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ELUCIDATION OF BIO-TARGETS AND BIOLOGICAL MECHANISMS OF VITAMIN C AGAINST LUNG CANCER USING NETWORK PHARMACOLOGY ANALYSIS

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ABSTRACT

Objective: With the highest cancer related mortality and morbidity, lung cancer has become a predominant problem world-wide. The antitumor property of vitamin C (VC) has already been demonstrated. However, the precise mechanism and the targets of VC against lung cancer are yet to be discovered. This study is conducted to identify potential targets and underlying mechanisms of VC against lung cancer by bioinformatics analysis of network pharmacology and molecular docking. Methodology: Targets of VC and lung cancer were obtained from various data bases. From the 142 pharmacological targets of VC, 136 overlapping targets with lung cancer were mapped. The 10 potential core targets of VC were identified from mapped proteins. These 10 core targets are subjected to Gene Ontology and Kyoto Encyclopaedia of Genes and Genomes pathway enrichment analysis. The top 20 biological processes, molecular functions, cellular components and pathways were presented. Results: EGFR, ADRA2C, CHRM2, CCR5, MAPK1, BDKRB2, DRD2, AGTR2, ADRA2A and ADRA2B were found to be the key targets involved. Molecular docking studies revealed that VC has highest binding affinity to B2 bradykinin receptor (BDKRB2). The mechanism of VC in lung cancer may be associated with regulation of catecholamine release by inhibiting BDKRB2 receptor. Conclusion: VC can suppress tumour development and progression in lung cancer. Further pre-clinical studies are required for VC before it is used as a therapeutic agent in lung cancer.

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INTRODUCTION

Lung cancer remains the most fatal malignancy with approximately 1.76 million deaths worldwide accounting for 18.4% of total cancer deaths [1]. Based on histology it was broadly classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with about 85% of lung carcinomas being NSCLC [2]. Resection of the lung, immunotherapy, chemotherapy, targeted therapy and radiation has been adopted for lung cancer treatment. However, increase of survival rate in advanced NSCLC has been a challenge [3]. Monoclonal antibody pembrolizumab was approved by US Food and Drug Administration (FDA) as the first-line treatment for metastatic lung cancer [4]. Pembrolizumab has also showed improvement in survival rate and quality of life in patients compared to chemotherapy [5]. However, the optimal dose and treatment duration of pembrolizumab remains uncertain [6]. Like many other monoclonal antibodies, pembrolizumab is also associated with some serious adverse reactions[7]. So, there is an immense need to explore pharmacologically bioactive components to battle lung cancer.

Vitamin C (VC) or ascorbic acid is an essential water-soluble vitamin with anti-oxidant, antiaging and cancer prevention properties [8]. VC intake may also improve the efficacy of chemotherapy and reduce the cancer treatment-related side effects [9]. Drug resistance is a major difficulty in lung cancer treatment. VC co-administered with other targeted therapeutics has shown to overcome this resistance [10][[11]. Thus, a combination therapy of targeted therapeutics and VC may improve the clinical outcomes in patients undergoing lung cancer therapy. However, the predictive targets and therapeutic mechanisms of VC in lung cancer are not yet studied. Therefore, our current study is designed to use the developing network pharmacology approach to reveal receptors and molecular mechanisms underlying the action of VC against lung cancer. Network pharmacology is a bioinformatics method which can assist us to predict the targets and mechanisms of a compound in various diseases [12]. In the present study, network pharmacology approach was applied to elucidate the core targets and the underlying molecular mechanisms of action of VC against lung cancer. The network constructed will aid us in understanding the pharmacological mechanism of VC in lung cancer in a biomolecular level.

METHODOLOGY

Pharmacological Targets for Vitamin C:

The Swiss Target Prediction tool [13], Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) [14], SuperPred [15] and Drugbank [16] databases were used to obtain the documented human target proteins of VC. From the attained target proteins, duplicate targets were removed.

Pathological Targets of Lung Cancer:

The target proteins associated with lung cancer were obtained from DisGeNET [17], GeneCards [18] and Online Mendelian Inheritance in Man (OMIM) [19] databases were used. From GeneCards, the targets with gene score > 1 were included in the study. Likewise, In DisGeNET, the targets with gene-disease association score > 0.1 were filtered. After the removal of the duplicate targets, a total of 16,188 targets were screened for lung cancer.

PPI Network Construction and Analysis to Identify Core Targets:

The correlative targets of VC against lung cancer were identified using a web tool to build Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/). Later, the Uniprot ID's of correlative targets were introduced into the STRING database (https://string-db.org/). A protein–protein interaction (PPI) network was constructed with the minimum required interaction score set to a high confidence at 0.700. The tsv.data of the network acquired from STRING database was analysed in Cytoscape_v3.8.0. The topological parameters were analysed and the core targets were filtered in Cytoscape. The lower limit of the degree filter was set at twice the value of average degree of freedom and the upper limit being the highest degree value from the topological data [20].

GO and KEGG Pathway Enrichment Analysis of Core Targets:

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed in R software (version 4.0.2). R-language packages of "clusterProfiler", "org.Hs.eg.db" and "enrichplot" were utilized to perform and visualise GO and KEGG pathway enrichment analyses. In clusterProfiler (version 3.16.0) [21] GO in biological process (BP), molecular function (MF) and cellular component (CC) were executed for core targets with $P < 0.05$ and $q < 0.05$ as cut-off values for enrichment and represented in bar plots and dot plots [22]. Benjamini–Hochberg procedure was used to correct P values. Furthermore, using enrichKEGG function in R, KEGG pathway enrichment analysis was performed with a cut-off value $P < 0.05$ and output was represented in dot plot and cnet plot.

Molecular Docking Of VC against the Potential Core Targets:

Molecular docking was carried out for VC against the core targets to assess their binding affinities and further screening of the targets. 3D structure of VC ligand was acquired from PubChem compound database at NCBI (https://pubchem.ncbi.nlm.nih.gov/) in SDF format. The 3D X-Ray diffraction structures of core target proteins were retrieved from Protein Data Bank (PDB). The hetero atoms present in the crude PDB files were removed. Kollman charges and polar hydrogen atoms were added to satisfy their appropriate charges in Autodock Vina. Active pockets in receptors were identified in Computed Atlas of Surface Topography of Proteins (CASTp) program (http://sts.bioe.uic.edu/castp/calculation.html) [23]. Subsequent to the preparation of ligand and receptor files in PDBQT format, the docking analysis was carried out in AutoDock Vina [24]. The poses of docked complexes were visualised in PyMOL version 1.7.4.4 (Schrodinger) molecular graphics system.

RESULTS

Prediction and Screening of VC and Lung Cancer Targets:

142 pharmacological targets were obtained for VC from Swiss Target Prediction tool, TCMSP, SuperPred and Drug Bank. Likewise, GeneCards and DisGeNET databases were used to obtain 16,188 targets for lung cancer. The constructed Venn diagram revealed 136 overlapping targets between VC and lung cancer (Fig. 1). The required information of screened 136 correlative targets was obtained from Uniprot database.

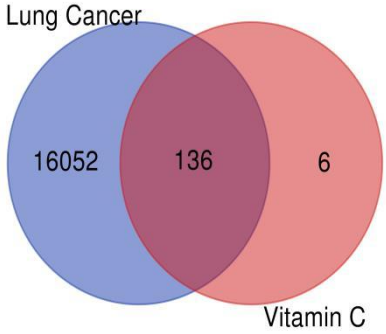

Fig. 1: Venn diagram highlighting the 136 overlapping targets between lung cancer and VC.

PPI Network Analysis and Core Targets:

PPI network was constructed for the 136 correlative targets in STRING database (Fig. 2a). The PPI network was later analysed in Cytoscape and the topological parameters of the mapped proteins were calculated. Number of nodes and edges in the PPI network were 115 and 685 respectively (Fig. 2b).

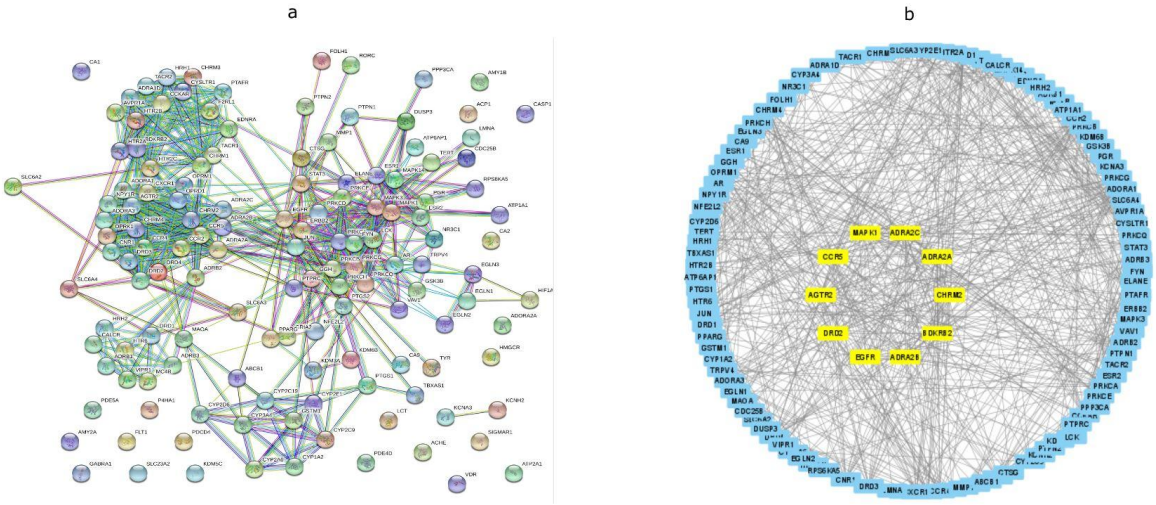

Fig. 2: PPI network of 136 overlapped genes (a) PPI network constructed in STRING database with a minimum required interaction score of 0.700 (high confidence) (b) PPI network of overlapped genes with core targets represented in yellow colour attained from Cytoscape_v3.8.0.

Target proteins are represented by the nodes and the interactions between them are represented by edges. The maximum degree of freedom was 35 whereas the average degree of freedom was 11.983. So, the lower limit of degree filter was set at 24 and the upper limit at 35. 10 core targets, type-2 angiotensin II receptor (AGTR2), alpha-2A adrenergic receptor (ADRA2A), alpha-2B adrenergic receptor (ADRA2B), alpha-2C adrenergic receptor (ADRA2C), B2 bradykinin receptor (BDKRB2), muscarinic acetylcholine receptor M2 (CHRM2), muscarinic acetylcholine receptor M5 (CCR5), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase-1 (MAPK1) and D(2) dopamine receptor (DRD2) were obtained (Fig. 3).

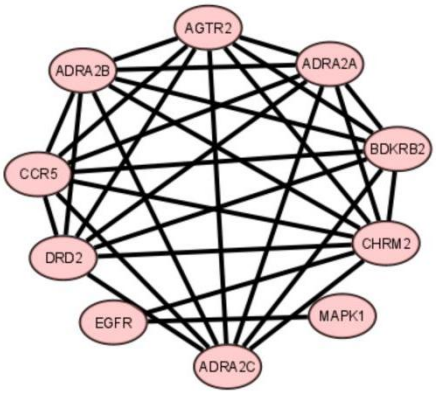

Fig. 3: Network of top 10 core targets of VC against lung cancer.

GO and KEGG Pathway Enrichment Analysis:

In R Studio, 10 core targets were analysed for GO and KEGG pathway enrichment analysis. The GO BP analysis revealed a total of 664 entries with $p < 0.05$ and $q < 0.0.5$. Biological Processes of the 10 core targets were mainly associated with secretion and transport of catecholamine and norepinephrine. The top 20 biological processes were showed in bar plot (Fig. 4a), (Table 1).

Table 1: Top 10 significantly enriched biological processes of VC against lung cancer.

S.No.	ID	DESCRIPTION	GENE RATIO	P.ADJUST VALUE	GENE NAME
1.	GO:0033604	negative regulation of catecholamine secretion	6/10	4.240273e-11	DRD2/AGTR2/ADRA2C/ ADRA2B/ADRA2A/ BDKRB2
2.	GO:1903522	regulation of blood circulation	8/10	8.671915e-11	EGFR/DRD2/CHRM2/ BDKRB2/AGTR2/ADRA2C/ ADRA2B/ADRA2A
3.	GO:0051953	negative regulation of amine transport	5/10	2.495094e-10	DRD2/AGTR2/ADRA2C/ ADRA2B/ADRA2A
4.	GO:0006939	smooth muscle contraction	6/10	1.991422e-09	DRD2/CHRM2/BDKRB2/ ADRA2C/ADRA2B/ADRA2A
5.	GO:0035296	regulation of tube diameter	6/10	5.883358e-09	EGFR/DBDKRB2/AGTR2/ ADRA2C/ADRA2B/ADRA2A
6.	GO:0097746	regulation of blood vessel diameter	6/10	5.883358e-09	EGFR/DBDKRB2/AGTR2/ADRA2C/A DRA2B/ADRA2A
7.	GO:0035150	regulation of tube size	6/10	5.883358e-09	EGFR/DBDKRB2/AGTR2/ ADRA2C/ADRA2B/ADRA2A
8.	GO:0019229	regulation of vasoconstriction	5/10	8.010140e-09	EGFR/DBDKRB2/ADRA2C/ADRA2B/ ADRA2A
9.	GO:0014061	regulation of nor-epinephrine secretion	4/10	8.873844e-09	AGTR2/ADRA2C/ADRA2B/ADRA2A
10.	GO:0050433	regulation of catecholamine secretion	5/10	8.873844e-09	DRD2/AGTR2/ADRA2C/ ADRA2B/ADRA2A

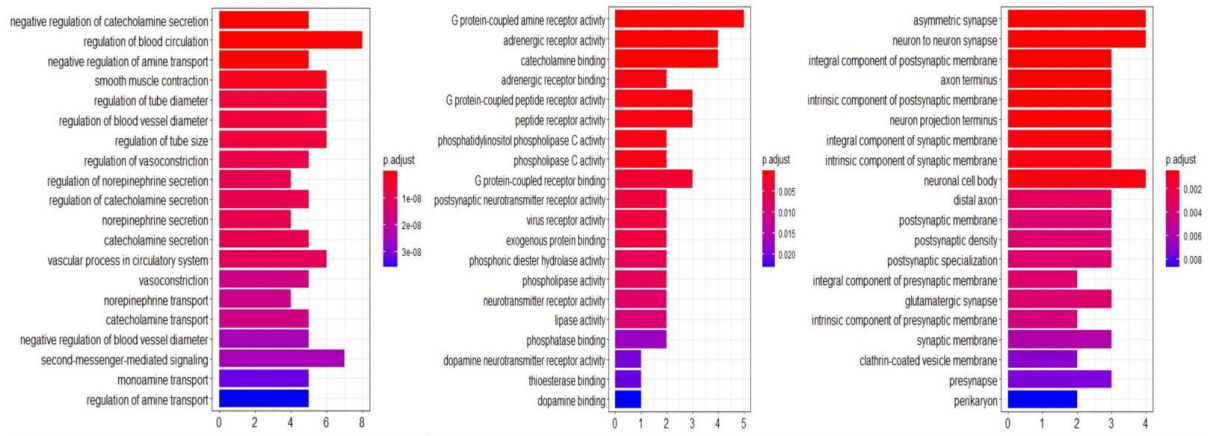

Fig. 4: Bar plots of top 20 GO enriched terms. (a) Biological Processes (b) Molecular Functions (c) Cellular Components.

46 entries of molecular functions ($p < 0.05$ and $q < 0.0.5$) for core targets were attained in GO analysis. These molecular functions included G protein-coupled amine receptor activity (GO:0008227), adrenergic receptor activity (GO:0004935) and other functions as shown in (Fig. 4b), (Table 2).

Table 2: 10 significantly enriched molecular functions of VC.

S.No.	ID	DESCRIPTION	GENE RATIO	P.ADJUST VALUE	GENE NAME
1.	GO:0008227	G protein-coupled amine receptor activity	5/10	3.988160e-03	DRD2/CHRM2/ADRA2C/ ADRA2B/ADRA2A
2.	GO:0004935	adrenergic receptor activity	4/10	3.988160e-03	DRD2/ADRA2C/ADRA2B/ ADRA2A
3.	GO:1901338	catecholamine binding	4/10	3.988160e-03	DRD2/ADRA2C/ADRA2B/ ADRA2A
4.	GO:0031690	adrenergic receptor binding	2/10	5.921712e-03	ADRA2B/ADRA2A
5.	GO:0008528	G protein-coupled peptide receptor activity	3/10	6.464359e-03	CCR5/BDKRB2/AGTR2
6.	GO:0001653	peptide receptor activity	3/10	7.912610e-03	CCR5/BDKRB2/AGTR2
7.	GO:0004435	Phosphatidyl inositol phospholipase C activity	2/10	8.719901e-03	BDKRB2/AGTR2
8.	GO:0004629	phospholipase C activity	2/10	1.715534e-02	BDKRB2/AGTR2
9.	GO:0001664	G protein-coupled receptor binding	3/10	1.973327e-02	BDKRB2/ADRA2C/ADRA2A
10.	GO:0098960	postsynaptic neurotransmitter receptor activity	2/10	2.055891e-02	DRD2/CHRM2

Asymmetric synapse (GO:0032279) and neuron to neuron synapse (GO:0098984) were revealed to be the chief cellular components among the total 40 entries ($p < 0.05$ and $q < 0.0.5$) obtained. The best 20 cellular components were represented in (Fig. 4c), (Table 3).

Table 3: Top 10 enriched cellular components of VC.

S.No.	ID	DESCRIPTION	GENE RATIO	P.ADJUST VALUE	GENE NAME
1.	GO:0032279	asymmetric synapse	4/10	0.0004675382	MAPK1,DRD2,CHRM2,ADRA2C
2.	GO:0098984	neuron to neuron synapse	4/10	0.0004675382	MAPK1,DRD2,CHRM2,ADRA2C
3.	GO:0099055	integral component of postsynaptic membrane	3/10	0.0004675382	DRD2,CHRM2,ADRA2C
4.	GO:0043679	axon terminus	3/10	0.0004675382	DRD2,CHRM2,ADRA2C
5.	GO:0098936	intrinsic component of postsynaptic membrane	3/10	0.0004675382	DRD2,CHRM2,ADRA2C
6.	GO:0044306	neuron projection terminus	3/10	0.0005631083	DRD2,CHRM2,ADRA2C
7.	GO:0099699	integral component of synaptic membrane	3/10	0.0006438634	DRD2,CHRM2,ADRA2C
8.	GO:0099240	intrinsic component of synaptic membrane	3/10	0.0007063883	DRD2,CHRM2,ADRA2C
9.	GO:0043025	neuronal cell body	4/10	0.0007175974	MAPK1,DRD2,CHRM2,ADRA2C
10	GO:0150034	distal axon	3/10	0.0028938117	DRD2,CHRM2,ADRA2C

41entries were obtained for KEGG pathway analysis with $p < 0.05$. These included pathways involved in various cancers along with neuroactive ligand-receptor interaction (hsa04080) and the other 20 top pathways were shown in plots (Table 4) (Fig. 5).

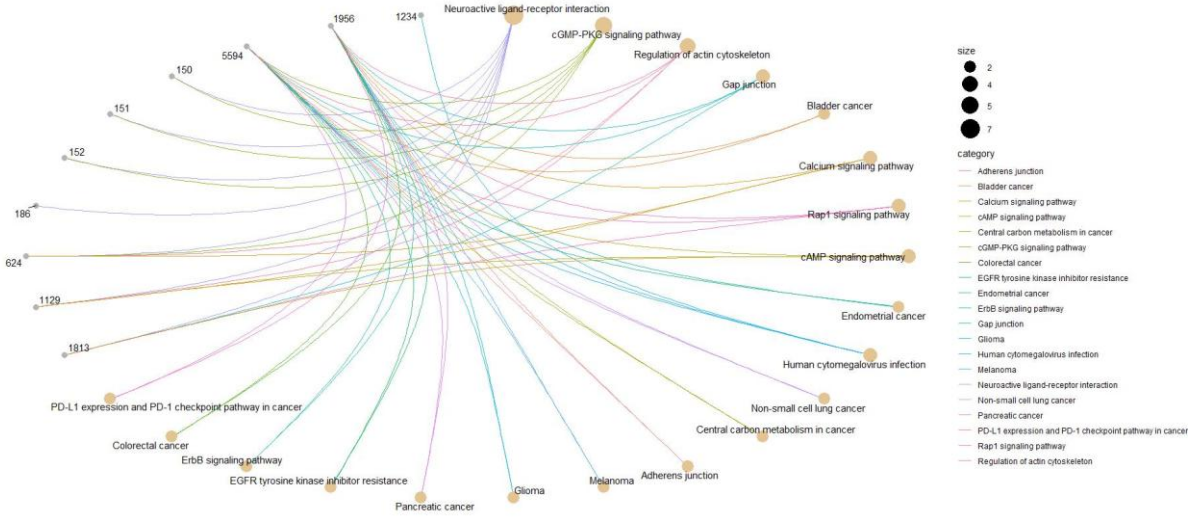

Fig.5 : Cnet plot illustrating core targets represented in entrez gene ID's linked to KEGG pathways.

Table 4: Significantly enriched KEGG pathways of VC against lung cancer.

S.NO	ID	DESCRIPTION	GENE RATIO	P.ADJUST VALUE	GENE NAME
1.	hsa04080	Neuroactive ligand-receptor interaction	7/10	3.076615e-06	DRD2,CHRM2,BDKRB2, AGTR2,ADRA2C, ADRA2B,ADRA2A
2.	hsa04022	cGMP-PKG signaling pathway	5/10	5.205992e-05	MAPK1, BDKRB2, ADRA2C,ADRA2B, ADRA2A
3.	hsa04810	Regulation of actin cytoskeleton	4/10	3.720619e-03	MAPK1,EGFR, CHRM2, BDKRB2
4.	hsa04540	Gap junction	3/10	4.446181e-03	MAPK1,EGFR, DRD2
5.	hsa05219	Bladder cancer	2/10	2.753931e-02	MAPK1,EGFR
6.	hsa04020	Calcium signaling pathway	3/10	2.774046e-02	EGFR,CHRM2, BDKRB2
7.	hsa04015	Rap1 signaling pathway	3/10	2.774046e-02	MAPK1,EGFR,DRD2
8.	hsa04024	cAMP signaling pathway	3/10	2.774046e-02	MAPK1, DRD2, CHRM2
9.	hsa05213	Endometrial cancer	2/10	2.774046e-02	MAPK1,EGFR
10.	hsa05163	Human cytomegalovirus infection	3/10	2.774046e-02	MAPK1,EGFR,CCR5

Molecular Docking:

The docking analysis revealed the exact targets of lung cancer to which VC binds. As shown in Table 5, the highest binding affinity was shown by BDKRB2 (-5.7 Kcal/mol).

Table 5: Binding affinities of core targets with VC.

S.No.	RECEPTOR NAME	BINDING AFFINITY (-Kcal/mol)
1.	EGFR	-4.5
2.	ADRA2C	-5.0
3.	CHRM2	-5.3
4.	CCR5	-5.0
5.	MAPK1	-5.1
6.	**BDKRB2**	**-5.7**
7.	DRD2	-3.6
8.	AGTR2	-4.6
9.	ADRA2A	-4.3
10.	ADRA2B	-4.6

This confirms that VC binds strongly to BDKRB2 in comparison to other core targets. Various poses of the docked complexes of 10 core targets with VC were viewed in PyMOL (Fig. 6).

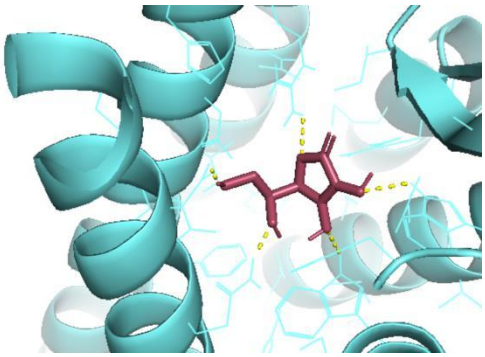

Fig. 6: Vitamin C binding to BDKRB2 target viewed in PyMol.

DISCUSSION

In both men and women lung cancer remains the primary cause of cancer death worldwide [25]. Although vitamin C intake and it's relation to lung cancer risk is extensively studied, it still remains ambiguous [26]. However, VC is currently being studied in treatment of lung cancer. In vivo studies on high dose VC and VC along with paclitaxel and carboplatin are being carried out [27]. In the present study, all documented bio-targets of VC against lung cancer were obtained, and 10 potential core targets were identified. Among the 10 core targets, ADRA2A, ADRA2B and ADRA2C are the subtypes of adrenergic receptors. Signals from adrenergic receptors are involved in regulation of apoptosis, angiogenesis and inflammation which leads to initiation and progression of tumours [28]. According to many reports, adrenergic receptors are also actively involved in metastasis of various cancers [29]. Type-2 angiotensin II (AT2) receptor is encoded by AGTR2 gene. While several previous studies suggest that AT2 receptor induces lung carcinogenesis by angiogenesis and increased cell proliferation [30], a study conducted by Pickel et al. demonstrates that over expression of AT2 receptor inhibits lung cancer proliferation by inducing apoptosis [31]. Hence, the role of AT2 receptor in lung cancer is still tentative. CHRM2 and CCR5 genes encodes muscarinic receptors 2 (M2) and 5 (M5) respectively. Previous studies reveal that, muscarinic receptors induce breast, colon, lung and prostate cancers [32]. Acetylcholine synthesized by bronchial epithelial and pulmonary neuroendocrine cells through expression of cholinergic autocrine loop stimulates muscarinic acetylcholine receptors and induces tumour growth in lung [33]. BDKRB2 gene encodes Bradykinin receptor B2 which is a G-protein coupled receptor for bradykinin in humans. Previous studies disclosed that BDKRB2 is involved in activation of ERK/MAPK pathway and angiogenesis in numerous cancers [34,35]. EGFR a tyrosine kinase receptor is a member of the erbB family. EGFR targeted drugs gefitinib and erlotinib have been approved in the treatment of advanced stage NSCLC [36]. Some reports also stated that EGFR expression is associated with reduced survival rate in NSCLC patients [37]. Unlike epinephrine and norepinephrine, dopamine inhibits angiogenesis and stimulated immune functions of tumours [38]. In a study conducted by Wu *et al*., overexpression of DRD2 also inhibited NF-κB signalling pathway further inhibiting the progression of NSCLC [39]. Although, DRD2 is one of the core targets, docking studies revealed that VC showed low binding affinity (-3.6 Kcal/mol) to DRD2. MAPK1 belongs to the family of MAP kinase proteins. Abnormal expression of MAPK1 has been observed in numerous cancers. MAPK1 is a key gene involved in tumour progression by facilitating cell proliferation and metastasis [40,41].

Bioinformatic-analysis of GO annotation and KEGG pathway enrichment disclosed the pharmacological mechanisms of VC in lung cancer. According to GO enrichment analysis, VC regulates the secretion and transportation of neurotransmitters, chiefly norepinephrine. Catecholamines play a vital role in metastasis through regulation of angiogenesis and tumour immunity [38]. The major issue in treatment of lung cancer is control and prevention of metastasis. The chief sites of metastasis for lung carcinomas are brain, bones and adrenal glands, the brain and adrenal glands being the two organs rich in catecholamines [42,43]. VC may help in control and prevention of metastasis in lung cancer through regulating catecholamine secretion and transportation. The significantly enriched KEGG pathways in this study are mostly associated with signalling pathways and various cancer pathways. The involvement of Rap1, cAMP, cGMP and ErbB signalling pathways suggests that VC action against lung cancer may be achieved by controlling both tumour development and progression. A network diagram of core targets and the pathways they are involved is constructed in Cytoscape (3.8.0) (Fig. 7).

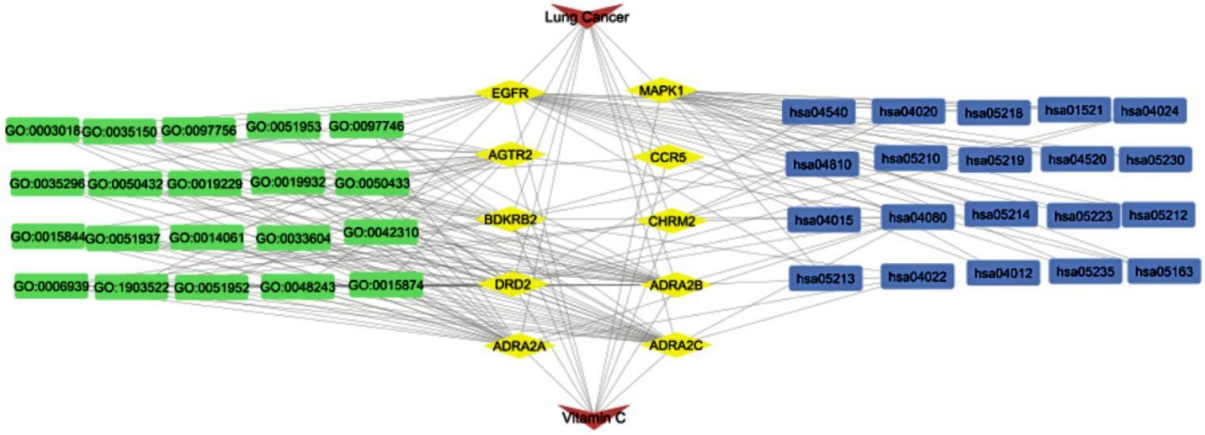

Fig. 7: PPI network of "disease-drug-target-pathway" of VC against lung cancer constructed in Cytoscape. The 10 core targets, GO biological processes and KEGG pathways represented in yellow, green and blue colours respectively.

CONCLUSION

In brief, this study used the network pharmacology analysis to identify the 10 core targets of VC against the lung cancer. From these core targets, BPs, MFs, CCs and pathways of VC against lung cancer were highlighted. VC can regulate several signalling pathways and play a vital role in treatment of lung cancer. Further VC can be very effective in prevention and control of metastasis when used in a combination therapy along with other chemotherapeutic agents. However, further experimental studies (*In vitro* and *In vivo*) should be conducted to validate the core targets and to demonstrate mechanism of VC in lung cancer treatment before using it as a therapeutic agent.

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Conflicts of interest:
The authors declare that no competing interest exists.

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ABBREVIATIONS

Vitamin C - VC
Small cell lung cancer - SCLC
Non-small cell lung cancer - NSCLC
Food and Drug Administration - FDA
Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform - TCMSP
Online Mendelian Inheritance in Man - OMIM
Gene Ontology - GO
Kyoto Encyclopaedia of Genes and Genomes - KEGG
Protein–protein interaction - PPI
Biological process - BP
Molecular function - MF
Cellular component - CC
Protein Data Bank - PDB

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