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Assessment of the Effects of Prolonged Ginkgo biloba Extract Therapy on Hepatocellular Carcinoma Using Network Analysis

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ABSTRACT

Extracts from Ginkgo biloba have been widely utilized in therapeutic applications for managing various conditions, including malignancies, cardiovascular issues, and neurological disorders. Given the potential adverse reactions linked to prolonged use of G. biloba extract, this study examined gene expression patterns in the livers of mice subjected to extended treatment through analysis of protein-protein interaction (PPI) networks. Information regarding the impact of G. biloba on mouse liver was obtained from the Gene Expression Omnibus (GEO) repository. Gene expression data from three sample categories—healthy controls, spontaneous hepatocellular carcinoma (HCC), and those exposed to G. biloba extract—were contrasted. Differentially expressed genes (DEGs) were evaluated using directed PPI networks to identify pivotal regulatory genes. In total, 23 key regulatory genes were linked to the administration of G. biloba extract. The evaluation revealed that Rhoc, Myc, Cdc20, Cdk1, Plk1, Bub1, Aurkb, Bub1b, Gsk3b, Incenp, Sgol2a, Rbl1, Aurka, Mapk7, and Ccnd1 were connected to hepatocellular carcinoma. The other significant regulatory genes were implicated in malignancies and additional pathologies. Rhoc and Myc emerged as central genes associated with extended intake of G. biloba extract. These results highlight a strong association between chronic exposure to G. biloba extract and elevated cancer risks, particularly hepatocellular carcinoma.

Keywords: Bioinformatics, Cancer, Gene expression, Ginkgo

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Introduction

Herbal remedies and plant-based therapies, known for their potential anti-neoplastic properties and reduced adverse effects, represent promising avenues in oncology research [1]. Among these, Ginkgo biloba L. stands out. Current investigations suggest that its extract possesses multiple health benefits, yet certain reports urge caution due to possible oncogenic risks with prolonged consumption. Malignancy represents a multifaceted disorder triggered by genetic alterations across various genes and signaling cascades, with its origins and development still not fully elucidated [2]. It remains a persistent degenerative condition with rising incidence worldwide [3]. Despite progress in fields like genomics, proteomics, and transcriptomics, understanding the roots and advancement of cancer continues to evolve [4].

No definitive treatment exists for cancer; conventional approaches such as surgical intervention, chemotherapeutic agents, and radiation therapy often carry substantial drawbacks [5]. Plant-derived compounds and traditional herbal treatments exhibiting tumor-suppressive effects with minimal toxicity offer viable options for advancing novel anticancer therapies [1]. The leaves of Ginkgo biloba are frequently employed in traditional remedies owing to their diverse bioactive and pharmacologic attributes. They are rich in compounds like flavonoids and terpene lactones [6]. Emerging evidence indicates anticancer potential of G. biloba extract via modulation of gene activity, reactive oxygen species scavenging, and inhibition of angiogenesis [7]. Its antioxidant mechanism involves

neutralization of reactive oxygen species [8], suppression of platelet activation, induction of apoptosis in tumor cells, and restraint of proliferation in liver cancer [9], as well as in gastric and pancreatic malignancies [10]. Extracts from Ginkgo biloba have been shown to elevate ING-3 levels while reducing FOXP1 in rodent models of liver cancer [11]. Han *et al*. reported that the extract restricts growth of Lewis lung carcinoma cells through regulation of catenin and Wnt3a [12]. Qian *et al*. found that G. biloba extract may arrest cell cycle advancement in human gastric tumor cells by lowering Cyclin D1 and c-Myc expression [13]. Conversely, Hoenerhoff *et al*. observed that chronic administration of the extract in mice resulted in higher rates of liver tumors, linked to mutations in H-ras and Ctnnb1, along with disruption of the WNT signaling pathway [14]. As illustrated by these instances, evidence on the influence of ginkgo extract on cancer development remains inconsistent and contradictory. Given the scarcity of studies exploring the underlying mechanisms by which ginkgo affects cancer induction or modulation, additional investigations into its chronic impacts are warranted. Leveraging genomic resources from established repositories and employing analytical tools for gene network mapping [15], the prolonged consequences of ginkgo extract on cancer promotion or inhibition can be explored. This investigation sought to evaluate the chronic influence of ginkgo extract on cancer risk, an area that has received limited attention.

Materials and Methods

Data acquisition

Searches for "Ginkgo biloba extract" and related genomic terms were conducted in Google Scholar to identify studies on its biological impacts. A specific report examining liver effects in B6C3F1 mice following two-year exposure to G. biloba extract was selected for further examination [14]. This report corresponded to dataset GSE29813 in the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29813>). The research involved three mouse cohorts: healthy controls, those with spontaneous hepatocellular carcinomas (HCC), and those with hepatocellular carcinomas associated with G. biloba extract (HCC-GBE), to assess alterations in hepatic gene expression due to extract exposure. The B6C3F1 mice under treatment received GBE administered via corn-oil gavage, five days per week, over a two-year period. Microarray analysis was performed to investigate hepatic responses to the extract in comparison to HCC states. Additional methodological information is available in the original work by Hoenerhoff *et al*. [14].

Pre-evaluation analysis

Gene expression data from the HCC and HCC-GBE cohorts were contrasted against the normal cohort employing the GEO2R tool to identify statistically significant differentially expressed genes (DEGs). Uniform Manifold Approximation and Projection (UMAP) visualization and a Venn diagram were utilized to highlight distinctions among the three examined cohorts. Significant DEGs were selected using criteria of adjusted p-value < 0.05 and absolute fold change > 2. Data preprocessing involved removal of unannotated and duplicate DEGs.

PPI network analysis

The identified significant DEGs were incorporated into directed protein-protein interaction (PPI) networks through the CluePedia plugin in Cytoscape software version 3.7.2. Interactions among the DEGs were established based on activation, inhibition, and expression relationships. Separate directed PPI networks were constructed for the GBE-induced HCC (GBE-HCC) and spontaneous HCC conditions. Network properties were evaluated using the “Network Analyzer” tool in Cytoscape, configured for directed edges. Networks were rendered with node sizes and colors reflecting out-degree centrality scores. Nodes exhibiting the highest out-degree values were designated as key regulatory players in the GBE-HCC and HCC networks. Overlapping key nodes between the two networks were classified as critical genes associated with hepatocellular carcinoma. Conversely, high-ranking nodes unique to the GBE-HCC network—and not prominent in the spontaneous HCC network—were interpreted as genes specifically modulated in the liver in response to prolonged G. biloba extract exposure, independent of the HCC pathology.

Results and Discussion

To assess potential variations in gene expression patterns across the HCC-GBE, HCC, and normal cohorts, a UMAP projection and a Venn diagram were generated. As illustrated in **Figures 1 and 2,** the three cohorts displayed clear separation based on overall gene expression profiles and the selected significant DEGs. In the UMAP visualization **(Figure 1)**, each data point was evaluated against 8 nearest neighbors to determine clustering patterns (number of neighbors (nbrs)=8). The plot revealed greater proximity between the spontaneous HCC and control groups compared to the HCC-GBE group.

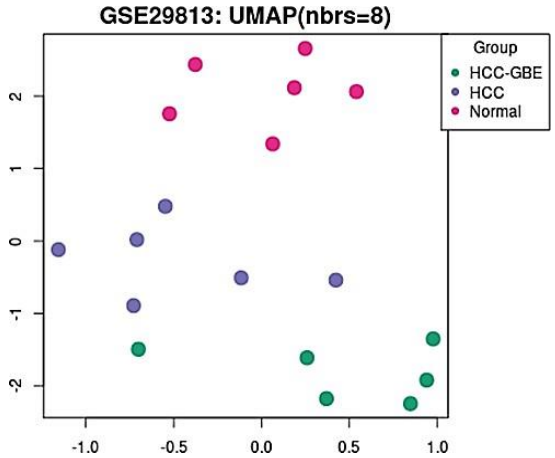

Figure 1. UMAP visualization of gene expression patterns across normal samples, hepatocellular carcinoma (HCC) samples, and HCC samples treated with Ginkgo biloba extract (HCC-GBE).

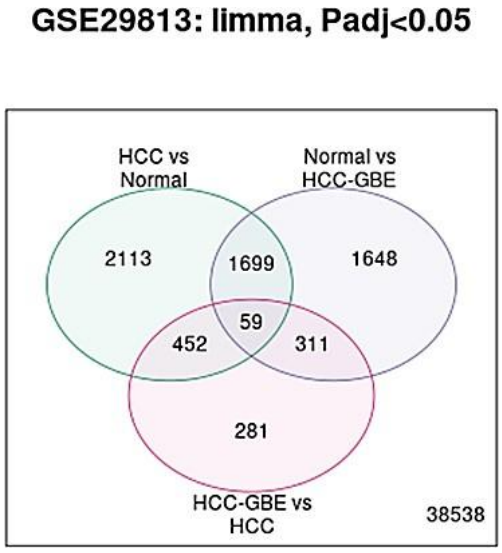

Figure 2. Venn diagram comparing the gene expression profiles across three groups: hepatocellular carcinomas (HCC), hepatocellular carcinomas induced by Ginkgo biloba extract (HCC-GBE), and normal liver tissue. The diagram highlights both shared and unique significantly differentially expressed genes (DEGs) among these pairwise comparisons.

These results underscore the molecular distinctions between the HCC and HCC-GBE groups. Following data preprocessing and filtering, a subset of 1,717 robust DEGs (out of an initial 38,538 dysregulated genes) emerged as key markers distinguishing the HCC-GBE group from healthy controls **(Figure 2)**. In parallel, 1,454 refined DEGs effectively separated conventional HCC samples from normal tissue. Of the 1,717 DEGs identified in the HCC-GBE analysis, 1,680 were successfully annotated and integrated using CluePedia. This enabled the construction of a directed protein-protein interaction (PPI) network, comprising 1,066 isolated nodes and 11 interconnected components linked by 1,960 edges. The core region of the primary connected component within this network is displayed in **Figure 3.**

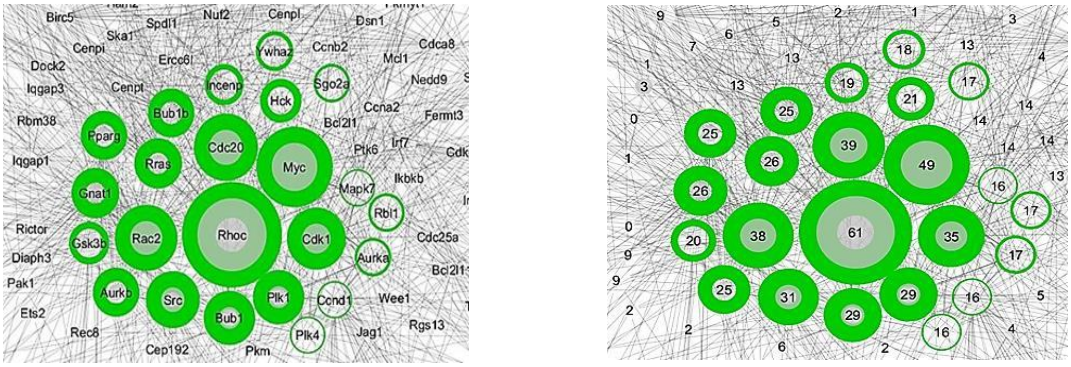

Figure 3. Central region of the largest connected component in the directed protein-protein interaction (PPI) network derived from the hepatocellular carcinoma induced by Ginkgo biloba extract (HCC-GBE) analysis. This network incorporates interactions involving activation, inhibition, and expression, with node positioning determined by out-degree centrality scores. Key hub genes are highlighted at the top of the schema, accompanied by their corresponding out-degree values below.

For the conventional HCC analysis, a directed PPI network was built using 1,429 annotated significant differentially expressed genes (DEGs). This network consisted of 932 isolated genes and 15 interconnected components. The primary connected component was arranged according to out-degree centrality **(Figure 4).** As detailed in **Table 1**, the genes Rhoc, Myc, Cdc20, Cdk1, Plk1, Bub1, Aurkb, Bub1b, Gsk3b, Incenp, Sgo2a, Rbl1, Aurka, and Ccnd1 were identified as shared hub DEGs present in both the HCC-GBE and HCC networks. In contrast, Rac2, Src, Gnat1, Rras, Pparg, Hck, Ywhaz, and Plk4 emerged as hub genes unique to the HCC-GBE network, absent from the conventional HCC network. To facilitate clearer visualization, the shared hub genes along with their respective out-degree values are illustrated in **Figure 5.**

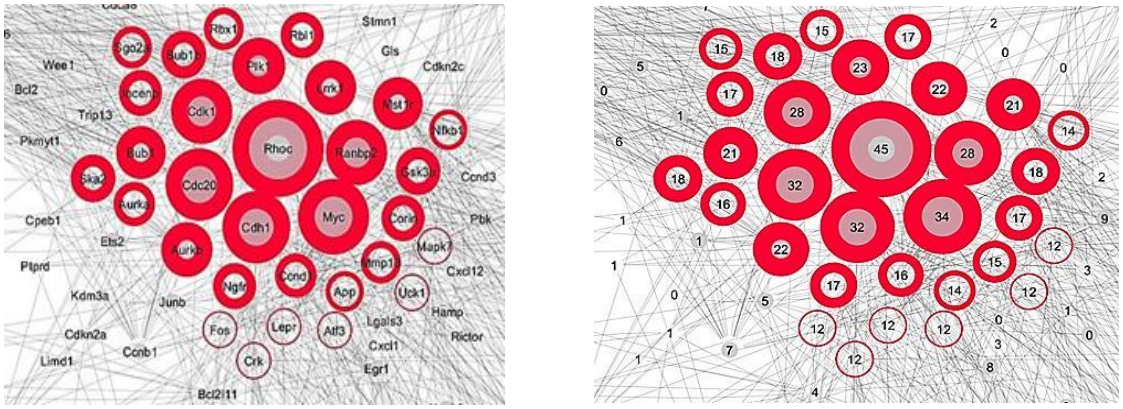

Figure 4. Central region of the primary connected component in the directed protein-protein interaction (PPI) network for the conventional hepatocellular carcinoma (HCC) analysis. The network incorporates interactions of activation, inhibition, and expression, with node arrangement based on out-degree centrality scores. Prominent hub genes are shown at the top of the schema, alongside their corresponding out-degree values below.

Extensive literature indicates that Ginkgo biloba extract exerts beneficial effects in the management of cardiovascular and neurological disorders, contributing to its widespread clinical use [16]. Research by Kim *et al*. has elucidated the involvement of ginkgo leaf extract in modulating cell proliferation and inducing apoptosis in gastric cancer cells [17]. However, a two-year gavage study involving Ginkgo biloba leaf extract revealed carcinogenic potential in mice, with elevated incidences of hepatocellular carcinoma (HCC) and hepatoblastoma [18].

As previously noted, the genes Rhoc, Myc, Cdc20, Cdk1, Plk1, Bub1, Aurkb, Bub1b, Gsk3b, Incenp, Sgo2a, Rbl1, Aurka, Mapk7, and Ccnd1—representing approximately 65% of the key hub genes in the HCC-GBE

analysis—have established roles in HCC pathogenesis. Statistical analysis revealed that the 23 hub genes in the HCC-GBE network exhibited a mean out-degree of 30 with a standard deviation (SD) of 11. Genes with out-degree values exceeding the threshold of mean + SD (i.e., >41), indicative of exceptionally high regulatory influence, were found to overlap completely between the HCC-GBE and HCC analyses, including top hubs such as Rhoc and Myc.

The Rho GTPase family comprises three isoforms—RhoA, RhoB, and RhoC—each with distinct physiological functions. RhoA primarily regulates actomyosin contractility, RhoB influences cytokine trafficking and cell survival, and RhoC is implicated in cell motility. Dysregulated expression of Rho proteins has been documented across various malignancies [19]. Specifically, Huang *et al*. demonstrated that RhoC positively correlates with migratory and invasive capabilities in HCC, key hallmarks of metastasis [20]. They proposed that modulating RhoC degradation could represent a promising therapeutic strategy for HCC. Notably, RhoC was upregulated in both HCC and HCC-GBE datasets. As illustrated in **Figure 5**, RhoC ranks as the foremost hub in the HCC-GBE network, achieving an out-degree value equivalent to mean + 3SD.

Ranking second among HCC-GBE hubs, Myc was upregulated following prolonged Ginkgo biloba extract exposure. Myc is frequently dysregulated in cancers, positioning it as a prime candidate for targeted therapy [21]. Min *et al*. reported that c-Myc overexpression in HCC is associated with diminished overall and disease-free survival, establishing it as both a prognostic biomarker and therapeutic target [22].

These observations collectively suggest that chronic administration of Ginkgo biloba extract may facilitate the initiation and progression of HCC. Consistent with the distinct gene expression profiles observed between HCC-GBE and conventional HCC **(Figure 1 and Table 1)**, approximately 35% of the hub genes in the HCC-GBE network—namely Rac2, Src, Gnat1, Rras, Pparg, Hck, Ywhaz, and Plk4—were absent from the HCC hub set. To assess the potential biological implications of these unique alterations, the functions of the five highest-ranked genes in this subset were examined through literature review.

Rac2 emerged as the leading unique hub, though it was neither classified as a hub nor a significant differentially expressed gene in the conventional HCC analysis. Existing studies predominantly link Rac2 expression to hematopoietic lineages, where it plays essential roles in the biology of neutrophils, lymphocytes, and related cell types. Mutations in Rac2 have been associated with various immunodeficiencies in humans [23].

The second-ranked unique hub, Src, contributes to metabolic reprogramming in cancer cells, processes intimately linked to differentiation, proliferation, and migration [24]. Ranked third, Gnat1 has been implicated in the pathogenesis of autosomal dominant congenital stationary night blindness [25]. Rras, a Ras family member, has been connected to Huntington's disease and nasopharyngeal carcinoma [26, 27]. Finally, Pparg (ranked fifth in this evaluation) has been evidenced to exacerbate malignancy in certain contexts [28, 29].

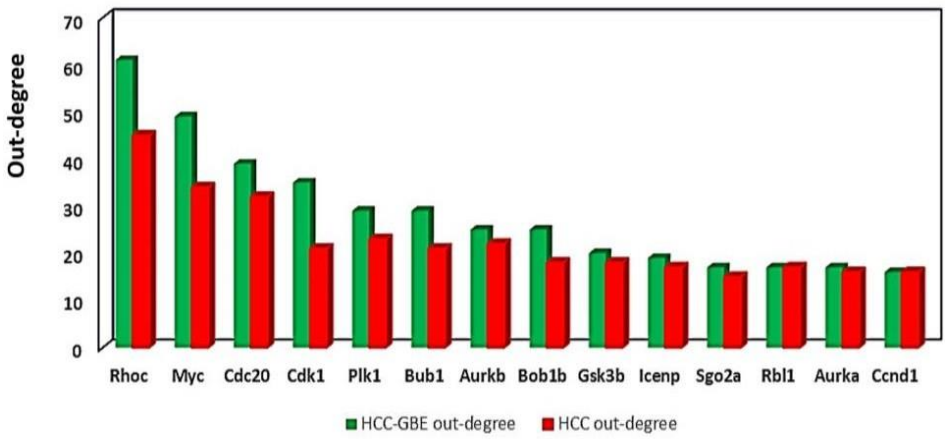

Figure 5. Shared key genes along with their corresponding out-degree values.

Table 1. List of actor genes of hepatocellular carcinomas induced by *G. biloba* extract (HCC-GBE) and hepatocellular carcinomas (HCC) analyses

No.	HCC-GBE analysis		HCC analysis	
	Central gene	**Out-degree value**	**Central gene**	**Out-degree value**
1	**Rhoc**	61	**Rhoc**	45

2	**Myc**	49	**Myc**	34
3	**Cdc20**	39	**Cdc20**	32
4	Rac2	38	Cdh1	32
5	**Cdk1**	35	Ranbp2	28
6	Src	31	**Plk1**	23
7	**Plk1**	29	Lrrk1	22
8	**Bub1**	29	**Aurkb**	22
9	Gnat1	26	**Cdk1**	21
10	Rras	26	Mst1r	21
11	**Aurkb**	25	**Bub1**	21
12	**Bob1b**	25	**Bub1b**	18
13	Pparg	25	Ska2	18
14	Hck	21	**Gsk3b**	18
15	**Gsk3b**	20	Ngfr	17
16	**Icenp**	19	Corin	17
17	Ywhaz	18	**Incenp**	17
18	**Sgo2a**	17	**Rbl1**	17
19	**Rbl1**	17	**Ccnd1**	16
20	**Aurka**	17	**Aurka**	16
21	**Mapk7**	16	**Sgo2a**	15
22	**Ccnd1**	16	Rbx1	15
23	Plk4	16	Mmp13	15
24	-	-	App	14
25	-	-	Nfkb1	14

The common genes between both HCC-GBE and HCC analyses are shown in bold face.

Conclusion

In summary, approximately 65% of the key DEGs identified in the HCC-GBE analysis overlapped with the primary driver genes from the HCC analysis. Rhoc and Myc emerged as central genes responding to prolonged Ginkgo biloba extract exposure, playing critical roles in HCC initiation and progression. The remaining 35% of HCC-GBE-specific genes were predominantly cancer-associated. These findings suggest that long-term or potentially high-dose use of Ginkgo biloba extract may contribute to the onset and exacerbation of HCC.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: The study received approval from Shahid Beheshti University of Medical Sciences under ethical code IR.SBMU.RETECH.REC.1403.192.

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