Virtual Screening and Reality Verification: Elemene Injectable Emulsion acts on the key targets and pathways of Colorectal Adenoma Cancerization

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Research Article

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Abstract

Purpose

Most colorectal cancer (CRC) is developed from intestinal adenomatous polyps. Therefore, it is urgent to find new therapeutic drugs to intervene intestinal adenoma development in CRC. ELEMENE INJECTABLE EMULSION (EIE) has been reported to exert antitumor activity in various digestive tumor diseases. However, the mechanism of EIE in preventing colorectal adenoma (precancerous lesions) from developing into CRC has not been systematically explored.

Methods

Using network pharmacology correlation analysis and molecular docking, the central target of EIE in preventing colorectal adenoma (CRA) from transforming into cancer through innate immunity was excavated and verified. The differentially enriched pathways of human CRA, CRC, and corresponding adjacent tissue samples were analyzed by reverse-phase protein array (RPPA) to verify the relevant mechanism. Colon cancer cells were intervened to observe the proliferation, apoptosis, and cell cycle in different concentrations of EIE. The predicted related targets were verified by RT-PCR (real-time PCR), and the pathways were confirmed by Western blot.

Results

The analysis results show that Retinoid X Receptor alpha (RXRa) was the key target gene, and the main pathway was PI3K/Akt. Molecular docking results show that β- Elemene, γ-Elemene, and δ- Elemene have a strong affinity for RXRa. RPPA technology was used to analyze the functional enrichment of the differentially expressed genes of the Adenoma Cancer sequence, Adenoma Paracancerous sequence, and Cancer-Paracancerous sequence. The enrichment results of the three groups of sequence differential genes showed that the PI3K/Akt signaling pathway was the most significant. In addition, based on HCT116 and THC8307 in vitro experiments, PI3K, p-PI3K, Akt, p-Akt, and RXRa proteins and the relative expression of RXRa mRNA in the EIE intervention group were studied, and the predicted results were verified.

Conclusion

This is also the first evidence that our data provide that elemene aims to target the PI3K-Akt signaling pathway and RXRa, a target gene to play its role in affecting the development of CRA in cancer through innate immunity.

1 Introduction

Previous clinical evidence has shown that CRA, the precancerous lesion of CRC, can develop into colorectal adenocarcinoma. The incidence of CRC is positively correlated with the incidence rate of CRA, and the incidence of the two diseases shows a younger trend (1, 2, 3). The primary treatment for CRA and
CRC in the early stage is endoscopic resection (4). There is no globally recognized drug to prevent CRA from developing into cancer. Therefore, in the prevention and treatment of CRC, it is urgent to find efficient and low toxicity drugs to improve this clinical dilemma.

EIE is an anticancer drug (5, 6) extracted from the natural herbal medicine wenyujin (Curcuma wenyujin, Y.H.Chen & C.Ling) to a clinical evidence-based basis. Wenyujin plays the characteristics of traditional Chinese medicine (TCM) in the prevention and treatment of cancer based on the principles of "enhancing immunity and fighting cancer" and "eliminating mass and preventing cancer" (7). Previous studies have shown that EIE effectively prevents and treats gastrointestinal cancer (8, 9, 10). Although elemene emulsion has been proven to reduce the invasive capacity of colorectal cancer cells by down-regulation of miR-155 mRNA expression (11), it can also inhibit the malignant proliferation and telomerase activity of colon cancer cells, induce apoptosis of colon cancer cells, and block cell cycle (12). However, the intervention of EIE on the Potential Molecular Mechanism of Human Colonic Adenoma Carcinogenesis is still poorly understood.

Network pharmacology is a frontier interdisciplinary drug systematic research. It is the first emerging discipline in traditional Chinese medicine to be formally formulated with international standards (13, 14). To comprehensively clarify the biological basis of EIE in the prevention and treatment of CRA carcinogenesis, combined with the characteristics of traditional Chinese medicine in EIE and related to the primary research in the process of colorectal "adenoma carcinogenesis" (15), we were conducted a network pharmacological study with EIE (main component: β- Elemene γ- Elemene and δ- Elemene (6)) to predict the mechanism of elemene acting on intestinal adenoma-cancer sequence related to innate immunity. The mechanism was verified by molecular docking technology. The colorectal adenoma VS cancer sequence was analyzed by RPPA (16). Combined with the in vitro experiment of EIE on CRC cells, these main mechanisms were verified, and the overall pharmacodynamic characteristics of elemene in preventing and controlling tumor occurrence and development through affecting innate immunity were discussed from a new perspective. It provides a basis for EIE to prevent and control CRC in the whole clinical process. Figure 1 shows the workflow of the prediction and analysis process.

2 Materials and Methods

2.1 Mechanism prediction according network pharmacology

2.1.1 Targets from public databases

Taking EIE as the research object, relying on TCMSP database (http://lsp.nwsuaf.edu.cn/tcmsp.php) (17), through Swiss target prediction database (http://www.swisstargetprediction.ch/) (18), STITCH Version 5.0 Database (http://stitch.embl.de/) (19), Target Prediction Data (http://prediction.charite.de/index.php?site=chemdoodle_search_target) (20) and Pharmmapper Database (http://www.lilab-ecust.cn/pharmmapper/) (21), and the corresponding targets of three effective active components of EIE
were retrieved. Through UniProt database (http://www.uniprot.org/)(22, 23), the targets' names were corrected and unified, the species were classified as Homo sapiens (humans), and EIE corresponding target database was constructed.

Taking colorectal adenoma (CRA), colorectal cancer (CRC), and innate immunity as research objects, relying on OMIM(https://omim.org/)(24), TTD(http://db.idrblab.net/ttd/) (25), PharmGkb(https://www.pharmgkb.org/)(26), DiGSeE(http://210.107.182.61/geneSearch/)(27) public database, retrieve the targets corresponding to the three research objects, and construct the targets database of the research objects. The targets data set of the study object was summarized in Supplementary Material(S1).

2.1.2 Seekning of key targets

To clarify the connection of target proteins involved with both EIE, CRA, CRC, and innate immunity, we intersected the potential targets of therapeutic targets, and the overlapping targets were considered EIE-treated targets of intestinal adenoma carcinogenesis through an innate immunity way. Putting the target database of research objects (β-elemene, γ-elemene, δ-elemene, and innate immunity, CRA, CRC) on the bioinformatics & evolutionary genomics platform(http://bioinformatics.psb.ugent.be/webtools/Venn/) (28). Seeking the key targets to prepare for further systems biology analysis and molecular docking. Summarize the obtained information in Supplementary Material(S2).

2.1.3 Construction of protein-protein interaction (PPI) network

To explain the biological role of the key targets, we uploaded the key targets to the genemania database(http://genemania.org/)(29), and obtained PPI information (PPI analysis results were listed in supplementary table(S3)). We were prepared for GO enrichment and KEGG pathway analysis. The correlation between the key proteins and proteins in the PPI network was analyzed through the gepia database(http://gepia.cancer-pku.cn)(30) to seek the most likely interacting protein targets.

2.1.4 Go enrichment and KEGG pathway

To illustrate the role of key targets in gene function and signal pathways, We used David 6.8 database(https://david.ncifcrf.gov/)(31). GO function and KEGG pathway enrichment analysis were performed on the most likely associated protein targets. Detailed GO and KEGG pathway enrichment analysis data are listed in Supplementary table(S4).

2.2 Molecular Docking

Molecular docking can reflect the binding energy between drug molecules and protein receptors, and can visualize the interaction between molecules(32). Therefore, to understand the affinity between the compounds and the targets, we used chem bio draw ultra (33) to draw the compound γ-elemene δ-elemene, and β-elemene's structure was then transformed into a three-dimensional structure with chembio3d ultra (34) and optimized using mmff94 force field. Download the crystal structure of the key
target from RCSB Protein Data Bank (www.rcsb.ORG) (35), and use autodock Vina 1.1.2 (36, 37) for molecular docking. The binding affinity information is summarized in supplementary table(S5).

2.3 Seeking Mechanism using RPPA technology

Reverse phase protein microarray (RPPA) technology is a highly sensitive and high-throughput detection method that can detect the expression and phosphorylation modification level of a variety of target proteins in minor quality samples. In recent years, it has been widely used in tumor research. For example, in the study of the tumor microenvironment, it could be found that there were differences in protein expression between tumor cells and normal cells, and between tumor stromal cells and normal stromal cells (38). It found key biomarkers with prognostic importance in the molecular typing of CRC (39). It was also widely used to evaluate tumor drug treatment effects and the study of a tumor-related drug resistance mechanism (40, 41).

2.3.1 Preparation of tissue samples

All patients were diagnosed with CRC by histological staining of intestinal tissue. Patients were staged according to the American Joint Committee on Cancer (AJCC) tumor lymph node metastasis (TNM) staging system (8th Edition). The pathological characteristics of the patients are summarized in Supplementary table(S6). Three intestinal tissue samples were collected from each of the five patients with primary colorectal cancer (primary CRC, adjacent to cancer, accompanied by intestinal adenoma polyps, n = 15), all of which were agreed by the patients, and the ethics committee approved the test scheme of the corresponding hospital. Intestinal tissue is the remaining part after completing the clinical diagnose requirements. It is preserved by Allprotect (Nucleic Acid and Protein Stabilization Reagent for Animal Tissue) reagent (beyotime, SH, CHN) and tested in strict accordance with the principles of the Helsinki Declaration, which does not violate the regulations of the people's Republic of China on the administration of human genetic resources. This experimental plan has been approved and approved by the Ethics Committee of Taicang Traditional Chinese Medicine Hospital, and has obtained the informed consent of all patients/or their legal guardians.

2.3.2 Differential protein data acquisition and pathway enrichment analysis by RPPA technology

The quality of the sample is controlled by protein quality (the protein content is more significant than 1.5 µg/µl). Serial gradient dilution (1/1, 1/2, 1/4, 1/8, 1/16) was carried out with fluent 480/780 (Tecan, Swit), and more than 350 protein microarray chips were prepared with 2470 arrayer high-throughput microarray printing equipment (Quanterix, MA, USA). On the autostainer link 48 automatic staining instrument (Agilent Technologies, Santa Clara, CA, USA), The antibody in the cancer signaling pano profiler cancer signal panoramic analysis panel (FynnBio, SD, CHN) was used to stain the microarray chip (one antibody stained one chip). After staining, the image signal was captured by a tissuescope le120 high-throughput chip scanner (Huron Digital Pathology, Inc., CAN). Finally, the image information were converted into digital information by Microvigene analysis software (MicroVigene Version 5608, VigeneTech, MA, USA) for
normalization and quality control. We analyzed the protein expression data obtained by RPPA technology, annotated and classified the differentially expressed proteins in the KEGG database, and presented the enrichment results in a significantly enriched KEGG scatter diagram. RPPA technology and data analysis are assisted by FYNBIO Technology (SD, CHN). The differentially expressed protein data are summarized in (S7).

2.4 Pathway and target verification based on experiment

2.4.1 Cell lines and cell culture

In this experiment, we used HCT-116 (human poorly differentiated colon adenocarcinoma cell) cell line (Fenghui Biotechnology Co., Ltd.) and THC-8307 (human highly differentiated colon adenocarcinoma cell) cell line (Fenghui Biotechnology Co., Ltd.). The cells were maintained in Dulbecco’s modified eagle’s medium (GIBCO, Thermo Fisher Scientific, Ma, USA), supplemented with 10% fetal bovine serum (GIBCO, Thermo Fisher Scientific, Ma, USA) and penicillin streptomycin (GIBCO, Thermo Fisher Scientific, Ma, USA), and then incubated in a humid atmosphere of 37°C and 5% CO2. The medium was changed every 24 hours and the logarithmic growth cells were recorded for the experiment.

2.4.2 Reagents and antibodies

EIE (Dalian Huali Jingang Pharmaceutical, DL, CHN). BCA (Pierce Rapid gold BCA protein assay kit) was purchased from Thermo Fisher (a53225, BJ, CHN), and ECL (supersignal) West femto substrate trial Kit was purchased from Thermo Fisher (34095, SH, CHN). The following antibodies were used: anti-rabbit IgG HRP-linked antibody (7074), anti-mouse IgG, HRP-linked (7076) antibody, p-pi3k (4228), Akt (4691) and p-Akt (4060) were obtained from the cell signaling technology. PI3K (Ab133595) and RXRa (Ab125001) were obtained from Abcam. Mouse anti-β-actin (A5441) was obtained from Sigma-Aldrich.

2.4.3 Cell proliferation

In 96 well culture plates, cells in the logarithmic growth stage were inoculated at the density of 5000 cells per well and cultured for 24 hours. Then replace the medium with a mixed medium containing different concentrations of EIE (add 100ul per well) and incubated in the incubator for 24, 48, and 72 hours. After incubation, suck out the medium of each well, add CCK-8 mixed medium and incubate for 3 hours, and determine the cell viability (beyotime, c0037) by cell counting kit-8 (CCK-8). After incubation, we used a MultiSkan GO microplate spectrophotometer (Thermo Fisher Scientific, MA, USA) to measure the absorbance of the samples at 450 nm.

2.4.4 Cell cycle and apoptosis

Flow cytometry detected the apoptosis and cell cycle of HCT116 and THC8307 cells induced by different concentrations of EIE were detected by flow cytometry. Harvested Cells, which were treated with different concentrations of EIE for 72 h, and then stained using Annexin V-FITC/PI stain kit following the manufacturer’s instructions (TransGen Biotech). The stained cells were analyzed with flow cytometry (BD, New Jersey, USA). For apoptosis, annexin V-fluorescein isothiocyanate/propidium iodide (annexin V-
FITC/PI) apoptosis detection kit (fa111, transgen biotech, BJ, CHN) was used according to the manufacturer's instructions. Apoptosis was detected by flow cytometry (accuri C6, BD, USA). For cell cycle analysis, the cells were fixed overnight with ice-cold 70% ethanol at 4 °C and then incubated with RNaseA and 100 UG/ml propidium iodide (PI) in the dark for 30 minutes at room temperature. The cell cycle was detected by flow cytometry.

2.4.5 RT-qPCR

Total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNA was synthesized using hiscript II Q select RT supermax for qPCR (+ gDNA wiper) kit (vazyme). Quantitative PCR (qPCR) and real-time PCR detection system (bio RAD) were performed using CFX96 Touch TM. All primers were synthesized by SANGON biotech (SH, CHN), and all primer sequences used in qPCR were shown in Supplementary table (S8). Quantified the transcription by levels of GAPDH as an internal control and analyzed the target gene fold-changes by the $2^{-\Delta\Delta Ct}$ method.

2.4.6 Western blotting

Cells were lysed with Ripa lysis buffer supplemented with the protease inhibitor (a32955, Thermo). Then, protein concentration was determined by BCA protein determination kit (a53225, Thermo). Gel electrophoresis (SDS-PAGE) was used to separate the total proteins of the samples. The proteins were transferred to the PVDF membrane and blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline containing Tween-20) at room temperature for 1 hour. The transferred membranes were incubated overnight with the primary antibodies against PI3K, p-PI3K, AKT, p-AKT, RXRa, and β-actin at 4°C. The membranes were then washed three times with TBS-T and incubated with secondary antibody anti-rabbit(or anti-mouse) IgG HRP-linked antibody (7074, 7076) at room temperature for 1 hour. The protein signals were recorded by the ECL chemiluminescence detection system (Bio-RAD).

2.5 Statistical Analysis

Data are expressed as percentage means and standard deviations (SDs), calculated from three independent experiments. The statistical analysis was performed using GraphPad Prism 8.0 (GraphPad). We determined statistical differences between groups using Students' t-tests. Statistical significance was declared at P-value < 0.05: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

3 Results

3.1 Establish data sets and seek key targets

We found the targets of the six research objects in the public database as presented below. There are 3279 targets corresponding to CRA, 4119 targets corresponding to CRC, and 3672 targets corresponding to innate immunity. β- Elemene has 79 corresponding marks, γ- Elemene has 213 related targets, δ- Elemene has 28 corresponding targets. Afterward, we took the intersection of the target data sets of the six research objects (Fig. 2A). We found that RXRa would be the key target of EIE, affecting innate
immunity to prevent and control the carcinogenesis and development of CRA, which was selected to prepare for further systematic biological analysis and molecular docking.

3.2 PPI Network

We uploaded the key target RXRa to the GENEMANIA database to obtain the protein interaction network of the target genes (Fig. 2-B). Twenty proteins are interacting with the key target RXRa in the network, of which 67.64% are physical interaction related proteins, 13.50% are co-expression related proteins, 6.35% are predicted related proteins, 4.35% are pathway-related proteins, and 1.40% are genetic interactions related proteins, shared protein domains related proteins accounted for 0.59%. The 20 interacting proteins in Fig. 2-B were analyzed with the key target proteins through the correlation analysis function in the GEPIA database (Fig. 2-C), and 17 closely related interacting proteins (P < 0.05) were selected (RARa, FAM120B, NR4A1, THRA, NR1H4, CCL23, CYP3A4, NCOA4, ZBTB16, PPARα, TBL1X, TBL1XR1, NR1I3, CXADR, VDR, NR1H2, and RARB).

3.3 GO and KEGG analysis results

The 17 closely related interacting proteins and key protein targets were uploaded to David 6.8 database for go and KEGG analysis. GO annotation analysis is carried out in three links: biological process, cell composition, and molecular function. Among them, 37 GO entries are involved in the biological process (Fig. 3-A), mainly involving steroid hormone-mediated signaling pathway, transcription from RNA polymerase II promoter, regulation of transcription, DNA template, cellular lipid metabolic process, retinoic acid receptor signaling pathway, internal receiver signaling pathway, negative regulation of cell promotion and signal transfer. There are six items related to cell composition (Fig. 3-B), mainly involving nucleoplast nucleus, transcriptional reporter complex, RNA polymerase II transcription factor complex, histone deacetylase complex, and spin microtubules. There are 26 items related to molecular function (Fig. 3-C), mainly involving steroid hormone receptor activity, RNA polymerase II transcription factor activity, organic activated sequence-specific DNA binding, sequence-specific DNA binding, zinc-binding, retinoid X receptor binding, transcription factor activity, sequence-specific DNA binding, retinoic acid receptor activity, etc. KEGG pathway enrichment analysis yielded three signal pathways (Fig. 3-D), including pathways in cancer, transcriptional misregulation in cancer, and thymoid cancer. These findings suggest that the regulation of retinoic acid and steroid hormone receptors or pathways in cancer may be the primary mechanism of EIE in innate immunity to prevent colorectal adenoma, carcinogenesis.

3.4 Molecular docking verification

To elucidate the mode of action of central genes (RXRa) and compounds at the molecular level. We docked γ-elemene, δ-elemene, and β-elemene to the active pocket of RXRa. The theoretical binding mode is shown in Fig. 4A. In its interaction mode, the small hydrophobic molecules γ-elemene, δ-elemene and β-elemene bind closely to the hydrophobic cavity of protein RXRa, and the two have good matching before, which makes it possible for small molecules to bind here. The affinity score between β-elemene and RXRa is -7.9kcal/mol, γ-elemene and RXRa is -7.7kcal/mol, δ-elemene and RXRa is -7.5kcal/mol. These hydrophobic interactions make the protein RXRa and the compound γ-elemene, δ-elemene, and β-
Elemene form stable complexes. The interaction of the above molecular docking studies with protein and compounds gives a reasonable explanation.

To further verify the discovery of molecular docking, we carried out a cell experiment in vitro. Two CRC cell lines intervened by EIE with different concentrations were treated. The changes in target proteins were determined by Western blot and PCR. The expression level of RXRa protein in THC8307 and HCT116 cells treated with EIE (60ug/ml) decreased significantly (P < 0.05) (Fig. 4B). In the quantitative test of RXRa mRNA levels using PCR, the mRNA level of RXRa in EIE (60ug/ml) treated THC8307 and HCT116 cells decreased significantly (P ≤ 0.0001), and the mRNA level of RXRa in elemene (20ug/ml) treated HCT116 cells also reduced significantly (P ≤ 0.0001) (Fig. 4C). (the supplementary picture of Fig. 4B is in the Supplementary Fig. 1)

3.5 Pathway enrichment analysis using RPPA Technology

The differentially expressed genes among CRC, CRA, and paracancerous tissues were detected by RPPA and annotated in the KEGG database. All nodes that significantly KEGG enrichment results were GeneRatio(the ratio of the total number of genes belonging to this pathway to the total number of differential genes), the larger the value, the bigger the node, indicating the more differential genes enriched in this pathway. The enrichment network diagram was sorted according to - log10 (P-value), showing the first 20 most enriched path information. The node color from green to red indicates that the p-value increases in turn. The enrichment results suggested that the larger the network enrichment node was and the closer the color was to green, the more significant the enrichment was.

We performed an enrichment analysis on significant KEGG terms between CRC and paracancerous tissues, important KEGG terms between CRC and CRA tissues, and significant KEGG terms between CRA and paracancerous tissue. Observing the effective enrichment results of pathways in these three groups of sequences, we found that the top of each pathway enrichment was the PI3K-Akt signaling pathway(Fig. 5). The PI3K-Akt signaling pathway happens to be the classic pathway in pathways in cancer. This enrichment was verified with the top KEGG enrichment in network pharmacological prediction (Fig. 3D).

3.6 EIE inhibits the Proliferation and induces apoptosis of CRC Cells

To evaluate the inhibitory efficacy of elemene on the proliferation of CRC cell lines, cell viability was determined by the CCK8 kit. We determined the IC50 of Elemene in two cell lines, including human poorly differentiated colon adenocarcinoma cells(HCT116) and human well-differentiated colon adenocarcinoma cells (THC8307) at 72 h. HCT116 and THC8307 cells were treated with Elemene at 0ug/ml,20ug/ml, and 60ug/ml.Cell proliferative capacity was detected at 24, 48, and 72h (Fig. 6A and Fig. 6B). The results indicated the viability of HCT116 and THC8307 cells was markedly reduced in a time- and dose-dependent manner.
3.7 EIE Inhibited Proliferation by triggering Apoptosis and inducing cell cycle arrest of CRC Cells

For preliminary assessment whether apoptosis was involved in Elemene-induced cytotoxicity in CRC cells, we detected the morphology of Elemene-treated CRC cells, found obvious morphological changes such as cytoplasmic vacuole formation and cell overlapping and nuclear structure disorders that are typical of apoptosis in Intestinal adenocarcinoma(Fig. 6C). (the supplementary picture of Fig. 6 is in the Supplementary Fig. 2)

To further investigate whether Elemene exhibited an antitumor effect on CRC, Annexin V-FITC/PI stain and FACS was conducted in both THC8307 and HCT116 cells after Elemene treatment for 72h(Fig. 7A). The results revealed that elemene induced apoptosis of CRC cells in a significant dose-dependent manner, and the apoptosis increased especially in the high concentration groups(Fig. 7B). Cell cycle analysis (Figs. 7C and B) showed that elemene high-dose intervention group significantly increased the proportion of G1 phase cells and decreased the proportion of G2 phase cells in CRC cells.

3.8 EIE Inhibits the PI3K/AKt Pathway in CRC Cells

The PI3K/AKt pathway is a key intracellular receptor signaling pathway for EIE against CRC. It was found by Western blotting that EIE could affect the expression of phosphorylation of key proteins in the pathway.(as shown in Fig. 8E). As shown in Fig. 8B and Fig. 8C, under the influence of different concentrations of EIE drugs, the protein levels of PI3K and AKt did not show significant statistical significance. But in Figs. 8A and 8D, the protein levels of p-PI3K and p-AKt were significantly decreased in a drug dose-dependent manner. Phosphorylated proteins represent the activation of molecules such as p-PI3K and p-AKt, indicating that the pathway is activated(42). After the PI3K-AKt signal is activated, it may transmit the signal to downstream signal targets such as RXRa. As shown in Fig. 4B, the protein level of RXRa was significantly decreased in a drug dose-dependent manner. These results indicated that EIE could induce apoptosis in CRC cells.(the supplementary picture of Fig. 8 is in the Supplementary Fig. 1)

4 Discussion

The latest cancer occurrence and death survey report shows that CRC ranks third (43) in the global cancer incidence rate and mortality. Compared with developed countries, the incidence of CRC in China continues to rise, and the prevention and control works are still imperative (44). Adenoma was considered the main precancerous lesion, which led to the development of CRC many years ago (45), and its presence may significantly increase the risk of CRC (46). In the process of mechanism research, scholars found that in the process of colorectal adenoma developing into cancer, whether it is the process of inflammatory cancer transformation or the inflammation caused by carcinogenesis, human innate immunity is involved in the process of adenoma carcinogenesis (47, 48, 49, 50, 51, 52). It is urgent to systematically interpret the mechanism of the transformation of precancerous lesions into cancer (53).
Therefore, the innate immune-related mechanism in the carcinogenesis of colorectal adenoma may be an essential research direction for the prevention and treatment of intestinal adenoma in CRC.

EIE, as a class II antitumor drug without cytotoxicity, has specific anticancer effects by enhancing the immune system (6). The nanostructured lipid carrier of EIE emulsion is a bright spot of this drug. Liposomes can load multiple molecular medicines to improve their anticancer properties (54). Although relevant, studies have proved in recent years that the main molecular drug components of EIE have evident biological characteristics of promoting apoptosis of intestinal adenocarcinoma cells (55, 56). However, there is no systematic study on the overall efficacy of EIE in preventing and treating CRC. Therefore, according to the drug characteristics of elemene emulsion injection and the disease characteristics of CRC, it is necessary to preliminarily explore the molecular mechanism of elemene liposome in interfering with the innate immunity of colorectal adenoma carcinogenesis.

This research found that EIE has an excellent therapeutic effect on CRC, mainly inhibiting RXRa expression and PI3K/Akt pathway, promoting tumor cell apoptosis. Firstly, according to network pharmacology, EIE intervened in the differentially expressed gene RXRa and core-dependent pathways in cancer related to the innate immunity of colorectal adenoma carcinogenesis, which was sought by the PPI network, GO and KEGG analysis. At the same time, we docked RXRa and three compound molecules in EIE. The fitting scores showed that the three compounds in EIE were the bioactive compounds of RXRa, which had the best affinity for CRC treatment. Then, the sequence of CRC and CRA was detected by RPPA technology, and the differential expression pathway PI3K / AKt was enriched. Do these results suggest that PI3K/Akt pathway and RXRa are innate immune mechanisms related to colorectal adenoma carcinogenesis? RXRa gene target is located downstream of PI3K/Akt pathway(https://www.kegg.jp/pathway/map04151 +K08524). PI3 K/Akt pathway is a crucial coordinator of immune cell activation and inflammatory balance (57). PI3K/Akt signaling pathway plays an essential role in regulating immune response and inflammatory factor release in vitro and in vivo by regulating the activation of downstream signaling molecules (58). Moreover, allelic variation in RXRa affects metachronous colorectal neoplasia (59). The retinoid X receptor (RXR) rela polymorphism may be associated with the risk of CRC (60). RXRa acts as an inhibitor of stem cell differentiation and triggers various interrelated signaling pathways to prevent carcinogenesis. In cancer cells, tumor inhibition can be activated through PI3K/Akt signaling pathway (61). Therefore, we hypothesized that EIE inhibits the occurrence and growth of tumors in the innate immune mode by regulating RXRa and inhibiting the expression of the PI3K/Akt signal pathway.

In vitro validation was carried out by using THC8307 and HCT116 cell lines. From the perspective of morphology and protein expression, it was confirmed that EIE mainly affected the apoptosis and cycle changes of human colon cancer by inhibiting RXRa transcription and expression and regulating the PI3K/Akt phosphorylation process, which further proved the feasibility of EIE in the prevention and treatment of human CRC (Fig. 9). Therefore, there was a theoretical basis for the bioactive components of EIE to inhibit the occurrence and development of CRC. However, this study also had some limitations. Firstly, due to technical conditions and cost constraints, we could not use models closer to the occurrence
and development of this disease, such as intestinal organoids, to simulate the carcinogenic process of adenomas. In addition, when operating in vitro experimental verification, we still need to edit specific gene targets to verify their direct effect and significance in this disease. Finally, there are still many mechanisms related to carcinogenic promoters or protein modification processes in this research process that have not been verified. Therefore, in the future, we will surmount these limitations, use the organoid intestinal adenoma carcinogenesis model to verify multiple mechanisms of carcinogenesis, deeply explore the disease evolutionary development principle of intestinal adenoma carcinogenesis, and look for the mechanism of EIE acting on the protein modification process of carcinogenesis from the essences of disease.

Although various factors limited this research, we were still excited by these data for the prediction of network pharmacology and the high-throughput of microfluidic technology. These prediction results were highly correlated. The critical nuclear molecule RXRa, which we focused on, was just the downstream gene of the intracellular pathway PI3K/Akt, which makes the subsequent verification very feasible. In vitro experiments proved the results of prediction and seeking. These findings make us believe that the changes of RXRa and PI3K/Akt are involved in colorectal adenoma carcinogenesis and may be the central mechanism for preventing colorectal adenoma carcinogenesis from the perspective of innate immunity. EIE acts on these mechanisms. Therefore, EIE is likely to become a drug that can block colorectal adenoma carcinogenesis and has been paid attention to clinically.

5 Conclusion

In this study, the network pharmacology and molecular docking analysis support that elemene may be a potential drug to intervene in the carcinogenesis of colorectal from the perspective of innate immunity. Furthermore, RPPA clarifies the possible mechanism of colorectal adenoma carcinogenesis. In vitro cell experiments verify that elemene may play an anticancer role in this mechanism. Our study identifies phosphorylation activation of the PI3K/Akt pathway as a key role in colorectal adenoma carcinogenesis. Concurrently, RXRa, which is downstream of the PI3K/Akt pathway, is also a significant target and plays a vital role in the occurrence of CRC.

EIE-induced RXRa and its transcriptional downregulation and mediated PI3K/Akt phosphorylation are the two highlights of this effect and mechanism. In the following research, we will continue to seek support to conduct a more systematic and in-depth study on the dynamic response network related to EIE intervention in colorectal adenoma carcinogenesis from the perspective of time and space to provide a clinical and theoretical basis for EIE in the prevention and treatment of colorectal adenoma carcinogenesis.

Abbreviations

CRC, Colorectal cancer; EIE, ELEMENE INJECTABLE EMULSION; RPPA, Reverse-phase protein array; RT-PCR, Real-time Polymerase Chain Reaction; PI3K, Phosphoinositide 3-kinase; AKt, Protein Kinase B; RXRa, Retinoid X Receptor alpha; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TCM,
Traditional Chinese Medicine; PPI, Protein-protein interaction; TCMSP, Traditional Chinese Medicine database and Analysis Platform; AJCC, American Joint Committee on Cancer; TNM, Tumor lymph node metastasis; V-FITC/PI, V-fluorescein isothiocyanate/propidium iodide; CCK8, Cell Counting Kit-8; FAM120B, Family With Sequence Similarity 120B; NR4A1, Nuclear receptor subfamily 4 group A member 1; THRA, Thyroid hormone receptor alpha; NR1H4, Bile acid receptor; CCL23, C-C motif chemokine 23; CYP3A4, Cytochrome P450 3A4; NCOA4, Nuclear receptor coactivator 4; ZBTB16, Zinc finger and BTB domain-containing protein 16; PPARA, Peroxisome proliferator-activated receptor alpha; TBL1X, F-box-like/WD repeat-containing protein; TBL1X; TBL1XR1, F-box-like/WD repeat-containing protein TBL1X; NR1I3, Nuclear receptor subfamily 1 group I member 3; CXADR, Coxsackievirus and adenovirus receptor; VDR, Vitamin D3 receptor; NR1H2, Oxysterols receptor LXR-beta; RARB, Retinoic acid receptor beta.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Ethics Committee of Taicang Traditional Chinese Medicine Hospital and was carried out in accordance with the Declaration of Helsinki.

Consent for publication

Written informed consent was provided by all the included subjects.

Competing interests

The authors declare that they have no competing interests. All other authors declare no financial relationship with any organization that might have an interest in the submitted work in the previous five years. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the figshare repository, https://figshare.com/articles/dataset/Supplementary_data/19561216. Further inquiries can be directed to the corresponding author.

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Authors’ contributions

All of the authors performed the study. TC and WCS presided bioinformatics analysis, YS designed Molecular Docking. TC and FL designed the experiments, analyzed and supervised the results, and corrected the manuscript. All the authors read and approved the final version of the manuscript.
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References


Figures
Figure 1

Whole study process
Figure 2

(A) The intersection of the target data sets of six research objects γ-elemene δ-elemene β-elemene Colorectal Adenoma Colorectal Cancer Innate Immunity. The yellow coils indicates the the number of γ-elemene targets, the brown coils indicates the number of β-elemene targets, the cerulean coils indicates the number of δ-elemene targets, the blue coils indicates the number of CRA targets, the red coils indicates the number of CRC targets, and the green coil indicates the numbers of innate immunity targets. (B) Protein
interaction network of core target genes, Showing 20 related genes, with 21 total genes, 0 attributes, and 419 total links. The core gene searched with are indicated with stripes. (C) We use the non-log scale for calculation and use the log-scale axis for visualization. The correlation between two proteins. The twenty proteins associated with RARX in Figure 2-B was used the non-log scale for calculation and use the log-scale axis for visualization.

Figure 3
GO and KEGG enrichment analysis results. (A) GO analysis: Biological process-related items, (B) Cell composition-related items, (C) Molecular function-related items. (D) The signal pathway was obtained by KEGG enrichment analysis.

Figure 4

Molecular docking and experimental validation of EIE binding core targets predicted by network pharmacology. (A) Molecular docking model diagram. β-Elemene with RXRa, γ-Elemene with RXRa, δ-Elemene with RXRa. All pictures (a-c) show the 3D docking of ligands in the active binding pocket, with the hydrophobic effect area, the ligands and proteins, and the essential interactions between the ligand atoms and amino acid residues of the proteins being displayed. (B) Cells were treated with the indicated concentrations of EIE for 72 h. Cell protein was extracted and detected by Western blotting with antibodies against RXRa and β-actin. The expression levels of RXRa were expressed as the mean ± SEM (n=3). *p < 0.05 compared with the control group. (C) The mRNA level of RXRa was quantified by RT-PCR (normalized to GAPDH) (**** p<0.0001)
Figure 5

Functional enrichment maps of differential gene pathways in colorectal adenoma, colorectal cancer, and Paracancerous tissue sequences. In the three network diagrams, the larger the node, the more the number of differential genes in the enrichment pathway, and the node’s color from green to red represents the gradual increase of the P-value.
As shown in Figure 6A and Figure 6B, the IC50 values were approximately 57.64μg/ml and 59.14μg/ml in THC8307 and HCT116 cells, respectively. Figure 6C, Morphology of HCT116, THC8307 cells were treated with the indicated concentrations of Elemene for 72h. Scale bar, 100 μm.
Figure 7

Effect of elemene on the biological behavior of CRC Cells. (A and B) Flow cytometry showed that high-dose elemene could promote the apoptosis of THC8307 and HCT116 cells. (C, D, and E) Cell cycle analysis showed that high-dose elemene may inhibit THC8307 and HCT116 cells in the early stage of DNA synthesis.
Figure 8

EIE inhibits the PI3K/Akt pathway in CRC cells. Representative Western blots showing the status of p-PI3K and p-Akt in THC8307 and HCT116.
Figure 9

Working model of EIE acting on PI3K/Akt signaling pathway to induce apoptosis of THC8307/HCT116 cells. Combining the network pharmacology analysis and our results, we hypothesized that EIE influences the PI3K/Akt signaling pathway to regulate the expression of RXRa, inhibit the proliferation of human colorectal cancer cells and trigger apoptosis.

Supplementary Files

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