancer-related death worldwide (1). With the development of minimally invasive techniques, minimally invasive surgery now occupies an absolute position in esophageal cancer surgery. Esophageal squamous cell carcinoma is dominant in East Asia, including China, while esophageal adenocarcinoma is dominant in Europe, which may be related to genetic susceptibility between different ethnicities and dietary conditions among different populations (2). Patients with esophageal cancer often only present with progressive dysphagia in the middle and late stages of disease due to the lack of specific clinical manifestations. Therefore, when patients present at the clinic, the indication for direct surgery has typically past, and even if surgery is possible, there is a risk of local and distant metastasis, which greatly increases the tumor burden on patients (3,4). With the development of neoadjuvant therapy in recent years, neoadjuvant therapy to achieve tumor shrinkage or even downstaging before surgery (thus increasing the radical resection rate of tumors) has become the optimal treatment option for patients with intermediate to advanced esophageal cancer (5). However, neoadjuvant therapy often causes adverse effects in patients, including anemia and hypoproteinemia (6), and a study has indicated that patients undergoing surgery following neoadjuvant therapy have an increased risk of pulmonary infection and anastomotic fistula (7). Therefore, it is particularly important to find a low-cost modality with few side effects to prevent or treat esophageal cancer.

Stromal interaction molecule 1 (STIM1) is a protein mainly found in the endoplasmic reticulum membrane and has a highly conserved protein structure (8). STIM1 is one of the key proteins of the store-operated calcium entry (SOCE) channel, which mediates the entry of extracellular Ca^{2+}

into the intracellular compartment to maintain the relative stability of Ca^{2+} inside and outside the cell. When the calcium pool is emptied for various reasons, such as drug action and hypoxia (9), Ca^{2+} dissociates from the EF-chiral structure (structural domain of STIM1), which induces a multimerization process in the N-terminal region of STIM1, resulting in a conformational change of STIM1 and its transfer to the inner cell membrane to couple with Orai1 and transient receptor potential canonical channels, thereby activating SOCE to allow the entry of extracellular Ca^{2+} into the intracellular compartment (10,11). STIM1 has a close relationship with a variety of tumorigenesis mechanisms; it can participate in the recruitment of endothelial cells by the bone marrow, thus promoting tumor angiogenesis (12), and downregulation of the STIM1 gene blocks the cell cycle in the S or G2 phase (13). It has also been shown that STIM1 expression is related to the differentiation and prognosis of esophageal malignant tumors, and the higher the expression of STIM1, the worse the prognosis, while downregulation of STIM1 expression significantly inhibits the proliferation and migration of esophageal cancer cells (14).

3,3'-Diindolylmethane (DIM; molecular formula, $C_{17}H_{14}N_2$; molecular weight, 246.3065) is a natural plant compound derived from cruciferous plants, including cauliflower and broccoli, and is a metabolite of indole-3-carbinol (I3C). After entering the digestive tract, I3C from cruciferous plants is readily hydrolyzed to DIM under acidic conditions to exert its biological effects (15). It has been demonstrated that DIM has antitumor activity against breast, nasopharyngeal, gastric and ovarian cancer (16,17). DIM can induce gastric cancer cell death by upregulating the expression of STIM1 protein (18), and it has been reported that DIM enhances sensitivity to radiotherapy in human esophageal cancer cells (19). However, whether DIM can directly inhibit the proliferation of esophageal cancer cells and through what possible pathway, and whether it can be regulated by STIM1 protein, has been rarely reported.

In the present study, the Cell Counting Kit-8 assay was used to verify whether DIM could inhibit the variation of TE-1 cells, and western blotting was applied to detect the expression of STIM1, Bcl-2 and Bax protein after DIM acted on TE-1 cells, to preliminarily explore the possible mechanism behind this.

Materials and methods

Prediction of DIM and esophageal cancer targets. '3,3'-diindolylmethane' was searched in the Comparative Toxicogenomics Database (CTD; https://ctdbase.org/detail.go?type=chem&acc=C016392) and the GeneCards database (https://www.genecards.org/Search/Keyword?queryString=3,3%27-diindolylmethane), selecting 'Homo sapiens' as the species. The disease key words, 'esophageal cancer', were also searched in the CTD (https://ctdbase.org/detail.go?type=disease&acc=MESH%3AD004938) and GeneCards database (https://www.genecards.org/Search/Keyword?queryString=esophagus%20cancer). The results files were downloaded as Excel files. The DIM and esophageal cancer targets were stored in two different Excel files and any duplicated content within each file was deleted.

Construction and analysis of a target network. The DIM and esophageal cancer targets were imported into Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/) to obtain the common targets. The common targets were then imported into STRING V12.0 (https://cn.string-db.org/cgi/input.pl) for network analysis, selecting 'Homo sapiens' as the species. The results were downloaded and saved as a tsv file, which was imported into Cytoscape 3.9.1 (Oracle Corporation) for visualization. Next, the PPI results were imported into Cytoscape and the Centiscape 2.2 plug-in was used. 'Degree', 'closeness' and 'betweenness' were selected to analyze the results, and 39 possible core targets were obtained.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses. The common intersection targets of DIM and esophageal cancer were entered into the Database for Annotation, Visualization and Integrated Discovery (https://david.ncifcrf.gov/), selecting 'Homo sapiens' as the species, for GO enrichment [Molecular Function (MF), Biological Process (BP) and Cell Composition (CC)] and KEGG pathway analyses. $P<0.05$ was considered to indicate statistical significance. The selected data were arranged in descending order, and the first 20 results were imported into SRplot (https://www.bioinformatics.com.cn/) to produce bubble diagrams.

Chemicals and reagents. DIM (cat. no. D9568; MilliporeSigma) was dissolved in dimethylsulfoxide (99%) and prepared as a 100 mM stock solution, which was stored at 4˚C. Thapsigargin (Tg; HY-13433; MedChemExpress), 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazole monosodium salt [Cell Counting Kit-8 (CCK-8)] and bicinchoninic acid (BCA) protein assay kit (cat. no. P0011) were purchased from Beyotime Institute of Biotechnology. Primary antibodies against STIM1 (1:1,000; cat. no. ab108994; Abcam), B-cell lymphoma-2 (Bcl-2; 1:1,000; cat. no. 3498S; CST Biological Reagents Co., Ltd.), Bax (1:1,000; cat. no. 50599-2-Ig; Proteintech Group, Inc.) and GAPDH (1:10,000; cat. no. ab8245; Abcam) were also purchased. Goat anti-rabbit HRP-conjugated (1:10,000; cat. no. ab6721) secondary antibody was purchased from Abcam.

Cell culture and drug treatment. The human TE-1 esophageal cancer cell line (esophageal squamous cell carcinoma; cat. no. TCHu 89) was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (Dalian Meilun Biology Technology Co., Ltd.), containing 20% fetal bovine serum (Shanghai ExCell Biology, Inc.), at 37˚C in a 5% CO_2 incubator. As according to the literature (18), TE-1 cells were treated with DIM at concentrations of 0, 40, 60 and 80 μM for 24 h. In addition, the STIM1 agonist toxic, Tg (1 μM), was used to pre-treat TE-1 cells for 10 min prior to DIM exposure.

CCK-8 assay. TE-1 cells (10,000 cells/well) were inoculated into 96-well plates and exposed to different concentrations of DIM (0, 40, 60 and 80 μM) for 24 h, once cells had sufficiently adherent to the wall of the plate. Then, 10 μl CCK-8 solution

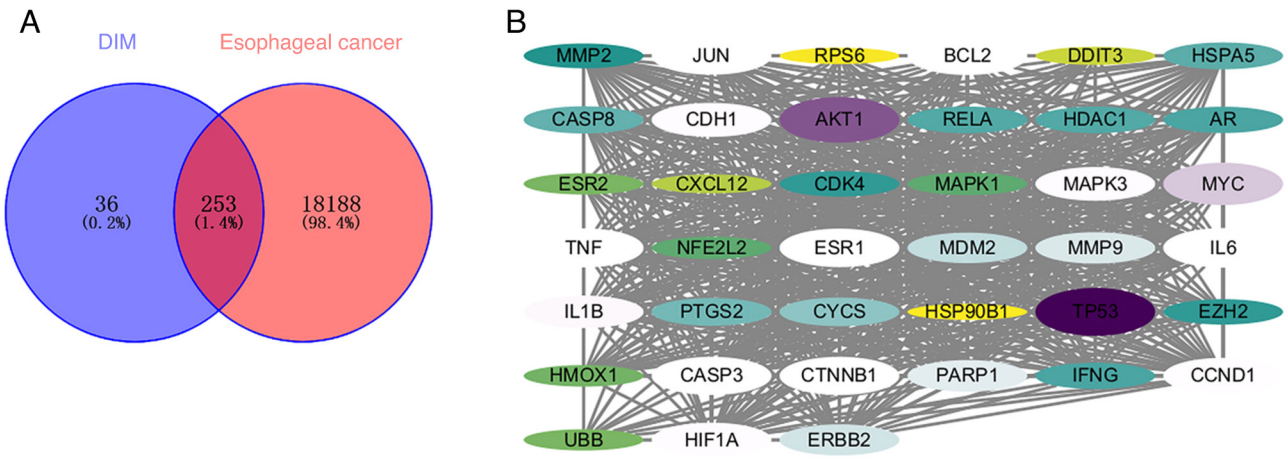

Figure 1. Pharmacology network of DIM in the treatment of esophageal cancer. (A) Venn diagram of esophageal cancer: The different colors represent the target sets of DIM and esophageal cancer, respectively, and the cross-section represents the elements shared by the two sets. (B) Possible core targets of DIM in esophageal cancer were screened by Centiscape 2.2. DIM, 3,3'-diindolylmethane.

was incubated with the cells for 1 h at 37˚C, protected from the light. The cell viability was evaluated by absorbance at 450 nm with a microplate reader.

Scratch test. TE-1 cells in the logarithmic growth phase were inoculated into 6-well plates. After 24 h of fully adherent cell growth, 200 μl pipette tips were used to draw a straight line through the cells with a width of ~1 mm, perpendicular to the plate surface. After washing the 6-well plate with PBS to remove non-adherent cells, different concentrations of DIM (0, 40, 60 and 80 μM) in serum-free medium were added to each well. The scratch width in each well was recorded under a light microscope at 0 and 12 h. The cell migration rate (%) was calculated as follows: (0 h scratch width-12 h scratch width)/0 h scratch width, and assessed using ImageJ V1.8.0 (National Institutes of Health).

Protein extraction and immunoblotting. After the TE-1 cells were washed 2-3 times with pre-cooled PBS (cat. no. MA0015; Dalian Meilun Biology Technology Co., Ltd.), the cells were lysed with cell lysis buffer (PMSF:RIPA, 1:100; PMSF cat. no. ST505; Beyotime Institute of Biotechnology; RIPA cat. no. P0013B; Beyotime Institute of Biotechnology). The resulting precipitate was scraped away and incubated on ice for 15 min, after which the supernatant was collected by centrifugation in an Eppendorf microcentrifuge at 4,747 x g for 15 min at 4˚C. The protein content was detected by the BCA kit, and the total protein amount in each group was adjusted to a consistent amount using 5X loading buffer (cat. no. P0015L; Beyotime Institute of Biotechnology). Equal amounts of protein (50 mg) were subjected to 12% SDS-PAGE, then transferred to polyvinylidene difluoride membranes (cat. no. FFP22; Beyotime Institute of Biotechnology). The membranes were incubated with 5% skimmed milk [prepared in tris-buffered saline (TBS) containing 0.05% Tween-20] at room temperature for 2 h, and then with STIM1, Bax, Bcl-2 and GAPDH antibodies at 4˚C overnight. The membranes were then incubated with the corresponding secondary antibody for 1 h at 4˚C. All primary and secondary antibodies were diluted with TBS containing 0.1% Tween-20 at 4˚C. Protein bands were visualized using BeyoECL Plus (cat. no. P0018S; Beyotime Institute of Biotechnology) and analyzed by ImageJ V1.8.0 (National Institutes of Health).

Statistical analysis. Each experiment was repeated three times to obtain more stable results. Statistical analysis was performed using GraphPad Prism 9.0 (Dotmatics). The results are presented as the mean ± SD. One-way ANOVA or Kruskal-Wallis followed by the Tukey or Nemenyi test post hoc tests, respectively, were used to assess the significant differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DIM and esophageal cancer protein interaction network. Through the retrieval of data from relevant databases, a total of 289 potential targets of DIM and 18,441 potential esophageal cancer targets were obtained. Through cross-analysis, 253 cross-targets were identified (Fig. 1A). To further determine the possible core targets of DIM acting on esophageal cancer cells, STRING was used to construct a protein-protein interaction (PPI) network of related cross-targets (Fig. 2). Next, the PPI results were imported into Cytoscape and the Centiscape 2.2 plug-in was used to analyze the results, and 39 possible core targets were obtained (Fig. 1B).

GO and KEGG pathway enrichment analyses. The GO-BP results showed that core targets mainly involved biological processes such as 'cell transcription', 'signal transduction', 'RNA polymerase II regulation', 'apoptosis regulation' and 'smooth muscle cell proliferation regulation' (Fig. 3A). The GO-CC results showed that core targets mainly involved cell components such as 'nucleolus', 'endoplasmic reticulum', 'cytoplasm' and 'chromatin' (Fig. 3B). The GO-MF results showed that core targets mainly involved molecular functions such as 'protein homodimerization activity', 'chromatin binding', 'protein kinase binding' and 'ubiquitin protein ligase binding' (Fig. 3C). The KEGG results showed that core targets were involved in a number of neurodegenerative diseases,

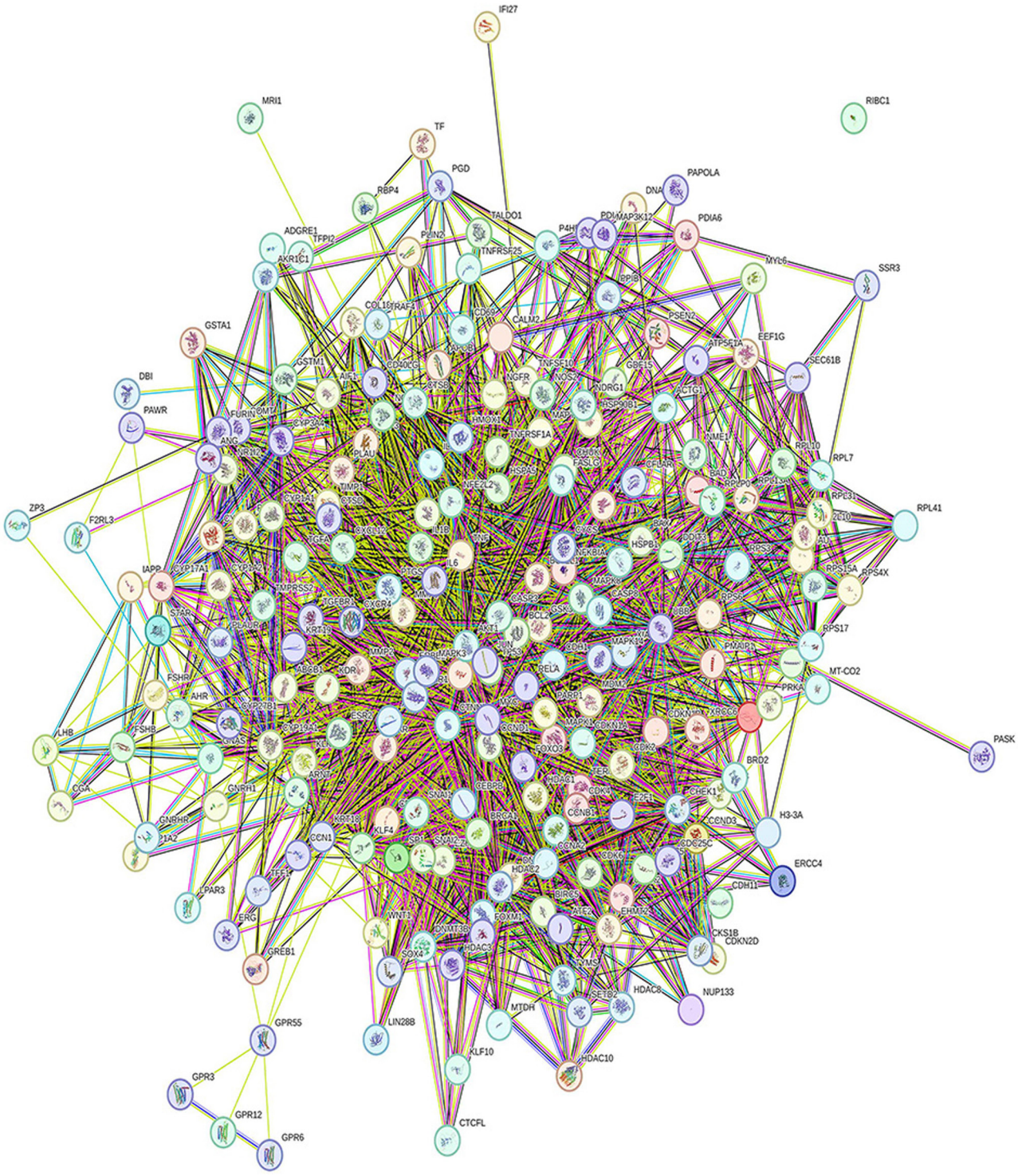

Figure 2. Protein-protein interaction network diagram of 3,3'-diindolylmethane in the treatment of esophageal cancer, as drawn by the STRING database (different circles represent nodes corresponding to various proteins, with the connections indicating diverse interactions between them).

'PI3K-Akt signaling pathway', 'Prostate cancer', 'Apoptosis', 'IL-17 signaling pathway' and 'Lipid and atherosclerosis' (Fig. 3D). The network analysis of signaling pathways and core targets showed the relationship between target pathways and core targets. For example, the 'PI3K-Akt signaling pathway' was related to the expression of 'Bcl-2' (Fig. 4).

DIM inhibits the viability of TE-1 esophageal cancer cells. Cells in the logarithmic growth phase were incubated with different concentrations of DIM (0, 40, 60 and 80 μM) for 24 h, then the cell viability was detected by the CCK-8 method. The results showed that DIM decreased the cell viability of TE-1 esophageal cancer cells in a concentration-dependent manner, which was statistically significant at DIM concentrations of 60 and 80 μM ($P<0.05$; Fig. 5B).

DIM inhibits the migration of TE-1 cells. To verify the effect of DIM on the migration of TE-1 cells, a scratch assay was

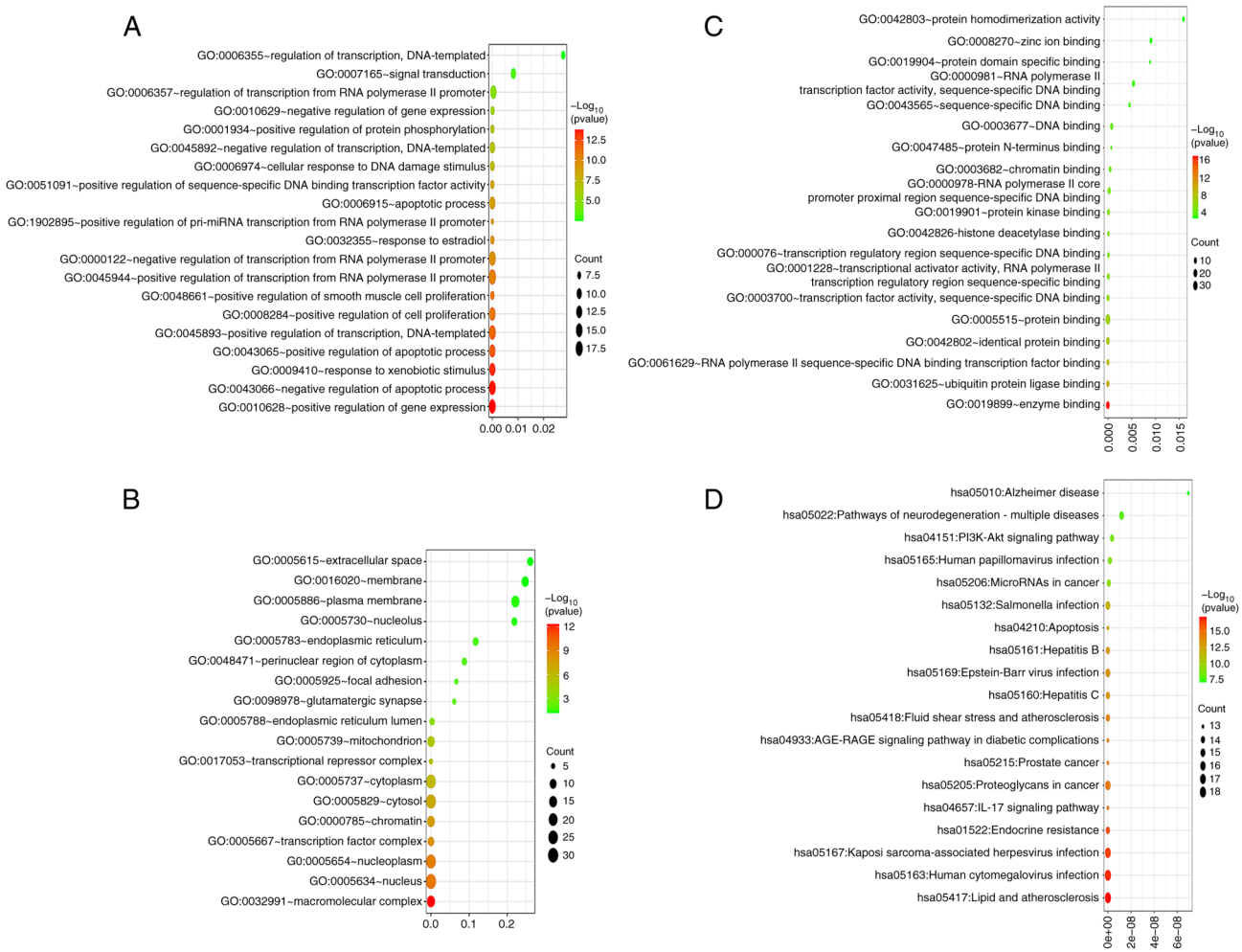

Figure 3. GO and KEGG pathway enrichment analyses were performed using the selected core targets. (A) The top 20 results of the GO-Biological Process analysis (P>0.05 hits were excluded). (B) The top 20 results of the GO-Cell Composition analysis (P>0.05 hits were excluded). (C) The top 20 results of GO-Molecular Function analysis (P>0.05 hits were excluded). (D) The top 20 results of the KEGG analysis (P>0.05 hits were excluded). GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

used. The results showed that DIM inhibited the migration of TE-1 cells in a concentration-dependent manner, which was statistically significant at DIM concentrations of 40, 60 and 80 μM (P<0.05; Fig. 6A and B).

DIM promotes apoptosis in TE-1 cells. To investigate the way in which DIM affects TE-1 esophageal cancer cell viability, the cells were treated with different concentrations of DIM (0, 40, 60 and 80 μM) for 24 h, then the protein expression levels of Bcl-2 and Bax were analyzed by western blotting. The results showed that DIM induced a concentration-dependent decrease in Bcl-2 protein expression in TE-1 cells, while it caused a concentration-dependent increase in Bax protein expression, both of which were statistically significant at DIM concentrations of 60 and 80 μM (P<0.05; Fig. 7A-C).

DIM upregulates the expression of STIM1 protein. The results of network pharmacology suggested that DIM may affect esophageal cancer cells by regulating the PI3K-Akt pathway. Endoplasmic reticulum stress is the upstream pathway of the PI3K-Akt pathway, and STIM1 is one of the key proteins of endoplasmic reticulum stress (20). Therefore, STIM1 was used as the next research object in the present study. While determining whether DIM could promote apoptosis, at the same time, the changes in STIM1 expression in TE-1 human esophageal cancer cells treated with different concentrations of DIM (0, 40, 60 and 80 μM) were also investigated using western blotting. The results showed that DIM downregulated the expression of STIM1 protein with a certain concentration dependence, which was statistically significant at DIM concentrations of 60 and 80 μM (Fig. 8A and B).

Tg counteracts the changes to STIM1, Bcl-2 and Bax protein levels included by DIM in TE-1 cells. Based on the aforementioned results, to further verify whether DIM promotes apoptosis and inhibits the viability of TE-1 cells by downregulating the expression of STIM1, cells were co-treated with Tg (to upregulate the expression of STIM1) and 80 μM DIM. After detecting cell viability using CCK-8, it was found that the inhibition effect of DIM on TE-1 cells was reverted, which was statistically significant (P<0.05; Fig. 9A). Following upregulation of STIM1 using Tg, the downregulation of bcl-2 and STIM1 was alleviated, while the role of

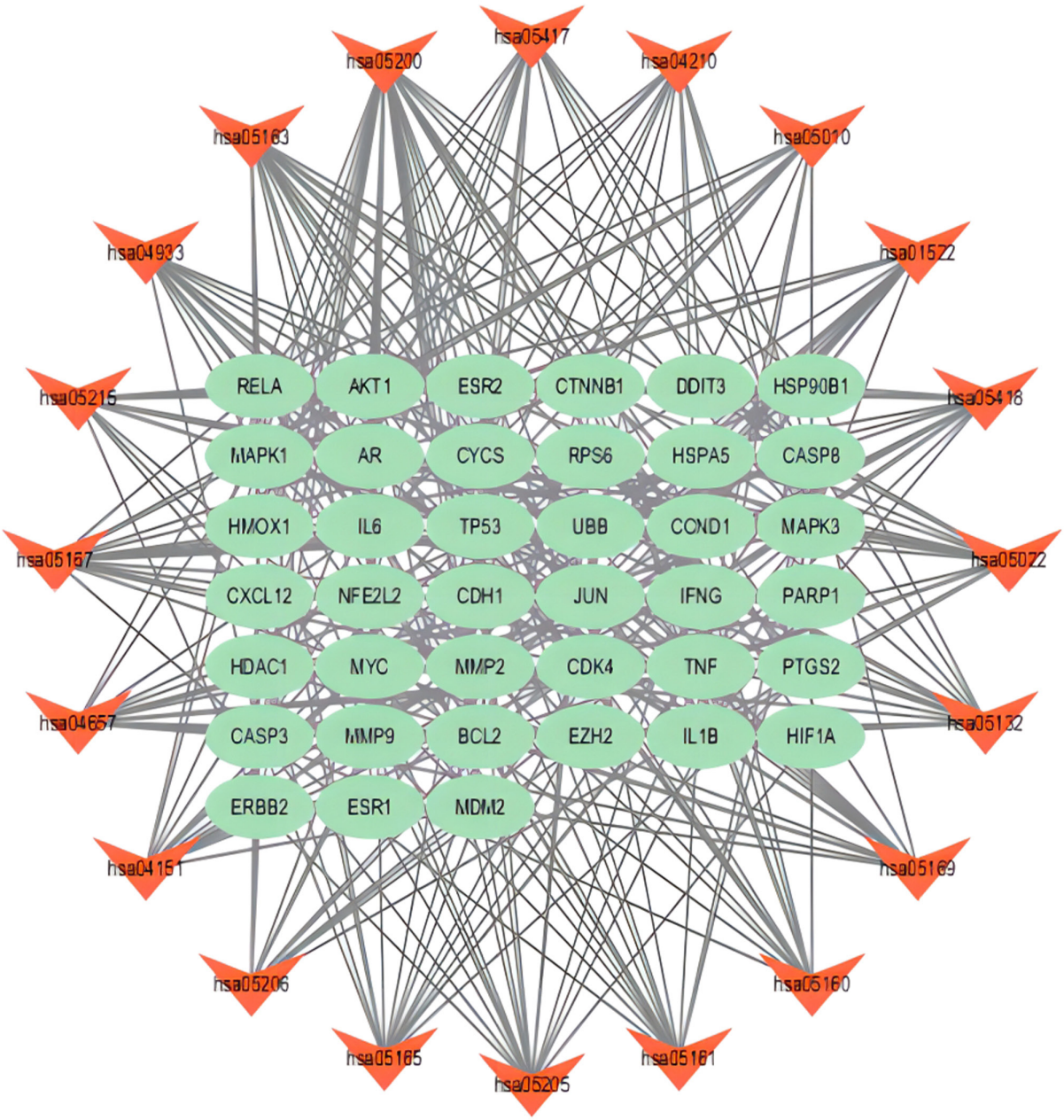

Figure 4. Relationship between DIM acting on the core target of esophageal cancer and the core targeting pathway.

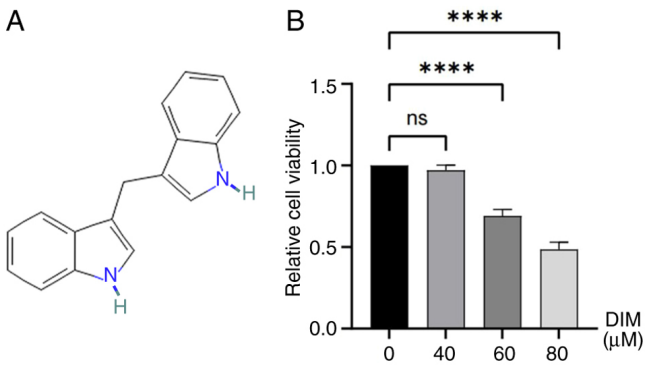

Figure 5. Effect of DIM on cell viability. (A) The structure of DIM. (B) TE-1 cells were treated with different concentrations of DIM (0, 40, 60 and 80 μM) for 24 h, then the cell viability was detected by Cell Counting Kit-8 assay. ****P<0.0001 vs. 0 μM, by Kruskal-Wallis followed by Nemenyi test. DIM, 3,3'-diindolylmethane; ns, not significant.

bax expression was suppressed compared with that in the DIM group, and the differences were statistically significant (P<0.05; Fig. 9B and C).

Discussion

Esophageal cancer is a highly aggressive gastrointestinal tumor and, since the clinical manifestations of patients in the early stage of disease are not typical, patients are often in the middle to late stages when they present with obvious symptoms. This leads to a poor prognosis if treating with surgery alone; therefore, comprehensive treatment of esophageal cancer has become the standard treatment option (21). However, the adverse effects of conventional neoadjuvant therapy may aggravate the tumor burden of patients further (6,7). In the present study, it was found that DIM, a natural phytochemical

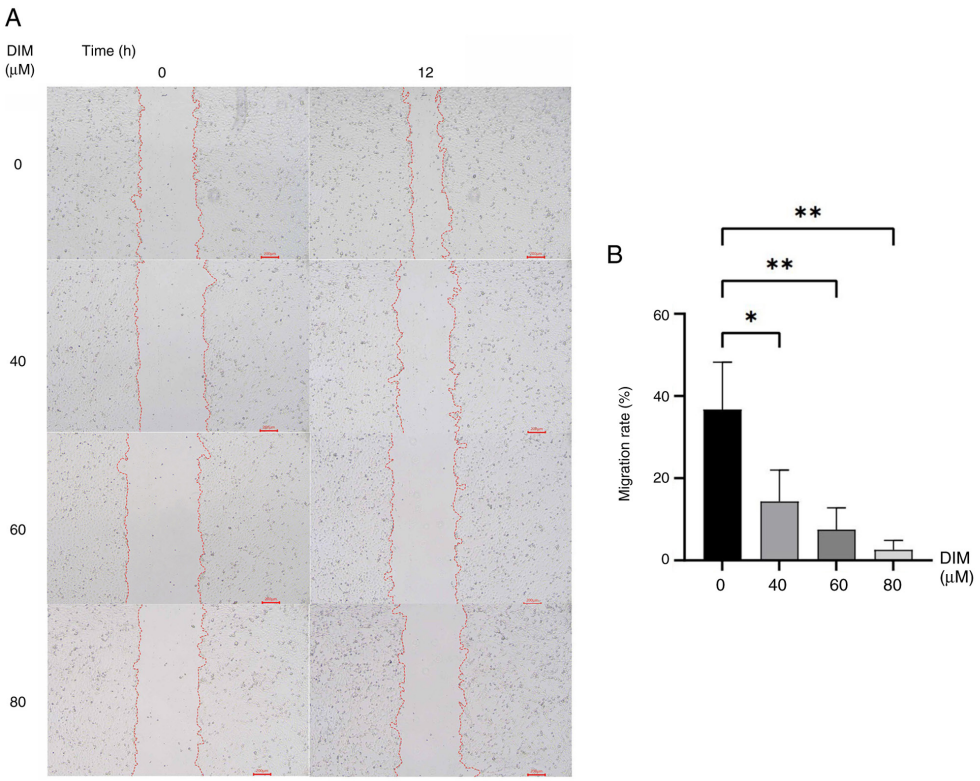

Figure 6. Effect of DIM on the migration of TE-1 cells. (A) Images of cells under the microscope after scratch formation. (B) Quantitative analysis of the scratch closure. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 vs. 0 μM, by one-way ANOVA followed by the Tukey test. DIM, 3,3'-diindolylmethane.

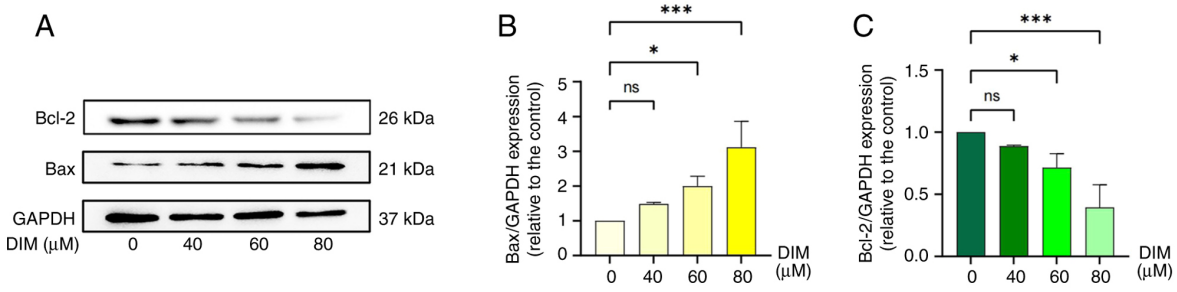

Figure 7. Effect of DIM on apoptosis in TE-1 cells. (A) Cells were treated with different doses of DIM (0, 40, 60 and 80 μM) for 24 h, then the levels of Bcl-2 and Bax were detected by western blotting. Semi-quantitative analysis of the (B) Bax and (C) Bcl-2 protein levels. data are presented as the mean ± SD of three independent experiments. *P<0.05, ***P<0.001 vs. the control (0 μM), by one-way ANOVA followed by the Tukey test. Bcl-2, B-cell lymphoma-2; DIM, 3,3'-diindolylmethane; ns, not significant.

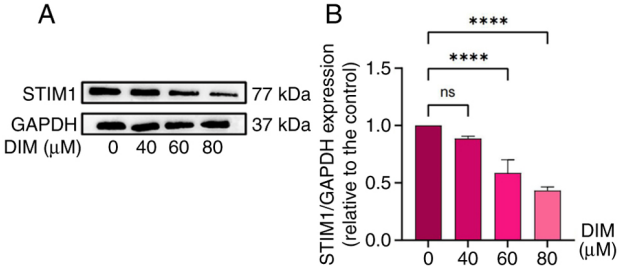

Figure 8. Effect of DIM on STIM1 expression in TE-1 cells. (A) Cells were treated with different doses of DIM (0, 40, 60 and 80 μM) for 24 h, then the levels of STIM1 were detected by western blotting. (B) Semi-quantitative analysis of STIM1 protein levels. Data are presented as the mean ± SD of three independent experiments. ****P<0.0001 vs. the control (0 μM), by one-way ANOVA followed by the Tukey test. DIM, 3,3'-diindolylmethane; ns, not significant.

from cruciferous plants, induced apoptosis and inhibited the viability of esophageal cancer cells by downregulating the expression of STIM1, which provides a new theoretical direction for the early prevention and treatment of esophageal cancer using DIM.

Through KEGG analysis, it was found that DIM may be involved in the apoptosis, IL-17 and PI3K-Akt signaling pathways. A study has shown that TP53 and IL-17 can affect the proliferation and migration of esophageal cancer cells (22). Furthermore, PI3K-Akt can affect the proliferation, invasion and metastasis of a variety of tumor cells. A number of mutations in PI3K-Akt are known to exist in patients with esophageal cancer, and inhibition of the PI3K-Akt pathway can reduce the resistance of esophageal cancer to chemotherapy drugs (23-25). In addition, the PI3K-Akt pathway can inhibit

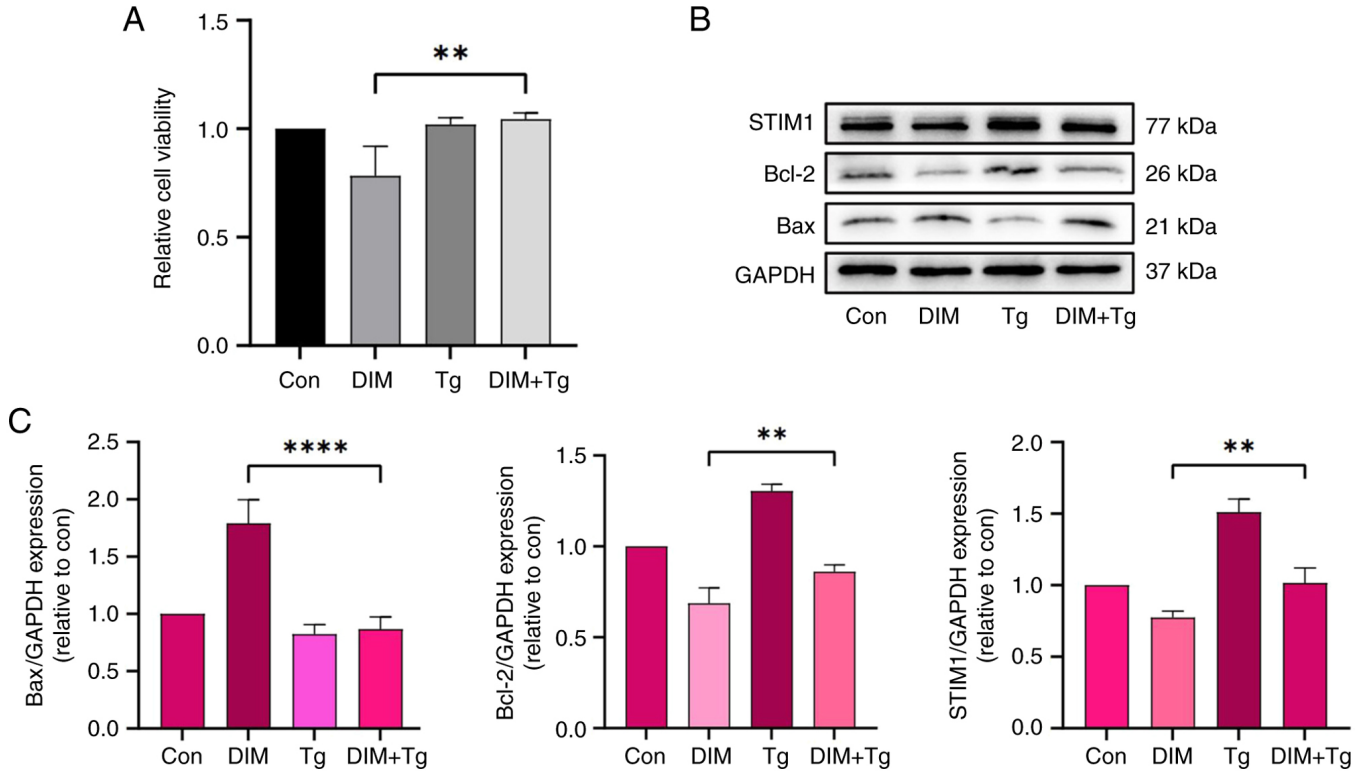

Figure 9. Effect of Tg on STIM1, Bcl-2 and Bax protein levels in TE-1 cells incubated with DIM (control group cells cultured without DIM for 24 h). (A) Cells were treated with DIM (80 μM) with or without Tg (1 μM) for 24 h, then cell viability was measured using the CCK-8 assay. Data are presented as the mean ± SD of three independent experiments. **P<0.01 vs. con, by Kruskal-Wallis followed by Nemenyi test. (B) The Bax, Bcl-2 and STIM1 expression levels were evaluated by western blotting following treatment with DIM and Tg. (C) Semi-quantitative analysis of the Bax, Bcl-2 and STIM1 protein levels following treatment with DIM and Tg. Data are presented as the mean ± SD of three independent experiments. **P<0.05, ****P<0.0001 vs. con, by one-way ANOVA followed by Tukey test. Bcl-2, B-cell lymphoma-2; con, control; DIM, 3,3'-diindolylmethane; Tg, thapsigargin.

endoplasmic reticulum stress and induce cell death (26). STIM1 participates in the stability of Ca^{2+} in the endoplasmic reticulum and participates in endoplasmic reticulum stress (27). Therefore, in the present study, the upstream regulatory signal of the PI3K-Akt pathway-endoplasmic reticulum stress signal, represented by STIM1, was studied to determine the effect of DIM on esophageal cancer cells (20). As a powerful pharmacological analysis tool, network pharmacology fully integrates the knowledge of system biology, pharmacology, information networks and computer science to explain the relationship between drugs, diseases, and argets (28). However, there were some limitations to the pharmacology network analysis performed in the present study. Due to the different inclusion criteria of studies, the comparability, integrity and reliability of data may differ between different databases, which may lead to certain differences in the relationship between data from different sources and between drugs and disease targets. In addition, the relationship between drug targets and disease targets is constantly updated, which may lead to a certain deviation in the results of the database. This may explain why STIM1 was not among the identified core targets.

Apoptosis is a type of programmed cell death. The Bcl-2 gene family is one of the important factors that causes apoptosis through the mitochondrial pathway, and Bax is one of its important members (29). Both Bcl-2 and Bax are located in the mitochondria and endoplasmic reticulum. The enhanced expression of Bcl-2 can promote cell apoptosis, while Bax has the opposite effect (29,30). In the present study, western blotting was used to demonstrate that DIM downregulated the expression of Bcl-2 and upregulated the expression of Bax in a concentration-dependent manner. After stimulation of STIM1 expression with Tg, this effect was prevented.

Through GO analysis, it was found that DIM may act on cellular components such as the endoplasmic reticulum. STIM1 is a transmembrane Ca^{2+}-binding phosphoprotein located on the endoplasmic reticulum membrane. STIM1 acts as a Ca^{2+} sensor in the endoplasmic reticulum cavity and participates in the regulation of the SOCE pathway (31). Studies have shown that SOCE has a certain role in the occurrence and development of tumors. In breast cancer cells, α-glucosidase inhibitors can significantly reduce the expression level of STIM1, thereby weakening the expression of SOCE to inhibit breast cancer cell viability (32). In addition, downregulation of STIM1 expression can inhibit the migration and invasion of thyroid cancer cells, and it can restore the expression of thyroid-stimulating hormone receptors (33). Tg is an irreversible endoplasmic reticulum Ca^{2+}-ATPase binder, which can induce endoplasmic reticulum protein folding disorder, leading to the accumulation of unprocessed proteins in the endoplasmic reticulum. This causes dysfunction of the endoplasmic reticulum and activation of SOCE, which is typically regarded as an agonist of endoplasmic reticulum stress (34). STIM1 is one of the key proteins of SOCE. Tg can empty the endoplasmic reticulum Ca^{2+} pool, thereby indirectly activating the expression of STIM1, which leads to extracellular Ca^{2+} entering the cell to maintain the balance of intracellular and extracellular Ca^{2+} (35). Therefore,

Tg can be regarded as an agonist of STIM1 protein. In the present study, the expression of STIM1 protein in TE-1 cells pretreated with Tg was restored compared with that of DIM alone. Therefore, we hypothesize that DIM can overload Ca^{2+} in the endoplasmic reticulum of TE-1 human esophageal cancer cells, thus causing apoptosis of these cells. However, further research is required to confirm this.

However, there are still some shortcomings to the present study. The aim of the present study was limited to the examination of esophageal squamous cell carcinoma and did not involve other pathological types of esophageal cancer. In addition, the present study only included experiments at the cellular level and was not extended to experimental mice and patients. The present study preliminarily verified that DIM affected the oncology-related characteristics of esophageal cancer cells by regulating the expression of STIM1 protein, which is a transmembrane protein located on the endoplasmic reticulum membrane that regulates calcium homeostasis. However, whether there is a change in the concentration of Ca^{2+} inside and outside of the endoplasmic reticulum during the anti-esophageal cancer activity of DIM requires further study. In addition, whether other tumor suppression methods, such as ferroptosis and pyroptosis, are influenced by DIM still needs further exploration. It has also been demonstrated that DIM has reproductive toxicity in mice, that is, it can inhibit the secretion of related sex hormones in male mice, thereby reducing the quality of sperm (36). At present, study of the antitumor activity of DIM remains in the cell and animal experiments stage, and no drugs for human consumption have been developed. However, DIM-related side effects still need to be further confirmed by a large number of clinical experiments after careful evaluation by pharmacologists and physicians.

In conclusion, the results of the present study indicated that DIM promoted apoptosis and inhibited the viability of esophageal cancer cells by downregulating the expression of STIM1. Therefore, the natural phytochemical, DIM, may be a potential substance for the early prevention and treatment of esophageal cancer cells.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CX YY, FL and YT designed the study. CX, YT FL, YY and XL assisted with the data analyses. CX, XL and JL performed western blotting. CX and SD performed the CCK-8 assay. CX and SD performed the scratch test. CX wrote the initial draft of the manuscript. CX and XL contributed to the analysis and interpretation of the data. FL and YY assisted in the preparation and critical review of the manuscript. CX YY, FL and YT confirm the authenticity of all the raw data. All authors agreed to be accountable for all aspects of the work. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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