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Original article

Tumorigenesis Inhibition of Carnosol From Rosemary: an Insight to Molecular Targeting

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Abstract

Background and objectives: Anti-proliferative activity of carnosol from Rosemary on malignancies has revealed its potential for cancer therapeutic purposes. Molecular studies such as proteomics could open a new insight in underling mechanisms of anticancer processes of carnosol treatment through analysis of the most relevant modulated proteins in cancer. **Methods:** Protein-protein interaction (PPI) network analysis of adult T-cell leukemia/lymphoma (ATL) treated with carnosol proteome was conducted. Cyroscape and its plug-ins explored the PPI network construction and its features including centrality and gene ontology. **Results:** Among 22 differentially expressed proteins (DEPs), 21 individuals were recognized by the STRING database. The queried DEPs and the added first neighbors formed a scale-free network. GAPDH, TPI1, ENO1, and PGK1 were identified as the hub-bottlenecks of the PPI network of carnosol-treated ATL. **Conclusion:** ALDOA, PFKFB3, PKM2, and LDHA and related metabolic processes are targets of anticancer compounds of rosemary extract; however, more investigation is suggested.

Keywords: carnosol; leukemia: lymphoma; network analysis; rosemary

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Introduction

Many herbal sources display health benefits including cancer preventive effects. One of which is rosemary (*Rosmarinus officinalis* L.) that can be a potential source of cytotoxic activity mainly related to its bioactive phenolic metabolites [1]. Treatment with rosemary extract has shown promising results in many cancers such as colon, pancreas, Adult T-cell leukemia/lymphoma (ATL), skin, and prostate cancers [2-6]. The anticancer properties of rosemary extract has been evaluated in-vitro and in animal models and has shown promising effects in reducing risk of malignancies [7]. One of the mechanism by which this plant shows anticancer properties is by MYC inactivation, which triggers apoptosis [8]. Further investigations in terms of molecular

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studies provide more information about underling mechanisms of anticancer properties of rosemary. According to study of gene and microRNA expressions, GCNT3 and its modulator were influenced by anticancer regulation of Rosemary. Based on this investigation, GCNT3 and miR-15b were differentially expressed as up-regulation and down-regulation, respectively. carnosic acid and carnosol, a phenolic diterpene, are components responsible for this effect on two cancers of colon and pancreas [2]. These two substances are constituents of rosemary that play anti-inflammatory and anti-oxidant roles against cancer cells. About 90% of antitumor properties of rosemary are related for these components [9]. The bioactive materials of rosemary apply distinct processes to block tumorigenesis in colon cancer. Carnosol inhibits chymotrypsin-like activity in 20S proteasome while carnosic acid elevates protein expressions in unfolded protein response (UPR) [10]. Human T-cell leukemia virus type 1 (HTLV-1) infection is an crucial risk factor for adult T-cell leukemia/lymphoma (ATL) since this type of cancer is known as a viral disease [11]. On the other hand, molecular studies of antitumor properties of herbal medicine have been of great interest [12]. Proteomics is a promising molecular approach in terms of detecting dysregulated proteins in cancer development for therapeutic targeting. Furthermore, it can explore therapeutic mechanism of herbals through discovery of modulated proteins in different types of tumors [13]. Bioinformatics can provide additional information for biomarker recognition with analysis of protein-protein interaction (PPI) networks [14]. In a PPI network of differentially expressed proteins (DEPs) in a treated-sample, proteins with centrality properties offer more importance in underlying mechanisms of therapeutic agents. Since central nodes are fundamental in network stability and function, they can be more promising candidates as therapeutic targets [15].

To gain better knowledge about mechanisms by which carnosol from rosemary shows apoptosic effects, a protein-protein interaction network analysis of carnosol-treated proteome of ATL was conducted.

Materials and Methods

Ethical considerations

This project was approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1400.295).

Data collection

A study by Ishida and et al., suggested that carnosol, the rosemary extract shows cytotoxic effect against ATL cell line via apoptosis. In the mentioned study, after 24 hours of treatment with the doses of 40 μM carnosol, Ms-based proteomics was carried out. The method of proteomics was via fluorescent two-dimensional electrophoresis [4].

In the present study, data were extracted from the published document by Yo-ichi Ishida and et al. [4]. Differentially expressed proteins were determined considering $p<0.05$ and fold change ≥ 1.3. The 21 DEPs were chosen for the network construction via Cytoscape 3.8.2 and STRING App. Three STRING database query methods are available through Cytoscape including protein name, disease name, and PubMed search. The confidence score of physical interactions ranged between 0 to 1 [16]. Data were included in STRING via protein query. The score cut off was 0.4 as a default option; however, in this study it was set to 0.5 to gain a better strength of connecting network. Cytoscape conducted two network constructions of DEPs in treated-carnosol. First network included only the query protein while the second contained neighbors as well. In the second network, 50 neighbor proteins were added and centrality analysis was carried out on this network. NetworkAnalyzer and CytoHubba performed the centrality analysis via topological algorithms [17,18]. NetworkAnalyzer computes topological attributes including node degrees, betweenness centrality, stress, shortest path, clustering coefficients as famous ones via graph algorithms. In this study, degree (K) and betweenness centrality (BC) were designated as topological parameters to assess network stability. CytoHubba as a Cytoscape application identifies different parameters of a PPI network centrality such as degree and betweenness centrality as well [17]. The combined information together from two plug-ins provide better resolution of central nodes. Lastly, functional enrichment study of hubs and bottlenecks clusters via STRING database expressed that some nodes were involved in statistically significant biological processes. STRING Application enrichment study can

explore gene ontology including biological process, cell component, and molecular functions as well as pathway analysis. By the use of "EnhancedGraphics" v.1.5.4 (http://apps.cytoscape.org/apps/enhancedGraphics), different charts types and gradients view of gene ontology can be retrieved [19]. The designated p-value for annotating terms was set to FDR<0.01.

Results and Discussion

A network was constructed of 21 identified differentially expressed proteins ($p<0.05$ and fold change ≥ 1.3) in treatment with carnosol through Cytoscape. First network includes 20 nodes and 33 edges without adding any other nodes (Figure 1). The second network contained additional nodes namely neighbor proteins. It consisted of 70 nodes and 1048 edges (data is not shown).

In the first network, 22 DEPs were subjected to the network query; however, 21 ones were identified as a constructed network through Cytoscape platform from String database. Seven of these proteins were not in the main component of the PPI network including ANXA1, ANXA5, MSN, PSMC6, NASP, BLVRB, and BLVRA. The second network was selected for the centrality analysis via NetworkAnalyzer and CytoHubba. NetworkAnalyzer measurements for degree and betweenness centrality are presented in Figure 2 and Table1.

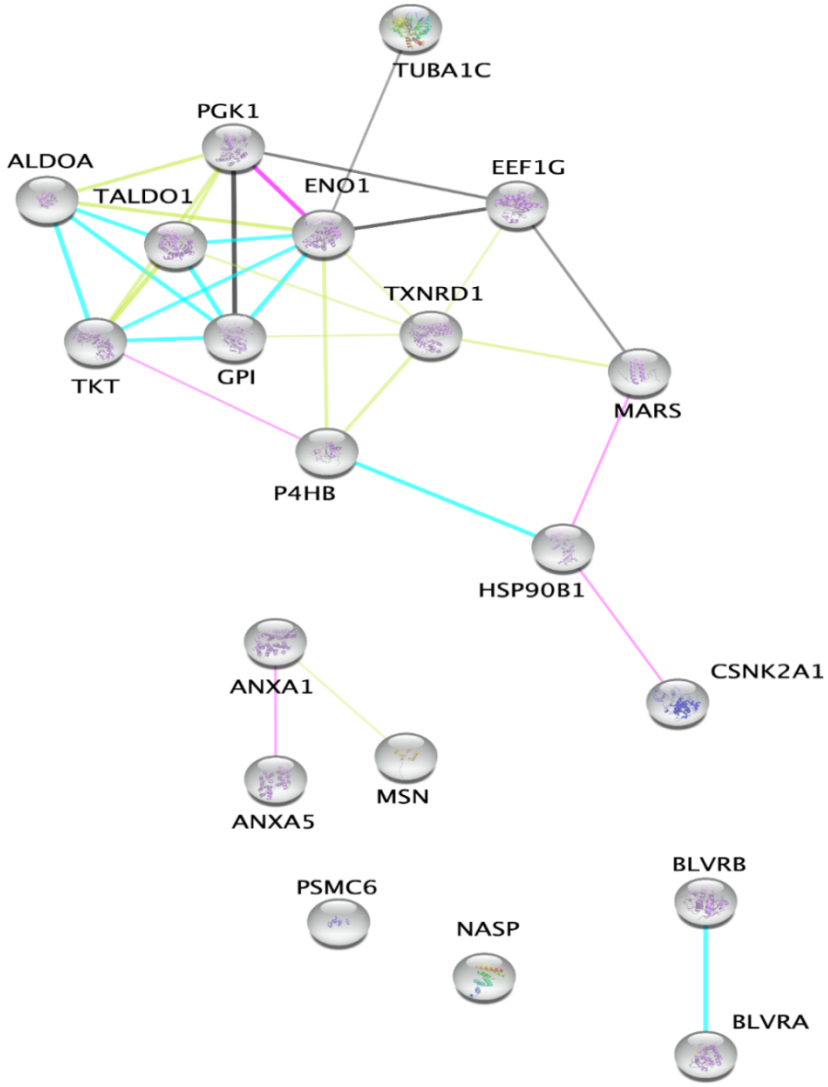

Figure 1. Protein-protein interaction network of differentially expressed proteins in carnosol -treated cells; confidence score cut off ≥0.5; edges represent different colors denoting the source of interaction relationships; blue: database; green: text mining; pink: experiment; black: coexpression

In Figure 2, most of the nodes are located on the left down corner of the plot and less in the right top the plot. Degree ranges from 0 to 70 and betweenness centrality ranges from 0 to 0.1

By the application of NetworkAnalyzer, high-degree and high-betweenness centrality nodes were measured. These topological parameters were assessed by assigning top 10%. The nodes with highest amounts of these centralities are known as hub-bottlenecks as shown in Table 1.

GAPDH, TPI1, ENO1, PGK1, PKM, TALDO1, and GPI are hub proteins among them the first four are considered as hub-bottlenecks of the PPI network. PGK1 (phosphoglycerate kinase 1) is marginally a hub-bottleneck and is implicated in carnosol modulatory impacts based on proteomics study. It is from queried proteins as significant differentially expressed proteins with elevated expressions. PGK1 in the main study indicated a fold change of 1.6 and $p \leq 0.001$. All query proteins in Table 1 are up-regulated. TALDO1 expresses higher regulation changes.

Centrality and enrichment analysis of seven hubs (links:21) and bottlenecks (links:18) of the PPI network via CytoHubba and String plug-ins, are performed in Figure 3, respectively.

In Figure 3, circle colors differ the most statistically significant biological processes in hubs: light green: (1.02E-14) glycolysis; dark green: canonical glycolysis (3.45E-14); and pink: nicotinamide nucleotide metabolic process (3.45E-14). For Bottlenecks the annotations are as follow: Pink: Canonical glycolysis (8.08E-05).

Malignancies prevention can be accelerated through identification of new therapeutic approaches, one of which is the complementary studies of proteome profile of treated-tumor samples. Bioinformatics, as a post-analysis of proteome data, can be helpful by application of PPI network analysis.

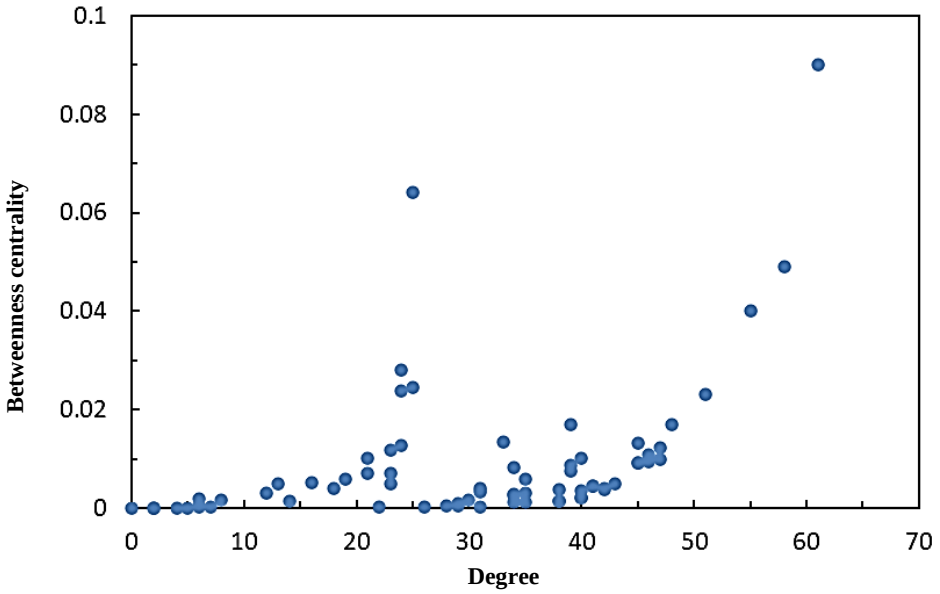

Figure 2. Scatter plot view of degree and betweenness centrality distribution in PPI network

Table 1. The list of top nodes ranked based on high-degree values

Row	Gene	Protein	K	BC	Query term	Regulation statues	Fold change
1	GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	61	0.09	-	-	-
2	TPI1*	Triosephosphate isomerase 1	58	0.05	-	-	-
3	ENO1*	2-Phospho-D-glycerate hydro-lyase	55	0.04	P06733	Up	1.3
4	PGK1**	Phosphoglycerate kinase 1	51	0.02	P00558	Up	1.6
5	PKM	Pyruvate kinase PKM	48	0.02		-	-
6	TALDO1	Transaldolase	47	0.01	P37837	Up	2.2
7	GPI	GPI-anchor transamidase	47	0.01	P06744	Up	1.3

* hub-bottlenecks; ** marginally hub-bottleneck; K: degree; BC: betweenness centrality

By this means, DEPs and candidate neighbors can be detected as worthy central agents in network constitution and strength. Cytoscape software analyzed the DEPs with the related neighbor proteins via corresponding plug-ins. The structure of the main component of the first network indicates acceptable interactions among query proteins. Second network with the addition of neighbor proteins may show presence of other central nodes that may be important in the network stability and ultimately as a part of underlying mechanism of treatment with carnosol. Centrality analysis of the second network from NetworkAnalyzer implies a scale-free PPI network since few numbers of nodes have high values of degree and betweeenness centrality as shown in Figure 2. Further evaluation with NetworkAnalyzer showed that GAPDH, TPI1, ENO1, PGK1 could be the hub-bottlenecks of the carnosol-treated PPI network. The last two proteins were from DEPs and both were up-regulated in the main proteomics study. It should be mentioned that many proteins may be targeted by carnosol but are not presented among the queried proteins or central nodes which are selected among the added first neighbors due to their centrality properties.

To provide insight for the role of these central proteins in the mechanisms of carnosol antitumor activity, literature survey can assist. The first hub-bottleneck is GAPDH that, as a housekeeping gene, has fundamental role in cancer development. Its deregulation has been constantly reported in different types of cancers. It participates mostly in regulation of the process of cell death [20]. Moreover, GAPDH shows overexpression in angioimmunoblastic T cell lymphoma (AITL) disease [21]. It is also reported that other herbal compounds such as green tea polyphenol (–)-epigallocatechin-3-gallate targets GAPDH in cancer [22]. Therefore, GAPDH may also be important in carnosol mechanism of action as well.

The next protein is TPI1, which likewise GAPDH is not from DEPs of the main study. Similarly, this protein is up-regulated in cancer [23-25]. However, no association has been pinpointed between these proteins as a possible target for herbal treatments in cancer.

ENO1 is up-regulated in the original proteomics study that is a key hub-bottleneck. Gang Wang et al have investigated the anticancer property of *Rhus chinensis* on SW620 colorectal cancer cells via network analysis. Based on this report, triterpenoids of *Rhus chinensis* extract targets ENO1 and several genes as like ALDOA, PFKFB3, PKM2, and LDHA in cancer cells to represent anticancer property [26]. PGK1 is also a hub-bottleneck DEP and its up-regulation in carnosol-treated samples was detected by proteomics study. This protein is a target of prostate cancer treated with curcumin as well [27]. Lack of expression of PGK1 has been related with development and treatment-resistance in cancer [28].

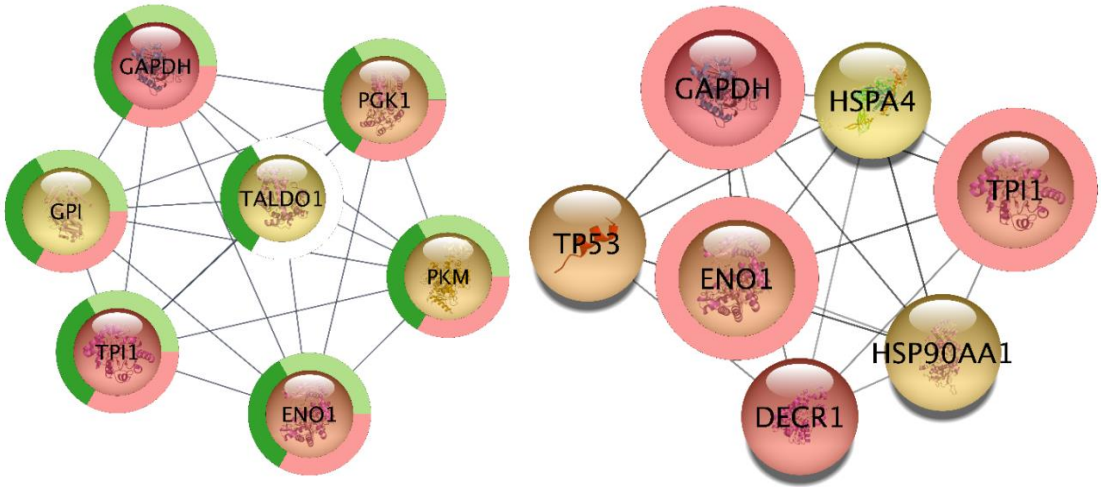

Figure 3. Left: hubs and **Right:** bottlenecks with the corresponding statistically significant enriched biological processes; color fading of each node implies on decline of centrality value. The rings around the nodes refer to different related biological processes. TP53, HSP90AA1, and DECR1are not involved in the determined biological term.

In order to recover what biological processes are enriched with hubs and bottlenecks, String App ontology analysis was carried out. The most statistically significant annotations were related metabolic processes.

These agents that are involved in metabolic processes could contribute to the development of antitumor mechanisms of carnosol. Consequently, the central nodes and the associated processes could be considered as key targeted elements in ATL by carnosol.

Conclusion

PPI network analysis suggests that carnosol can exhibit cancer preventive activity through targeting GAPDH, TPI1, ENO1, PGK1 as central proteins. Since ENO1 and PGK1 are the two queried proteins relative to GAPDH and TPI1 as the two first neighbors, it seems that ENO1 and PGK1 are critical targeted proteins. Future studies could provide more knowledge to establish the importance of candidate proteins in carnosol-treated ATL.

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Author contributions

Mona Zamanian Azodi designed and supervised the study; Babak Arjmand, Mostafa Rezaei Tavirani, Mahmood Khodadoost, Mohammad Rostami Nejad and Nayebali Ahmadi were involved in data collection and analysis; all authors approved the final draft of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

PPI: protein-protein interaction; ATL: adult T-cell leukemia/lymphoma; DEPs: differentially expressed proteins; UPR: unfolded protein response; HTLV-1: human T-cell leukemia virus type 1; K: degree; BC: betweenness centrality