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原 著 論 文

Genome-wide transcriptome analysis to evaluate the potential functions of liquid of fermented plant extract
―Regulation of cholesterol metabolism in liver cells―

ゲノムワイドなトランスクリプトーム解析による植物発酵液の潜在的機能性の検討
―ヒト肝細胞に対するコレステロール代謝調節―

Wang Jinyun（早稲田大学人間科学研究科）[1]，Xie Kun（早稲田大学人間科学学術院），
原　太一（早稲田大学人間科学学術院）

Jinyun Wang (Graduate School of Human Sciences, Waseda University) [1],
Kun Xie (Faculty of Human Sciences, Waseda University),
Taichi Hara (Faculty of Human Sciences, Waseda University)

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Abstract

Liquid of fermented plant extract (LFPE) is a functional plant food which originates from Japan. It can be produced by fermentation, using probiotics, of various plants such as fresh vegetables, fruits, mushrooms, and herbs. Many studies have revealed the health promoting effects of LFPE due to its abundant nutrients and active substances, however, the potential mechanisms underlying these effects are still unclear. Thus, in the present study, we recruited genome-wide RNA sequencing analysis to investigate alterations in gene expression and metabolic pathway activation. in cells with LFPE supplementation. Differentially expressed genes (DEGs) were identified and subjected to Gene Ontology and reactome pathway analysis, and results showed the DEGs were mainly enriched from sterol metabolism and oxidoreductase activity. Furthermore, protein-protein interaction analysis revealed that the genes encoding MVD, HMGCS1, DHCR7, FABP3, and ACAT2 were most significantly altered in expression level. In summary, the genome-wide analysis revealed the most significantly altered genes by LFPE supplementation in cells, and further analysis showed that the DEGs were enriched in sterol and cholesterol biosynthesis and metabolism. These effects may be associated with the modulation of oxidoreductase activity by LFPE supplementation.

Key Words：differentially expressed genes, liquid of fermented plant extract, HepG2 cell

1. Introduction:

Extending healthy lifespans has becoming a worldwide health concern. Aging is associated with a loss of metabolic homeostasis and plasticity, which is causally linked to multiple age-related pathologies. Studies have demonstrated that prevention is better than cure

[1] Jinyun Wang (Graduate School of Human Sciences, Waseda University): wangjinyun@ruri.waseda.jp

for aging associated diseases (Koizumi, Oku et al. 2019). Therefore, due to the minor adverse side effects, consumption of functional food has been considered as an available resource for extending good health, rather than medicine. Foods exhibit three principal functions. The first is nutritional function, which is to provide essential nutrition for survival (for instance, proteins, carbohydrates, and vitamins) and is the most important property, especially in areas of poverty where there are food deficits. The second function is the taste and sensory properties of foods. The third function of food is to regulate biological functions.

Although functional foods are not as sufficient as medicines in disease therapy, they exhibit health-promoting functions, especially for long-term prevention (Ohnishi, Yano et al. 2020). A variety of foods (such as fruits, vegetables, fish, oils, and nuts) contain ingredients that are functional components (including polyphenols, terpenes, flavonoids, alkaloids, sterols, anthocyanin, and unsaturated fatty acids). Both *in vivo* and *in vitro* studies have showed these functional food components play an important role in disease prevention, such as for cancer, depression, T2DM, obesity, asthma, and cognitive dysfunction (Mirmiran, Bahadoran et al. 2014). The bioactive compound resveratrol showed beneficial effects in the prevention of different types of cancer, such as breast cancer (Abdel-Latif, Al-Abd et al. 2015), cervical cancer, (Tomoaia, Horovitz et al. 2015) kidney cancer (Raghubeer, Nagiah et al. 2015), hepatic cancer (Dai, Lei et al. 2015) and bladder cancer (Wu, Li et al. 2014). Moreover, these functional components have also been shown to reduce oxidative stress and inflammation (Anderson 2003). Thus, functional foods have been developed as complementary therapies to prevent diseases or when patients seek to relieve symptoms (Ernst and Pittler 2001).

Liquid of Fermented Plant Extract (LFPE) is a novel type of functional food that contains a high concentration of functional components. Briefly, the products were made by using the different vegetables and fruits as raw materials, fermented, aged and sterilized for several months at the appropriate temperature and humidity. Since the production process of different products is not exactly same, the sample was supported in this study was Liquid of Fermented Plant Extract SW, thereafter abbreviated as LFPE. According to our previous study(Yano, Oyy et al. 2020) in human fibroblast cells, LFPE exhibits a suppressive effect that protects cells from oxidative stress and increasing COL3A1 expression, suggesting that it may also affect collagen biosynthesis. Several other studies have validated the many advantages of LFPE including scavenging reactive oxygen species (Serafini and Peluso 2016), inhibiting allergen reactions (Takata, Kinoshita et al. 2011), and preventing oxidative stress. Therefore, further investigating the mechanisms of LFPE on health is necessary. RNA sequencing (RNA-seq) technology has significantly progressed in recent decades and has become a crucial transcriptome profiling tool in many aspects of research and therapy (Hong, Tao et al. 2020), including biomarker discovery and characterization of disease progression and evolution (Stark, Grzelak et al. 2019). Here, we used RNA-seq to analyze changes in transcription factor levels and differentially expressed genes (DEGs) in OUMS-36T-1 cells supplemented with LFPE, and then explored changes in the biological processes (BPs), molecular functions (MFs), and cell components (CCs) based on Gene Ontology (GO) enrichment analysis. Moreover, pathway analysis and protein-protein interaction (PPI) network analyses were performed to identify the activated or inactivated pathways and specific proteins involved. The altered genes were validated by qPCR in HepG2 cells. This study revealed the potential capabilities about LFPE and provides directions for developing new functions of LFPE in the future.

2. Materials and methods

2.1. Reagent preparation

HepG2 cells and OUMS-36T-1 cells were purchased from the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). The liquid of fermented plant extract was provided by Yagumo kousan corporation. RNA extraction was performed using a ReliaPrepTM RNA cell Miniprep system (Promega, Madison, WI, USA). Reverse transcription was conducted using ReverTra Ace® qPCR RT Master Mix with a gDNA remover

kit (TOYOBO, Tokyo, Japan). RT-PCR reagents were purchased from TAKARA corporation (Tokyo, Japan). The LFPE processing procedure was described in our previous paper(Yano, Oyokawa et al. 2020).

2.2. Cell culture and liquid of fermented plant extract treatment

HepG2 cells were cultured at 37°C in 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Wako, 044-29765). OUMS-36T-1 cell was seeded in a 6-well plate at a density of 1×10^5 cells for 24 h and treated with medium containing 1%stock LFPE solution for 24 h.

LFPE solution was prepared using the following steps: The original LFPE solution was centrifugated at 15000 rpm for 10 min. The supernatant was extracted and filtered using 0.45 μm filters to remove insoluble substances. The extracted liquid was sterilized using a 0.22 μm filter and used as the stock solution.

2.3. RNA extraction and sequencing

Total RNA was extracted using a ReliaPrepTM RNA cell Miniprep system (Promega, Madison, WI, USA) according to the manufacturer's protocol. mRNA was isolated using oligo (dT) beads. A cDNA library was constructed using the NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. The final cDNA library was prepared after several rounds of purification, terminal repair, A-tailing, sequencing adapter ligation, size selection, and PCR enrichment. RNA sequencing was performed on the libraries by Novogene (Beijing, China) using a NovaSeq 6000 System. Reads were mapped to reference sequences using TopHat 2.

2.4. Bioinformatic analysis

METASCAPE (https://metascape.org/gp/index.html#/main/step1) was used for systematic analysis. All raw data were submitted and annotated using existing human gene databases. Predicted target genes were analyzed using GO enrichment analysis of BPs, CCs, and MFs with the following criteria: $p<0.01$, minimum count = 3, and enrichment factor>1.5. Genes coding for protein and process pathways were used for reactome pathway analysis.

2.5. Reverse transcription and RT-qPCR

The extracted total RNA was converted into cDNA using the ReverTra Ace® kit. The mRNA expression of selected genes was determined using RT-qPCR with the following reaction mixture contained 12.5 μL of TB Green Premix Ex Taq II, 2 μL of cDNA, 1 μL of 10 μM

Table 1. The primer sequences for real-time qPCR.

Gene name	Primer sequence 5'-3'
GAPDH	F: GAAGGTGAAGGTCGGAGTCA
	R: TGGACTCCACGACGTACTCA
FABP3	F: GTG GAG TTC GAT GAG ACAACA GC
	R: TGG TCT CTT GCC CGT CCC ATT T
HMGCS1	F: CTA GCA CAG TAC TCA CCT CAG C
	R: GGT GCC ACA CCA GTT CTT GA
MVD	F: TGG TGA GCG CTG AGA AGA AG
	R: GTG GAA CTG GTT GCT GTC CT
ACAT2	F: TGA GCA AGG CTC CTC ACT TG
	R: ACA GAA CTG CAA CCT TGT CCT
DHCR7	F: TCA CCA ATG ACA GAA CCG CA
	R: AGC TGT ACT GGT CAC AAG CC

1:1 forward and reverse target primers, and 8.5 μL of nuclease-free water. The primers for MVD, HMGCS1, DHCR7, ACAT2, FABP3 and GAPDH are listed in Table 2. Reactions were performed in independent triplicate experiments. Gene expression was normalized to the geometric mean of the housekeeping gene GAPDH as an internal control. Data were analyzed using the 2-ΔΔCT method.

2.6. Statistical analysis

Results were expressed as the mean ± SD. Significant differences between treatments were analyzed with Student's t-tests using SPSS 24.0 (SPSS Inc., Chicago, IL, USA). Significant differences ($p<0.05$) are indicated using asterisks.

3. Results

3.1 RNA-seq analysis of differentially expressed genes (DEGs)

The OUMS-36T-1 cells is a Japanese derived, hTRT gene transfected normal human embryo fibroblast cell line. Since the mainly users of the LFPE were Japanese, OUMS-36T-1 cells were selected for the initial analysis. To investigate the potential alterations in gene expression caused by LFPE treatment in the OUSW-36T-1 cell line, we extracted total RNA from the 1% concentration of LFPE and no-treatment (control) groups, and then performed RNA-seq analysis followed by gene annotation in comparison with published human gene databases (Metascape). A total of 13423 clear reads were successfully filtered and mapped onto the human genome. Among them, 1109 genes were differentially expressed in each group, of which 661 were expressed in the control group only and 448 were expressed in the LFPE treatment group only (Figure 1A). A total of 78 DEGs were displayed using a volcano plot (Figure 1B) using a threshold of $p<0.05$, |log2FoldChange|>0.4. Of these, 20 were downregulated and 58 were upregulated. To evaluate the potential influence of LFPE, DEGs that showed |log2FoldChange|<1.3 were excluded by a stricter threshold, and the remaining DEGs were selected for further analysis. The remaining genes are displayed to highlight significantly upregulated (blue) and downregulated (red) genes in the volcano map (Figure 1B). The results depicted in Figure 1B may include false positives, but those false positives are subsequently excluded based on the results of the PPI analysis and GO analysis, which are discussed later.

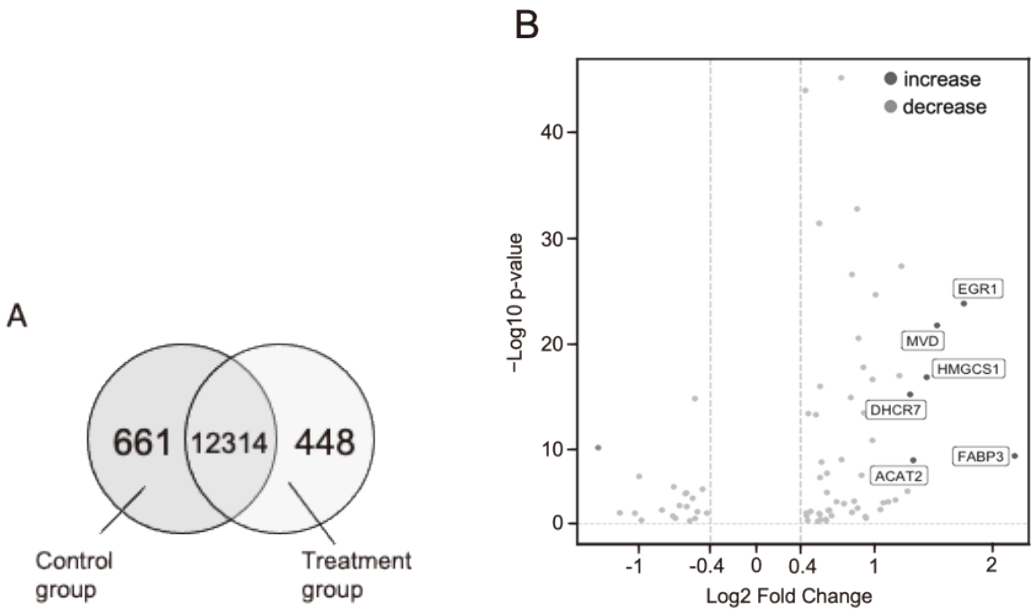

Fig 1. Gene annotation and volcano plot of transcriptome analysis.
(A) Gene annotation. 13423 genes were successfully mapped onto the human gene database. (B) Volcano map of 78 differentially expressed genes (DEGs) ($p<0.05$, |log2FoldChange|>0.4). Significantly upregulated and downregulated DEGs are represented by blue and red dots, respectively. The x-axis represents the statistical significance of differential expression, the more significant the difference in expression, the higher the value on the x-axis. The y-axis represents the change in gene expression, expressed as the log2 fold change (log2FC).

3.2 GO enrichment analysis

In the field of bioinformatics, several computational methods have been proposed to analysis the potential meaning of alteration. The Gene Ontology (GO) is organized into three main categories, namely Biological Process(BP), Molecular Function (MF), and Cellular Component (CC), to provide a comprehensive and structured framework for describing gene attributes. Biological processes(BPs), which described the larger-scale biological events or processes that occur within a cell or an organism. The results described the ordered set of activities that contribute to particular biological phenomenon. Molecular functions (MFs), which focused on the specific molecular activities or functions performed by genes or proteins. The results of MFs focused on the enzymatic, binding, or structural activities of proteins. Cellular components (CCs),which described the location relative to cellular structures in which a process product performs a function. Unlike the other aspects of GO, the results of cellular components refer to the cellular anatomy. In this study, we performed GO enrichment analysis comparing LFPE-treated cells with control cells in terms of differences in three parts. The most enriched BPs were sterol and cholesterol biosynthetic and metabolic processes (Figure 2, Table 1), whereas the most enriched CCs were the lytic vacuole, lysosome, and endosomal and lysosomal membrane. The most enriched MF was oxidoreductase activity. In addition, we performed pathway analysis to identify the pathways that correlated the most with the significant BPs. As shown in Figure 3 and Table 2, DEGs were significantly enriched in the sterol biosynthetic process.

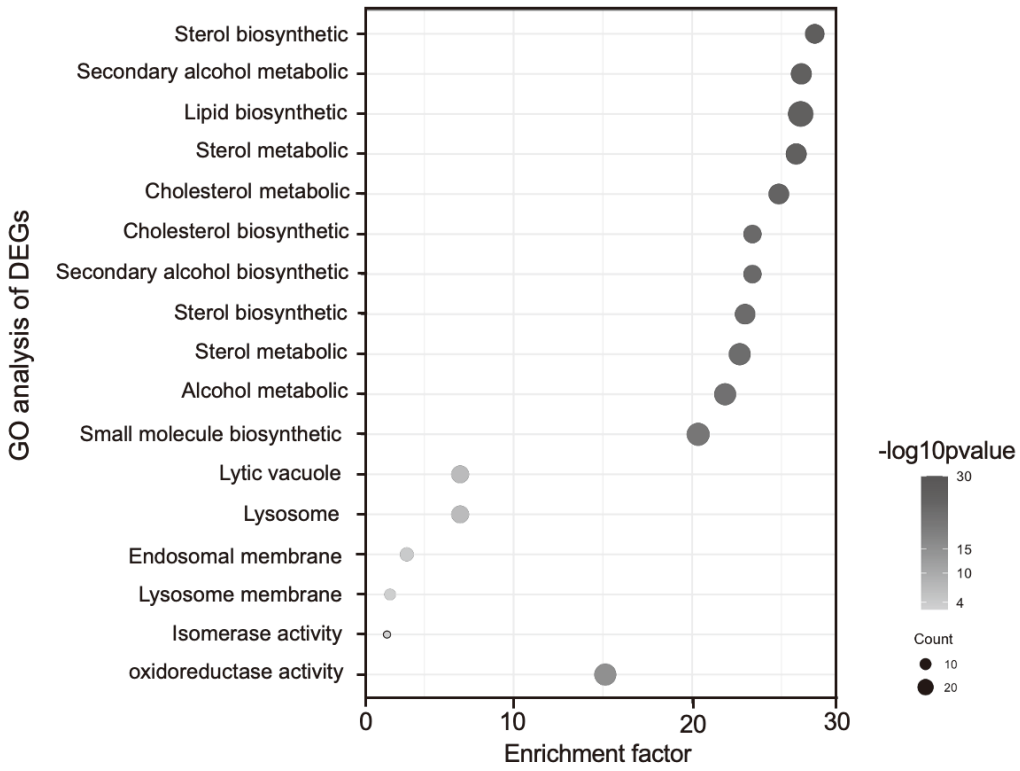

Fig 2. Gene Ontology (GO) analysis of LFPE-related DEGs.
Gene Ontology (GO) analysis is a bioinformatics method for functional annotation and analysis of genes or gene products, which provides a standardized and structured vocabulary to describe the biological functions, molecular activities, and cellular components associated with differentiated genes (DEGs). The data represented the biological process that DEGs may involve, and p-value indicated that the enrichment of the DEGs, the higher the p-value the more of the DEGs are involved.

Table 2. Gene Ontology (GO) analysis of LFPE-related DEGs

	Go number	Description	Log10 P -value
Biological processes	GO:0016126	sterol biosynthetic process	-26.87
	GO:1902652	secondary alcohol metabolic process	-26.12
	GO:0008610	lipid biosynthetic process	-26.08
	GO:0016125	sterol metabolic process	-25.83
	GO:0008203	cholesterol metabolic process	-24.85
	GO:0006695	cholesterol biosynthetic process	-23.38
	GO:1902653	secondary alcohol biosynthetic process	-23.38
	GO:0006694	steroid biosynthetic process	-22.97
	GO:0008202	steroid metabolic process	-22.67
	GO:0006066	alcohol metabolic process	-21.85
	GO:0044283	small molecule biosynthetic process	-20.34
Cellular components	GO:0000323	lytic vacuole	-7.00
	GO:0005764	lysosome	-7.00
	GO:0010008	endosomal membrane	-4.01
	GO:0005765	lysosomal membrane	-3.08
	GO:0098852	lytic vacuole membrane	-3.08
Molecular Functions	GO:0016491	oxidoreductase activity	-15.14
	GO:0016215	acyl-CoA desaturase activity	-7.09
	GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	-6.14
	GO:0016628	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	-5.91
	GO:0016614	oxidoreductase activity, acting on CH-OH group of donors	-5.62

3.3 Pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Wiki pathway associated enrichment analysis were performed to identify the potential activated pathways using select DEGs from the LFPE treatment group. As shown in figure 4, DEGs were significantly enriched in the cholesterol metabolism (Top 1, WP4718), steroid biosynthesis(Top 2,Ko00100), and oxidoreductase pathways(Top 3 Go:0016491,Top 9 Go:0055114,Top11 Go:0016705). These results showed that LFPE may have the potential to activate nutritional metabolism. LFPE also appears to play a role in modulating oxidoreductase activity, which may correlate with previous findings of its antioxidant effects.

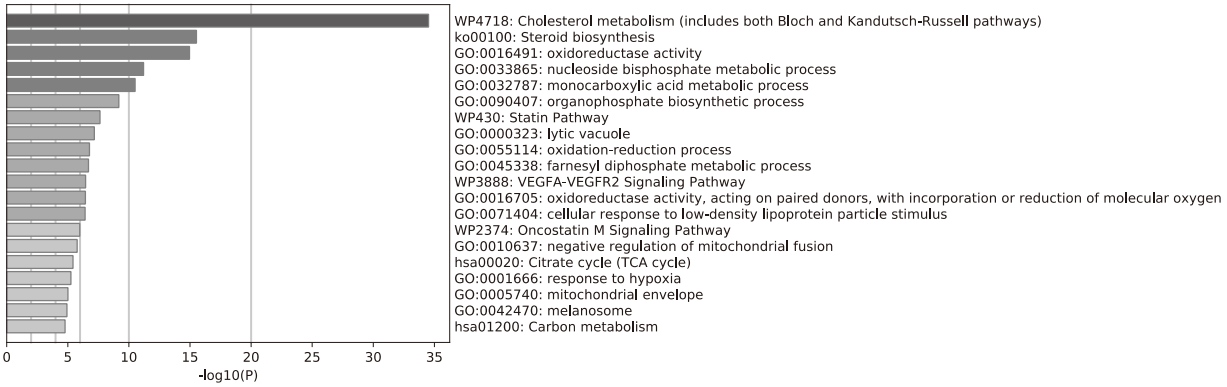

Fig 4. The columns represent different significantly enriched pathway results from databases. The length of the bar and the depth of the color represent the significance.

3.4 Protein-protein Interaction (PPI) Enrichment Analysis

Understanding indirect interactions between proteins provides a comprehensive description of potential mechanisms and functions. To identify hub genes of DEGs and relationships between proteins involved in the cholesterol metabolism pathway, protein-protein interaction (PPI) network analysis was performed. The STRING, BioGrid, OmniPath8, InWeb_IM databases were used in combination for the PPI enrichment analysis. As shown in Table 3, some indirect genes were predicted to regulate cellular sterol biosynthesis. As shown in Figure 5, DHCR7, MVD, ACAT2, HMGCS1, FABP3 were significantly associated with the sterol

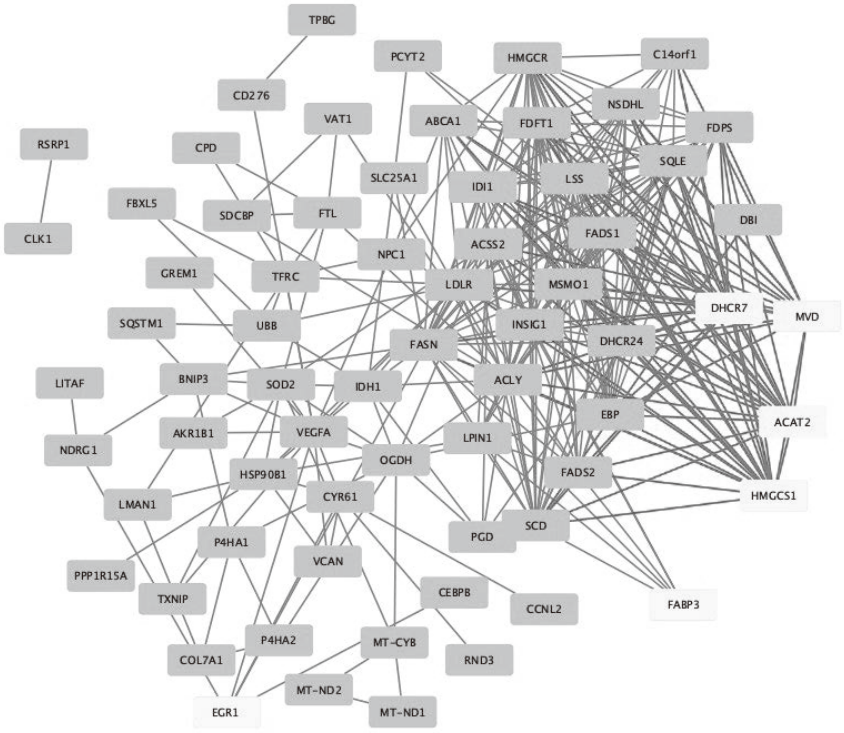

Fig 5. The PPI analysis of DEGs. Protein-protein interaction (PPI) enrichment analysis is a computational method used to analyze and interpret protein interaction data to gain insights into biological processes and functional relationships between proteins. Yellow background proteins presented here indicated the prediction interaction of the most differentiated genes (DEGs) encoded proteins, and blue background proteins represented the proteins that associated with the DEGs encoded proteins.

Table 3. Predicted hub proteins from protein-protein interaction

Gene ID	log2.Foldchange	p-value	name	Description
ENSG00000108828	0.53838	4.50E-08	VAT1	vesicle amine transport protein 1 homolog
ENSG00000087086	0.5313	3.94E-32	FTL	ferritin, light polypeptide
ENSG00000137575	0.5364	0.00013516	SDCBP	syndecan binding protein (syntenin)
ENSG00000131473	0.59714	1.76E-08	ACLY	ATP citrate lyase
ENSG00000120437	1.3273	1.04E-09	ACAT2	acetyl-CoA acetyltransferase 2
ENSG00000131069	0.92867	0.00031774	ACSS2	acyl-CoA synthetase short-chain family member 2
ENSG00000142657	0.43463	0.0005216	PGD	phosphogluconate dehydrogenase
ENSG00000160752	1.2787	8.92E-07	FDPS	farnesyl diphosphate synthase
ENSG00000079459	1.2277	4.45E-28	FDFT1	farnesyl-diphosphate farnesyltransferase 1
ENSG00000104549	0.8898	2.75E-08	SQLE	squalene epoxidase
ENSG00000133935	1.1159	8.95E-06	ERG28	chromosome 14 open reading frame 1
ENSG00000172893	1.3013	6.12E-16	DHCR7	7-dehydrocholesterol reductase
ENSG00000160285	1.0087	2.29E-25	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)

biosynthetic process. Consequently, based on our bioinformatic data, these findings suggest that LFPE has the potential to regulate the sterol and lipid biosynthetic processes. Although the expression of genes involved in sterol and lipid biosynthesis is significantly low in OUMS-36T-1 cell lines, the bioinformatic data suggested that LFPE may play an important role in cellular sterol and lipid biosynthesis process.

3.5 DEGs validation using RT-PCR

To verify the accuracy of the predictive analysis results, we performed qPCR analysis in HepG2 cell treated with LFPE. The 5 most significantly altered genes in the pathway analysis results were subsequently analyzed, and the results had a same trend as the RNA-seq results (Figure 6). The MVD and HMGCS1 genes encoded the enzymes that involved in mevalonate pathway, and increased expression of MVD and HMGCS1 may increase the protein levels of mevalonate diphosphate decarboxylase and Hydroxymethylglutaryl-CoA synthase which are mainly present in the liver of mammals, and are controlled the conversion of mevalonate to cholesterol. And the cholesterol is further converted to steroid hormones, bile acids, and vitamin D. Therefore, these data indicated that LFPE supplementation enhanced the mevalonate pathway in HepG2 cells, and LFPE has interfered the cellular sterol and lipid biosynthesis process.

4. Discussion

Genome-wide transcriptome analysis has been developed as a novel next generation analysis method to explore the differentiated genes in organisms (Higgs and Lehman 2015). The present study investigated the change of the genes after LFPE supplementation by using genome-wide RNA sequencing. Results showed that DEGs were enriched in the sterol and cholesterol metabolic and biosynthetic processes, indicating that LFPE supplementation may affect cellular endogenous sterol biosynthesis. Several reports have showed that providing

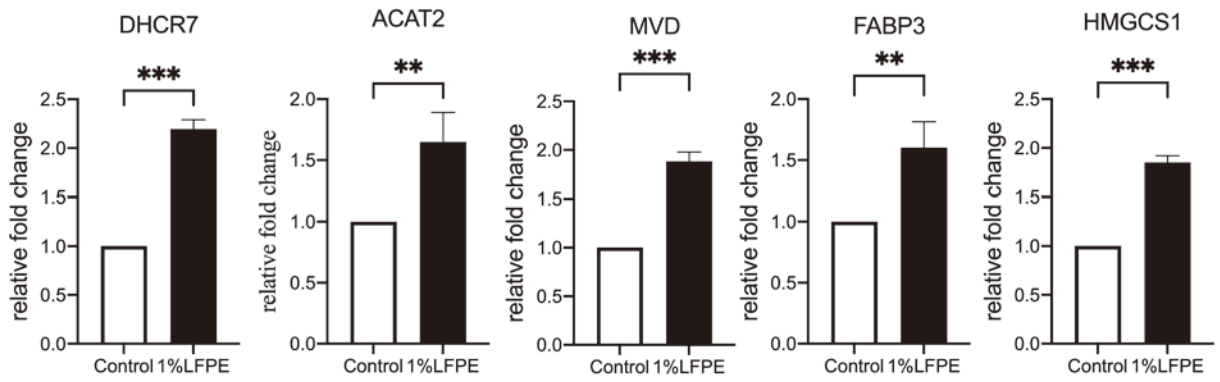

Fig 6. The most significant changed genes in RNA-seq analysis which were further confirmed in HepG2 cells treated with LFPE HepG2 cells were treated with 1% LFPE solution for 24h. The data represent the mean ± SD (n=3). The ** indicate significance ($p<0.01$) and *** indicated extreme significance ($p<0.001$) among the groups analyzed with Student's t-tests using SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

fermented plant-based food to the hypercholesterolemic individuals could reduce body mass index and decrease total cholesterol and low-density lipoprotein cholesterol (Chiu, Chen et al. 2017). Another study showed that fermented wheat germ extract supplementation acts as a redox modulator that alleviated endotoxin-triggered oxidative stress (Mackei, Vörösházi et al. 2020), and the redox balance change could modulate endogenous steroid metabolism since steroid synthesis requires proton donors or acceptors (Poli, Leoni et al. 2022) (Biswas and Maitra 2021). Consistently, GO analysis of molecular function in the present study revealed that oxidoreductase activity, including paired donor interactions and incorporation or reduction of molecular oxygen, was significantly changed after LFPE supplementation. As indicated in Table 2, Molecular function of oxidoreductase activity involving CH-CH groups that recruit NAD+ or NADP+ significantly enriched after LFPE supplementation. To our knowledge, endogenous steroid metabolism includes the cholesterol biosynthesis are the essential biosynthesis pathway for construction of cell membranes. The enzyme HMG-CoA reductase is considered to be the rate-limiting step in cholesterol biosynthesis. In the conversion of HMG-CoA to mevalonate, a two-electron reduction occurs, leading to the formation of mevalonate. Therefore, LFPE may regulate the oxidoreductase activity of HMG-CoA reductase. This reduction reaction catalyzed by HMG-CoA reductase can be summarized as follows:

$$\text{HMG-CoA} + 2\ \text{NADPH} + 2\ \text{H}^+ \rightarrow \text{mevalonate} + \text{CoA-SH} + 2\ \text{NADP}^+$$

These data suggested that LFPE which contain high levels of polyphenols and flavonoids (Feng, Zhang et al. 2017), act as an antioxidant agent involved in modulation the redox reaction *in vitro*. Moreover, our results showed LFPE supplementation significantly increased relative expression of MVD and HMGCS1 genes, the gene of MVD and HMGCS1 encoded the enzymes that involved in mevalonate pathway, consistent with the results that LFPE regulate endogenous cholesterol biosynthesis.

Cholesterol metabolism is an essential and complex biological process involved in the regulation of basic cell functions, such as regulating the cellular phospholipid membranes (de Meyer and Smit 2009) and the synthesis steroid hormones (Qamar and Bhatt 2015), and an imbalance in cholesterol regulation leads to the dysfunction of organs (Xu, Zhou et al. 2020). In our study, the most enriched genes were identified to be involved in vital steps of endogenous cholesterol metabolism. For example, ACAT2 gene regulation can improve the lipid toxicity of excess cholesterol and fatty acids (Wang, Bian et al. 2017), and is selectively expressed in the small intestine and liver in order to effectively absorb cholesterol from the diet and assemble lipoproteins(Farese 1998, Chang, Chang et al. 2001, Rudel, Lee et al. 2001). Therefore, the significantly elevated expression of ACAT2 in our results suggest that LFPE improved the assembly of

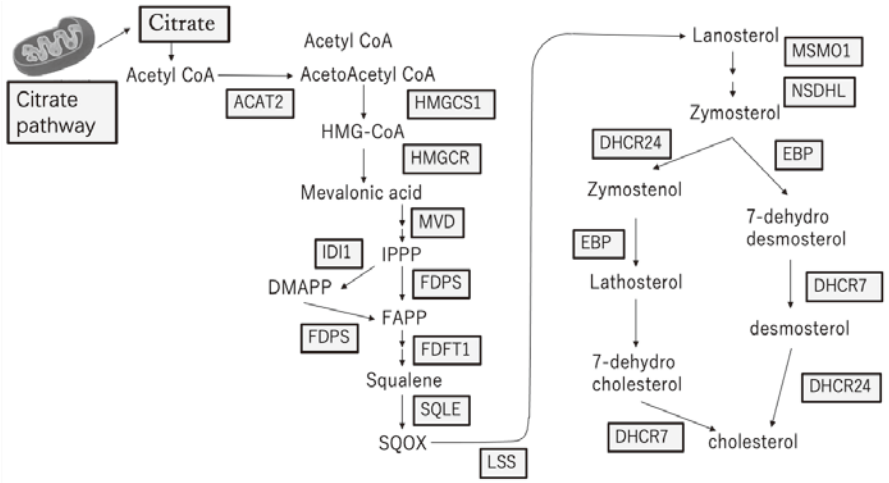

Fig.7 Cholesterol metabolism pathway .Cholesterol metabolism pathway, which also called as mevalonate pathway, In the mevalonate pathway, the starting molecule is acetyl-CoA, which undergoes a series of sequential reactions catalyzed by different enzymes to produce isopentenyl pyrophosphate (IPP). IPP serves as a precursor for the synthesis of numerous molecules, including cholesterol, ubiquinone, dolichol, and several other essential isoprenoids. Throughout the pathway, a series of reduction reactions take place, where electrons are gained by certain intermediates. For example, in the conversion of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) to mevalonate, a two-electron reduction occurs, leading to the formation of mevalonate. This reduction is catalyzed by the enzyme HMG-CoA reductase, which is a rate-limiting step in the pathway and a target for cholesterol-lowering drugs called statins.

lipoproteins and may accelerate the conversion of acetyl-CoA to acetoacetyl-CoA in the mevalonate pathway. Moreover, HMGCS1 catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form 3-hydroxy-3-methylglutarylcoenzyme A, and accordingly, MVD mRNA expression was significantly increased more than 1.5-fold than that in the control cells, indicating that LFPE supplementation interfered the normal lipid metabolism of the cells.

Cholesterol plays essential roles in the stability and architecture of the plasma membrane and as a precursor for bile acid and steroid hormone synthesis in mammals (Cruz, Mo et al. 2013). Cells continuously lose cholesterol to extracellular circulation. Regulation of synthesis, influx and efflux keeps cellular cholesterol levels precisely controlled. Such a loss can be quite rapid, up to 0.1% of total cholesterol per minute (Miyazaki, Sakai et al. 1995). The release from the plasma membrane occurs by desorption of cell surface cholesterol into lipoproteins or is induced after high-density lipoproteins bind to membrane receptors (Rader, Alexander et al. 2009). Some tissues, mainly the liver and the intestine, release cholesterol to the circulation mostly as esters by synthesizing and secreting lipoproteins (Olofsson, Asp et al. 1999). Thus, the high expression of genes that are enriched in the steroid biosynthesis pathway in HepG2 cells treated with LFPE, indicates an enhancement in cholesterol synthesis. However, cholesterol is not solely beneficial to the cell. Excess cholesterol is toxic and therefore cells have to employ a number of safety mechanisms to limit the concentration of free cholesterol. Consistently, we observed the increased mRNA expression of ACAT2 and FABP3, which regulate the transportation of endogenous lipids and control lipoprotein assembling, suggested that these released cholesterols were metabolized to form bile, together with phospholipids. Meanwhile, due to deficient feedback inhibition of *in vitro* cell culture (Ikonen 2008), the LFPE activated cholesterol synthesis was maintained longer than in *in vivo* investigation (Ikonen 2008). The present study provides novel potential effects, indicated that supplementation 1% LFPE to cells significantly enhanced cholesterol synthesis, and subsequently interfere the whole lipid metabolism of the cells, although the specific mechanisms still need to be studied further *in vivo*.

Collectively, genome-wide analysis revealed the most significantly altered genes following LFPE supplementation in cells, and further analysis showed that the DEGs were enriched in sterol and cholesterol

biosynthetic and metabolic processes. Furthermore, these effects may be associated with the modulation of oxidoreductase enzyme that involved in mevalonate pathway by LFPE.

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Conflicts of Interest: The sponsors, Yagumo kousan Co.Ltd., had no role in the design, execution, interpretation, or writing of the study. The authors declare no conflict of interest.

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