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Preliminary Study on the Anti-Proliferative Mechanism of Hesperetin against MDA-MB-231 Cells Based on RNA-Seq Analysis

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Abstract

Background: Hesperetin is a natural dihydroflavonoid compound with multiple pharmacological activities. Previous studies have shown that hesperetin targets multiple cellular proteins, such as cell cycle regulation, apoptosis, metastasis, tyrosine kinases, growth factor receptors, estrogen metabolism, and antioxidant-related proteins, to inhibit tumor formation.
Methods and Results: In this study, we treated the triple-negative breast cancer cell line MDA-MB-231 with hesperetin for 24 h and extracted total RNA for RNA-seq analysis, which revealed that 1203 genes were up-regulated and 1997 genes were down-regulated. GO clustering and KEGG enrichment analysis indicated that most of these genes played important roles in tumorigenesis and various cellular signaling pathways. Four genes related to tumor cell apoptosis and migration, including *NR4A1*, *CSF2*, *KDR*, and *LRRK2*, were selected for real-time RT-PCR analysis for validation. The mRNA level changes were consistent with the transcriptome sequencing results. Further western blot analysis of changes in the expression of the orphan nuclear receptor NR4A1 and the apoptosis-related protein Bcl-2 showed that they may be involved in hesperetin-induced apoptosis in MDA-MB-231 cells. The current research results provide a preliminary basis for further elucidating the molecular mechanism of hesperetin against breast cancer and for developing future clinical drugs based on hesperetin.(**International Journal of Biomedicine. 2026;16(2):157-162.**)

Keywords: breast cancer • hesperetin • transcriptome sequencing • orphan nuclear receptor • molecular mechanism

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Introduction

As a naturally occurring dihydroflavonoid compound commonly found in citrus fruits, hesperetin (4-methoxy-3,5',7'-trihydroxyflavanone, C16H14O6, MW 304.2713) has been demonstrated to possess various pharmacological activities, including antitumor, anti-inflammatory, antioxidant, antiviral, antibacterial, antifibrotic, anti-aging, and neuroprotective effects.[1] In recent years, it has garnered widespread attention from numerous researchers. Hesperetin effectively scavenges hydroxyl radicals and singlet oxygen in the human body, upregulates or downregulates dysfunctional immune cells, and activates or inhibits multiple target molecules such as p38/MAPK, reactive oxygen species, Bcl-2, as well as related cellular signaling pathways, thereby inducing various programmed cell death pathways including apoptosis, autophagy and necroptosis, exerting its diverse pharmacological functions.[2-4] However, due to its low bioavailability, relatively poor solubility, and instability, there have been increasing reports in recent years on coating, modification, and solubilization technologies for hesperetin.[5] These studies provide further support for enhancing the higher application value and clinical development prospects of hesperetin.

Breast cancer is a heterogeneous disease involving multiple genetic and environmental factors. In recent years, it has become one of the tumors with the highest incidence and mortality rates globally, and it has been the most diagnosed malignant neoplasm and the second leading cause of cancer mortality among Chinese females in 2020.[6] Current treatments for breast cancer primarily involve surgical interventions, antibody-drug conjugate (ADC) therapy, immunotherapy, and chemoradiotherapy, based on specific diagnostic biomarkers and prognostic criteria.[7] Although these approaches show certain efficacy for patients with early-stage detection, the high individual heterogeneity of breast cancer, particularly in triple-negative breast cancer (TNBC, characterized by negative estrogen receptor and progesterone receptor expression, and HER2 expression below 2+ or FISH-negative), presents significant challenges. Due to the strong drug resistance and high metastasis risk in these cases, effective therapeutic targets

remain scarce, leading to limited survival outcomes. Research on the molecular mechanisms influencing the proliferation and migration of breast cancer cells, especially TNBC, is now urgently needed to identify new treatment targets for this aggressive subtype.

As a natural compound with broad-spectrum antitumor effects, hesperetin can exert cytotoxicity against various tumor cells while exhibiting minimal toxicity to normal cells.[8,9] In preliminary studies, we treated a triple-negative breast cancer cell line MDA-MB-231 with hesperetin and observed its dose- and time-dependent inhibition of tumor cell proliferation and migration, along with induction of autophagy and apoptotic cell death. Based on this, this study employed transcriptome sequencing (RNA-Seq) to analyze differentially expressed genes, aiming to identify functional genes influencing cell proliferation, migration, and apoptosis, and to further validate their roles through experimental validation, with the expectation of uncovering the cellular signaling pathways mediating hesperetin's anticancer effects. This research could provide more data and theoretical insights for molecular-level exploration of its mechanisms.

Materials and Methods

Cell Culture and Hesperetin Stock

Breast cancer MDA-MB-231 cells were preserved at Henan University of Technology. The cells were cultured with RPMI 1640 medium containing 10% FBS at 37°C in a 5% CO2 incubator. Hesperetin was purchased from Macklin Inc. (Shanghai, China) and dissolved in DMSO solution to prepare a concentration of 600 mM as a storage solution.

Cell RNA Extraction and Purification

MDA-MB-231 cells were treated with hesperetin (400 μM final concentration), and after 24 hours of drug treatment, total RNA was extracted from the cells using the TRIzol reagent (Thermo Fisher, no. 15596018, CA, USA) according to the manufacturer's instructions. An untreated blank group was set up. Three replicates were set for each sample group. The extracted RNA was measured for concentration using a NanoDrop 2000 (Thermo Fisher Scientific), and its integrity was assessed using an Agilent 2100 and a LabChip GX (PerkinElmer LabChip GX).

Transcriptome Sequencing

After binding mRNA with oligo (dT) magnetic beads twice, interrupting mRNA, synthesizing the first strand of cDNA, synthesizing the second strand of cDNA, purifying the product, repairing the end of the cDNA strand and adding an A base, purifying the linker product and fragment selection, PCR amplification and product purification, the library was subjected to quality inspection. Double-end sequencing was performed using Illumina Novaseq 6000 (transcriptome sequencing process was entrusted to Beijing Tsingke Biotech Co., Ltd., China).

Data Filtering and Quality Control

The raw image data files obtained from high-throughput sequencing are converted into sequencing raw data (Raw Data) using CASAVA Base Calling analysis. The base quality value (Quality Score or Q-score) is an integer that maps to the probability of base-calling errors. This experiment requires an average Q30 ratio of 85% or above. mRNA fragmentation results were simulated by mapping read positions across various mRNA transcripts, and the randomness of mRNA fragmentation was assessed. Furthermore, the saturation of gene numbers with different expression levels was simulated using the Mapped Data for each sample, and saturation testing was performed on transcriptome sequencing data.

Data Assembly and Gene Expression Analysis

We applied HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts, https://daehwankimlab.github.io/hisat2/,version:hisat2-2.2.1) to align Clean Reads with the reference genome to obtain positional information on the reference genome or gene, as well as unique sequence features of the sequencing sample. The number of Mapped Reads in different regions (exons, introns, and intergenic regions) of the specified reference genome was counted. StringTie (version number stringtie-2.1.6, developed by Johns Hopkins University in collaboration with the University of Texas Southwestern Medical Center) was used for transcript assembly and prediction of expression levels, and FPKM (Fragment Per Kilobase of transcript per Million fragment mapped, per kilobase transcript fragment/million mapped reads) was used as an indicator to measure transcript or gene expression levels.

Screening and Analysis of Differentially Expressed Genes

The DESeq2 software was applied to perform differential gene expression analysis between sample groups. A fold change greater than 2 and a false discovery rate (FDR) parameter less than 0.05 was used as the screening criteria (using the Benjamin Hochberg correction method to correct the significance p-values obtained from the original hypothesis test, and using FDR as the key indicator for differential gene screening). Moreover, hierarchical clustering analysis and functional annotation of the selected differentially expressed genes were performed, along with GO functional and KEGG pathway enrichment analyses of the expressed genes.

RT-qPCR

Based on transcriptome sequencing analysis, four differentially expressed functional genes, *CSF2*, *KDR*, *NR4A1*, and *LRRK2*, which affect cell proliferation, apoptosis, and autophagy, were randomly selected for RT-qPCR validation analysis. The brief process is to collect MDA-MB-231 cells treated with hesperetin and extract total RNA using a cell total RNA extraction kit (Beijing Qingke Biotechnology Co., Ltd., Beijing). After reverse-transcribing it into cDNA, the RT-qPCR reaction system was prepared according to the procedures of the SYBR qPCR Master Mix kit (Nanjing Novozymes Biotechnology Co., Ltd.), and the reaction was detected using the Eppendorf PCR System instrument. The data were calculated using the $2^{-\triangle\triangle Ct}$ method (experiment was repeated three times in parallel). The primer sequences required for the experiment were as follows:

CSF2-F: GCCTCACCAAGCTCAAGGG, *CSF2*-R: TCATCTGGCCGGTCTCACTC

KDR-F: CGGTCAACAAAGTCGGGAGA, *KDR*-R: TCCCACATGGATTGGCAGAG

NR4A1-F: CCTCGCCTTGGTTGGAGG, *NR4A1*-R: CTCGGTGCTGGTGTCCCATA

LRRK2-F: CCTGGATCTTTCAACTCGTCG, *LRRK2*-R: GTCCCAAACGGTCAAGCAAG

Western Blot

After protein extraction from MDA-MB-231 cells treated with hesperetin, subsequent immunoblotting was performed using conventional methods.[10] The brief procedures are as follows: The proteins were first analyzed by SDS-PAGE (labeled with color prestained markers) and then transferred onto nitrocellulose membranes (NC membrane, Beijing Solarbio Technology Co., Ltd.). After blocking the NC membranes with a 5% skim milk powder solution diluted with TBS buffer for 1 h, the membranes were incubated with primary antibodies against NA4R1 or Bcl-2 for 16-18 h at 4 ℃, respectively. After washing three times with TBS buffer, the NC membranes were further incubated with secondary antibody (sheep anti-mouse IgG produced by LiCOR Bioscience, USA) for 4 h at 4 ℃. After washing with TBST buffer three times (7 minutes each time), the blots in the NC membranes were scanned and saved using an Odyssey infrared laser imager.

Results

Quality Control of Sequencing Data

To investigate the effect of hesperetin treatment on gene expression in MDA-MB-231 cells, we set up three blank control groups (C1, C2, and C3) and three MDA-MB-231 cell groups (T1, T2, and T3) treated with hesperetin (400 μ M) for 24 h each for RNA-seq analysis in this study. The correlation analysis of biological replicates showed that the r-values between the dosing groups T1, T2, and T3, and the blank groups C1, C2, and C3 were close to 1.000, while the r-values between the two groups were 0.773~0.681 (Figure 1) (according to the Pearson correlation coefficient r-index, r>0.95 is significant correlation, and 0.5 ≤ r < 0.8 is moderate correlation). The results indicated a strong correlation between intra-group duplicate samples in the hesperetin treatment group and the control group, while the correlation between the two groups was weak, indicating that the experimental sample selection is reasonable and the conditions are reliable.

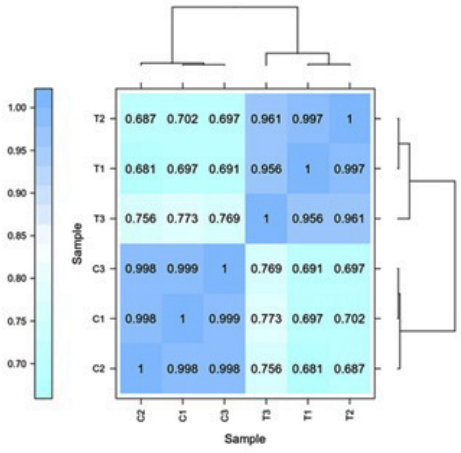

Fig. 1. *Correlation analysis of gene expression between different samples. C1, C2, and C3 denote different control duplicates, while T1, T2, and T3 stand for three hesperetin treatment samples, respectively.*

In addition, by comparing the sequencing base information with the human genome, the total Clean data of the sequencing samples was 37.71 Gb, and the average Q30 ratio was above 93.64% (Table 1). The sequencing results indicate that the data quality of this study is qualified, and all samples meet the basic requirements for library sequencing.

Table 1.
Statistical analysis of sequencing data quality control.

Sample ID	Clean reads	Clean bases	GC(%) GC Content	Q30 value% ≥ Q30
Control 1 (C1)	21563838	6464239479	49.70	93.64
Control 2 (C2)	21278783	6377945170	50.01	95.03
Control 3 (C3)	20657474	6192076317	49.83	94.31
Test 1 (T1)	20275484	6077236081	49.56	94.94
Test 2 (T2)	19147049	5739575817	49.28	95.00
Test 3 (T3)	22887520	6860538159	49.48	95.36

Note: Clean reads, total number of pair-end reads in Clean Data; GC content, the percentage of G and C bases in the total bases in Clean Data; ≥Q30%, the percentage of bases with a Clean Data quality value greater than or equal to 30.

Overall Analysis of Differentially Expressed Genes

The screening criteria for differentially expressed genes were based on a multiple difference of ≥ 2 (equivalent to an absolute value of log2FC ≥ 1) and a q-value<0.05 (p-value correction). The number of differentially expressed genes between the experimental and control groups was counted. Among them, 3200 genes were differentially expressed (DEGs), including 1203 up-regulated and 1997 down-regulated genes, indicating that hesperetin affects the transcription levels of multiple genes. Cluster analysis was performed on genes with the same or similar expression patterns (Figure 2A), and the control and experimental groups showed good reproducibility. The volcano plot (Figure 2B) visually displays the overall distribution of differential gene expression levels and fold differences between two groups.

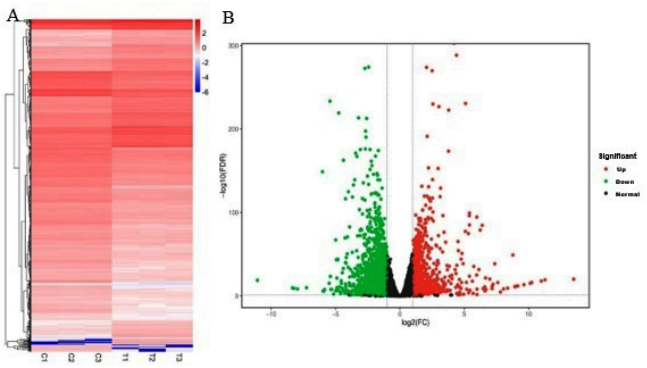

Fig. 2. *Cluster heatmap of differentially expressed genes in hesperetin-treated MDA-MB-231 cells (**A**, Different columns represent different samples and different rows represent different genes. Colors represent the log10 expression levels of genes in the samples) and volcano plot (**B**, Green dots represent down-regulated differentially expressed genes, red dots represent up-regulated differentially expressed genes, and black dots represent non-differentially expressed genes).*

Clustering of Differentially Expressed Genes and KEGG Annotation

Further Gene Ontology analysis was conducted on 3200 differentially expressed genes induced by hesperetin in MDA-MB-231 cells, which were classified into three functional categories: Molecular Function (MF), Cellular Component (CC), and Biological Process (BP). Among them, there are relatively more differentially expressed genes involved in intracellular metabolic processes and molecular functions such as cell membrane binding, catalysis, and regulation of molecular signaling pathways.

On this basis, further KEGG (Kyoto Encyclopedia of Genes and Genomes) annotation analysis was performed on differentially expressed genes in MDA-MB-231 cells treated with hesperetin. The results showed that the differentially expressed genes had the highest number of genes involved in anti-tumor related pathways, with 105 genes, accounting for 8.95% (Figure 3, marked in red). In addition, 29 genes (2.47%) were related to cell apoptosis. There are 60 genes related to the MAPK signaling pathway, all of which can regulate cell proliferation and apoptosis. Besides that, hesperetin treatment of cells also led to differential expression of 70 genes (5.97%) in the PI3K-Akt pathway, most of which are related to cellular autophagy, migration, and apoptosis. These data provide direction for further in-depth study on the molecular anti-proliferative mechanism of hesperetin on MDA-MB-231 cells.

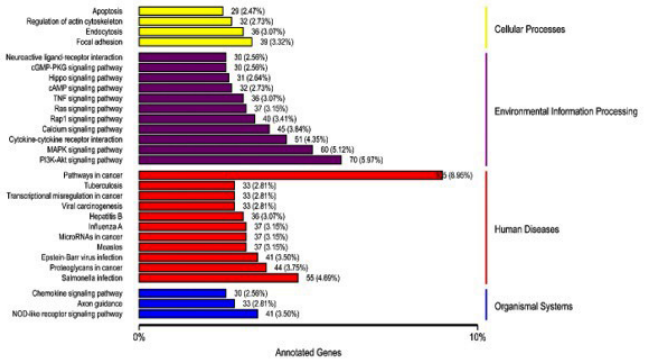

Fig. 3. *Classification of differentially expressed genes KEGG in hesperetin-treated MDA-MB-231 cells. The vertical axis shows the name of the KEGG metabolic pathway, and the horizontal axis shows the number of genes annotated to this pathway and their proportion to the total number of annotated genes.*

Enrichment Analysis of Differentially Expressed Genes in KEGG Pathway

Using the metabolic pathways represented in the KEGG database as units of analysis, hypergeometric testing was applied to identify pathways that are significantly enriched in differentially expressed genes compared to the entire genome background. Figure 4 shows the top 20 pathways with the lowest Q-values in the KEGG enrichment analysis (the lower the Q-value, the more significant the enrichment). These pathways involve TGF-β signaling pathway, NOD like receptor signaling pathway, microRNAs in cancer, longevity regulation pathway (multi species), JAK-STAT signaling pathway, anti-folate resistance, ECM receptor interaction, toxoplasmosis, steroid biosynthesis, relaxin signaling pathway, Kaposi sarcoma associated herpesvirus infection, cancer pathway, IL-17 signaling pathway, measles, small cell lung cancer, herpes simplex virus 1 infection, whooping cough, TNF signaling pathway, legionellosis, AGE-RAGE signaling pathway in diabetes complications, etc. Among them, the proportion of differentially expressed genes involved in pathways in cancer is the highest (Figure 4), which again confirms the disturbance of MDA-MB-231 tumor cell-related signaling pathways caused by hesperetin treatment, providing guidance for further identification of functional genes that affect tumor cell proliferation and death.

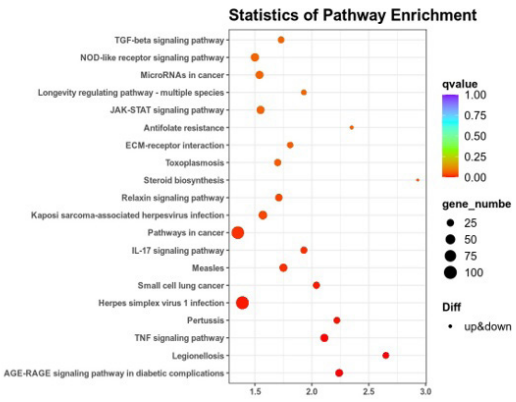

Fig. 4. *Scatter plot of KEGG pathway enrichment for differentially expressed genes in hesperetin-treated MDA-MB-231 cells.*

RT-qPCR and Western Blot Validation of Differentially Expressed Genes

Based on the differential gene GO clustering and KEGG pathway enrichment analysis results, we further selected four genes related to tumor cell apoptosis and migration, including nuclear receptor 4A1 (NR4A1), colony stimulating factor 2 (CSF2), kinase insert domain receptor (KDR), and leucine rich repeat kinase 2 (LRRK2), for RT-qPCR analysis to further verify the reliability of transcriptome sequencing. The results showed that after treatment with hesperetin in MDA-MB-231 cells for 24 h, the mRNA levels of NR4A1, CSF2, and KDR were upregulated, while the mRNA level of LRRK2 decreased (Figure 5A), which were consistent with the trend of RNA-seq analysis, proving the high reliability of sequencing in this study.

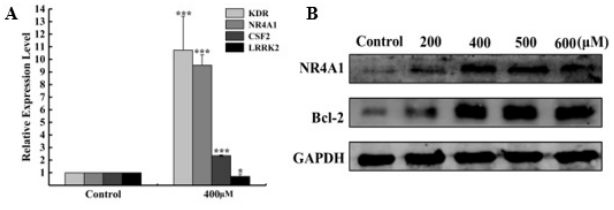

Fig. 5. A*. Changes in mRNA level (CSF, LRRK2, KDR, and NR4A1) of relevant differential genes detected by fluorescence quantitative PCR in hesperetin-treated MDA-MB-231 cells (*p<0.05, ***p<0.001).* ***B****. Western blot analysis of NR4A1 and Bcl-2 protein expression. tect protein expression.*

To further verify the expression changes of key functional genes and the potential anti-proliferative mechanism in

hesperetin-treated MDA-MB-231 cells, the NR4A1 gene was selected for western blot analysis to assess its expression. Previous research confirmed that the orphan nuclear receptor NR4A1 can induce mitochondrial apoptosis by exposing the BH3 domain of the anti-apoptotic protein Bcl-2, leading to conformational changes in Bcl-2. This study found that after hesperetin treatment, NR4A1 and Bcl-2 expressions increased in a dose-dependent manner (Figure 5B). It is speculated that the synergistic effect of the two proteins is involved in the hesperetin-induced apoptosis process of MDA-MB-231 cells.

Discussion

As an important natural dihydroflavonoid compound, hesperetin has been shown to exhibit strong anti-tumor effects, although the specific molecular mechanisms remain to be further explored. Previous studies showed that hesperetin can inhibit the proliferation of breast cancer cells by acting as an epigenetic modulator of gene expression.[12,13] In addition, hesperetin can inhibit breast cancer stem cells and, when combined with other agents, achieve a synergistic anti-tumor effect by targeting p53, PPARG, Notch, and other signal transduction pathways.[14]

In order to further explore the anti-proliferative molecular mechanism on breast cancer cells, we selected the triple-negative breast cancer cell line, MDA-MB-231, as a cell model, and used transcriptome sequencing to study the differential expression of functional genes after hesperetin treatment, aiming to screen important functional proteins involved in regulating cell proliferation, migration, and influencing various cell signal pathways. As a mature high-throughput RNA sequencing technology, RNA-seq can obtain millions of readings in each experimental sample, enabling high-resolution study of any cellular transcriptome changes and obtaining valuable clues about the organism's response to specific stimuli.[15,16] Our current research showed that, compared with the blank control group, the numbers of upregulated and downregulated genes were 1203 and 1997, respectively, in hesperetin-treated cells. Subsequent gene cluster analysis and KEGG pathway enrichment analysis revealed that the largest number of genes are involved in tumorigenesis; furthermore, numerous genes are involved in modulating signaling pathways, such as tumor cell proliferation and apoptosis. This suggested that hesperetin treatment can affect tumor cell survival-related functional genes, thereby inhibiting the proliferation of MDA-MB-231 cells.

Previous studies have shown that hesperetin targets multiple cellular proteins, such as cell cycle regulation, apoptosis, metastasis, tyrosine kinases, growth factor receptors, estrogen metabolism, and antioxidant-related proteins, to inhibit tumor formation.[17] Based on transcriptome sequencing data analysis, four differentially expressed genes (*NR4A1*, *CSF2*, *KDR*, and *LRRK2*) related to tumor cell proliferation, migration, and apoptosis were further screened and validated by RT-qPCR. The trends in mRNA levels were consistent with transcriptomic sequencing, further demonstrating the reliability of RNA-seq in this study. On this basis, the orphan nuclear receptor NR4A1 was also selected for western blot analysis to assess its translation level. We found that in hesperetin-treated MDA-MB-231 cells, both NR4A1 and the apoptosis-related protein Bcl-2 were upregulated in a dose-dependent manner. Previous literature has shown that NR4A1 can be located in the gene body and 3'UTR regions of immediate early genes in cells, inhibits the transcriptional elongation of RNA polymerase II, and that its ectopic expression enhances the tumorigenesis of breast cancer cells.[18] Preliminary studies on retinoids and apoptosis inducers have suggested that their cytotoxic activity is NR4A1 dependent and involves drug-induced NR4A1 nuclear export and mitochondrial pro-apoptotic NR4A1-Bcl-2 complex formation.[19,20] Orphan members of the nuclear receptor family, lacking the BH3 domain, can bind to Bcl-2 and regulate its effects on cell apoptosis and autophagy.[21]The endogenous apoptotic pathway of cells (also known as the mitochondrial pathway) is initiated by various intracellular signals, mainly regulated by the Bcl-2 family, which comprises pro-apoptotic and anti-apoptotic proteins that together control the permeability of the mitochondrial outer membrane.[22] In this study, we found that NR4A1 and Bcl-2 exhibit a dose-dependent upregulation, which may play an important role in hesperetin's anti-proliferative effect on breast cancer cells. In fact, our recent study has shown that hesperetin indeed induces apoptosis and autophagy in MDA-MB-231 cells. In the future, further exploration can be conducted to determine whether the two proteins exhibit a synergistic effect and participate in hesperetin-induced mitochondrial apoptosis in MDA-MB-231 cells. However, current research undoubtedly lays the foundation for such future study.

Conclusion

Through RNA-seq analysis, combined with RT-qPCR and western blot validation, we preliminarily identified several differential gene expression changes that affect the death of breast cancer cells MDA-MB-231 induced by hesperetin, discussed the possible functional role of NR4A1 protein in it, and provided a preliminary basis for the future in-depth study of the molecular mechanism of hesperetin's anti-tumor effect and the development of clinical drugs based on hesperetin.

Author Contributions

Yu-Zhen Ma: Investigation, Methodology, Data curation, Writing – original draft.
Shuang-Shuang Sun: Investigation, Methodology, Data curation, Writing – original draft.
Guang-Zhou Zhou: Conceptualization, Supervision, Funding acquisition. Writing – review and editing.
All authors have approved the final article.

Conflict of Interest

The authors have declared no conflict of interest.

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