Analysis of the role of DAMTC in lung adenocarcinoma cells based on the DNA microarrays

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Abstract. The present study aimed to investigate the effect of 7, 8-diacetoxy-4-methylcoumarin (DAMTC) on lung adenocarcinoma cells (A549) and analyze the molecular mechanism underlying DAMTC-treated lung adenocarcinoma. Gene expression profile GSE29698 was downloaded from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) in 3 DAMTC-treated A549 samples were analyzed and compared with 3 DAMTC-untreated samples using the limma package. Gene Ontology (GO) and pathway enrichment analyses of DEGs were performed, followed by the functional annotation and protein-protein interaction (PPI) network construction. Finally, pathway crosstalk analysis was conducted. A total of 500 upregulated and 389 downregulated DEGs were identified. The upregulated and downregulated DEGs were enriched in different GO terms and pathways, including metabolic process, p53 signaling pathway and metabolic pathways. A total of 9 DEGs were determined to have node degrees >16 in the PPI network, including interleukin 6 (*IL6*), MDM2 oncogene, E3 ubiquitin protein ligase (*MDM2*), cell division cycle 42 (*CDC42*) and MYC associated factor X (*MAX*). Furthermore, numerous DEGs were identified to function as transcription factors and tumor suppressor genes, including histone deacetylase 3 and *MAX*. Additionally, apoptosis, tight junction, and endocytosis pathway were determined to cross-talk with small cell and non-small cell lung cancer. The DEGs (*IL6*, *MDM2*, *CDC42* and *MAX*) involved in different pathways, including the p53 signaling pathway and endocytosis, may be the potential targets for DAMTC in lung adenocarcinoma. The elucidation of the underlying mechanism of the DAMTC effect may make it a potential drug.

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Introduction

Lung cancer remains the leading cause of cancer-associated mortality, annually resulting in >1,000,000 mortalities globally in 2012 (1). Additionally, ~1,200,000 new cases are diagnosed every year and prognoses of lung cancer are poor, as estimated worldwide in 2012 (2). Lung adenocarcinoma is the predominant histological subtype of non-small lung cancer (3). The mean 5-year survival rate of lung adenocarcinoma is <15% in the urban areas of China as estimated in 2015, primarily due to the late-stage detection and a paucity of late-stage treatments (4). The availability of effective anticancer drugs is considered to be the key for improvement in the treatment of lung adenocarcinoma.

7, 8-Diacetoxy-4-methylcoumarin (DAMTC) is a thioderivative of 4-methyl coumarin (5). Coumarins belong to the flavonoid class of plant secondary metabolite, which have been demonstrated to exhibit diverse and beneficial biological activities, including antitumoral, anticoagulant and anti-inflammatory properties (5). Natural coumarins or synthetic analogs have attracted intense interest for their applicability as drugs (6). They have been determined to have a variety of therapeutic applications, including antitumor and anti-human immunodeficiency virus therapy (7), and antioxidant (8) and antibacterial (9) applications. Lacy and O'Kennedy (10) demonstrated that genistein and esculetin could exert the most potent inhibitory effect on cell growth of two cell lines, A549, a lung carcinoma cell line, and MCF-7, a breast carcinoma cell line. Previously, the coumarin derivative DAMTC was indicated to inhibit cellular proliferation and induce apoptosis on human non-small cell lung cancer A549 cells (11). Furthermore, the observations of Goel *et al* (12) reported that upregulation of the nuclear factor-κB, p53 and Akt pathways, and downregulation of the mitogen activated protein kinase (MAPK) and Cox-2 pathways were involved in the molecular mechanism of apoptosis induction by DAMTC in A549 cells. However, the mechanisms of the anti-proliferative effects of DAMTC in lung adenocarcinoma are incompletely defined, and further insights into the mechanisms are required.

Previously, Goel *et al* (1) used the integrated proteomics and transcriptomics approach, and identified that DAMTC could regulate cell motility and cytoskeletal reorganization in lung adenocarcinoma. In the present study,

differentially-expressed genes (DEGs) were identified in DAMTC-treated lung adenocarcinoma, compared with DAMTC-untreated controls, using the same gene expression profile. Comprehensive bioinformatics were used to analyze the significant pathways and functions, and to construct the protein-protein interaction (PPI) network, to determine the critical DEGs. Furthermore, the putative interactions between signaling pathways were analyzed. The present study aimed to investigate the potential molecular mechanism underlying DAMTC-induced apoptosis and inhibition of cell motility in lung adenocarcinoma.

Materials and methods

Microarray data and data preprocessing. The gene expression profile of GSE29698, deposited by Goel *et al* (1), was downloaded from the Gene Expression Omnibus database in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) based on the platform of GPL6884 Illumina HumanWG-6 v3.0 expression beadchip. A total of 6 specimens were applied, including 3 specimens of DAMTC-treated lung adenocarcinoma cells (A549) and another 3 specimens of DAMTC-untreated A549 cell lines as controls.

The gene expression profile data were preprocessed using the limma (13) package in Bioconductor. Following background correction, quantile normalization and probe summarization, the gene expression matrix of specimens was received.

DEGs screening. Unpaired Student's t-test (13) in limma package was used to identify the DEGs in the DAMTC-treated A549 cell group, compared with the control group. False discovery rate (FDR) (14) was performed for multiple testing correction using the Benjamini and Hochberg method (15). The threshold for the DEGs was set as FDR <0.01 and $|\log_2$ FC (fold change) $|\geq 2$.

Functional and pathway enrichment analysis of DEGs. Gene Ontology (GO) (16) categories, including biological process (BP), molecular function (MF) and cellular component (CC), of the selected DEGs were enriched from GO databases using Database for Annotation Visualization and Integrated Discovery (DAVID) (17). Additionally, the pathways of selected DEGs were enriched using DAVID from Kyoto Encyclopedia of Genes and Genomes (KEGG) (18) analysis. $P<0.05$, as determined by the hypergeometric test (19), was selected as the threshold.

Functional annotation of DEGs. Identification of tumor-associated genes (TAGs) and understanding their functions can be critical for investigating the roles of genes involved in tumorigenesis. The tumor suppressor gene (TSGene) database (http://bioinfo.mc.vanderbilt.edu/TSGene/) is a comprehensive literature-based database that provides detailed annotations for each TSG. The TAG database (http://www.binfo.ncku.edu.tw/TAG/) is designed to utilize information from well-characterized oncogenes and tumor suppressor genes to accelerate cancer research. According to the data information regarding transcription factors (TFs) from the TRANSFAC database (20), functional enrichment of the DEGs for transcription regulation was assessed. Additionally, the selected DEGs were mapped into the TSGene and TAG database to extract the genes that had transcriptional functions or functioned as TAGs.

PPI network construction. The PPI network is represented by an undirected graph with nodes indicating the genes and edges indicating the mapped interactions of the proteins encoded by the genes (21). The PPI network of the selected genes was constructed by using data from the Retrieval of Interacting Genes (STRING) database, which is a comprehensive database containing functional associations between proteins that are experimentally derived, as well as associations predicted by comparative genomics and text mining (22). The interaction pairs with the PPI combined score >0.4 were selected in this network, which corresponded to a medium-confidence network (23).

Pathway crosstalk analysis. If more significant protein interactions are detected between two pathways, these two pathways are probable to influence or interact with each other (cross-talk). Understanding the crosstalk between pathways is momentous for understanding the function of cells and more complex systems (24). In the present study, the KEGG pathway data and protein interaction data based on the PPI network were combined to investigate pathway crosstalk in the DAMTC-treated A549 cells. The detail is included in the studies by Li *et al* (24) and Liu *et al* (25).

Results

Identification of DEGs. For the dataset GSE29698, a total of 889 DEGs were identified in DAMTC-treated A549 cell groups, compared with DAMTC-untreated controls, including 500 upregulated genes and 389 downregulated genes. The heat-map of the DEGs is depicted in Fig. 1. The results demonstrated that the DEGs expression pattern could significantly distinguish the DAMTC-treated A549 cell samples from controls.

GO and pathway enrichment analysis of the DEGs. Functional and pathway enrichment analysis indicated that upregulated and downregulated DEGs in DAMTC-treated A549 cell groups were significantly enriched in different GO terms and KEGG pathways (Tables I and II). GO analysis demonstrated enrichment in upregulated DEGs involved in BP, including transcription, DNA-dependent and regulation of cellular metabolic process. Significant CC ontology terms of upregulated DEGs were associated with intracellular part. The majority of significant MF ontology terms of upregulated DEGs were associated with binding function and enzyme activity, including DNA binding and phosphatase activity. Furthermore, significant BP ontology terms of downregulated DEGs were associated with tissue morphogenesis, and morphogenesis of an epithelium. CC ontology terms of downregulated DEGs were also associated with intracellular part. Significant MF ontology terms of downregulated DEGs were associated with organic cyclic compound binding, and RNA polymerase II transcription corepressor activity (Table I).

Additionally, upregulated DEGs were enriched in different pathways, including the p53 signaling pathway, and fructose and mannose metabolism pathway. Downregulated DEGs

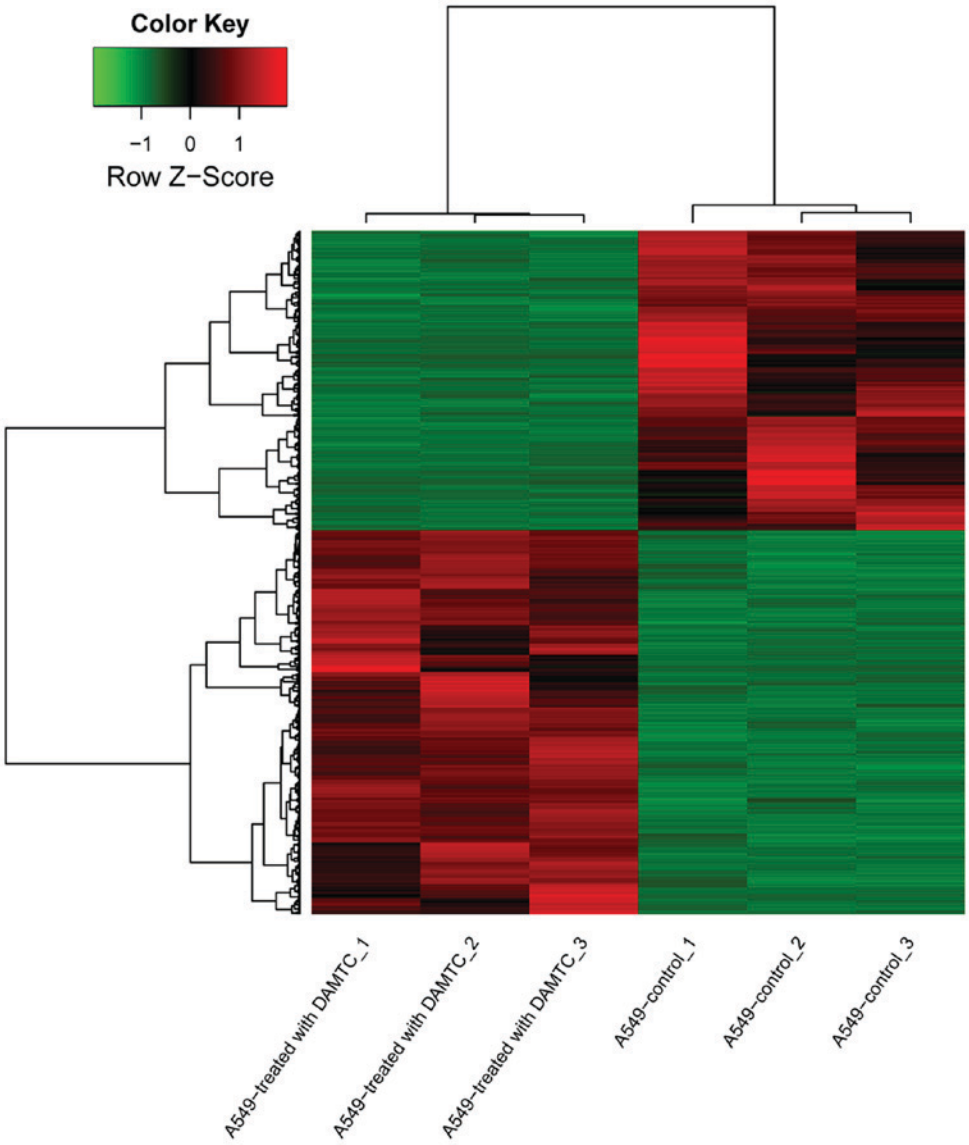

Figure 1. Heat-map of differentially-expressed genes. For each gene (row), red indicates a higher expression and green a reduced expression relative to the mean level of expression of the gene across the samples (columns), while black indicates genes not differentially expressed. DAMTC, 7, 8-diacetoxy-4-methylcoumarin.

were also identified to be involved in different pathways, including ubiquitin mediated proteolysis, and metabolic pathways (Table II).

Function annotation of DEGs. The expression change of TFs, TSGs and other TAGs in DAMTC-treated A549 cell groups were observed (Table III). The upregulated functional genes included 34 TFs and 26 TSGs. Subsequently, *MAX* was identified to be a TF, while tumor protein p53 inducible nuclear protein 1 and bone morphogenetic protein 2 were identified to be TSGs. Additionally, histone deacetylase 3 (*HDAC3*) was identified as both a TF and a TSG. Additionally, the downregulated functional genes included 27 TFs and 18 TSGs. Among them, sex determining region Y-box 4 and signal transducer and activator of transcription 1 (*STAT1*) were identified to be TFs.

PPI network and the pathway crosstalk analysis. Based on the STRING database, the PPI network was constructed (Fig. 2). The results demonstrated that a total of 9 genes had a node degree >16, including interleukin 6 (*IL6*; degree, 37), MDM2 oncogene, E3 ubiquitin protein ligase (*MDM2*; degree, 27), *STAT1* (degree, 23), ataxia telangiectasia mutated (degree, 20), *HDAC3* (degree, 19), cell division cycle 42 (*CDC42*; degree, 18), cytochrome c, somatic (degree, 17), bone morphogenetic protein 4 (degree, 17) and *MAX* (degree, 17).

The result of pathway crosstalk analysis is depicted in Fig. 3. The majority of the significant pathways were determined to have crosstalk with small cell lung cancer and non-small cell lung cancer. The apoptosis, tight junction and endocytosis pathways were determined to be cross-talking with small cell lung cancer and non-small cell lung cancer. Additionally, the bacterial invasion of epithelial cells, adherens junction, shigellosis and mechanistic target of rapamycin kinase signaling pathways had cross-talk with non-small cell lung cancer. In addition, small cell lung cancer and non-small cell lung cancer were also determined to have cross-talk.

Table I. The enriched GO terms of differentially-expressed genes in 7, 8-diacetoxy-4-methylcoumarin-treated lung cancer groups.

Expression changes	Category	GO-ID	Name	Count	P-value
Upregulated	BP	GO:0006351	Transcription, DNA-dependent	153	3.30×10^{-11}
		GO:0031323	Regulation of cellular metabolic process	194	7.80×10^{-11}
		GO:0060255	Regulation of macromolecule metabolic process	187	8.24×10^{-11}
		GO:0051252	Regulation of RNA metabolic process	143	1.56×10^{-10}
		GO:0019222	Regulation of metabolic process	207	2.08×10^{-10}
	CC	GO:0005622	Intracellular	387	2.45×10^{-10}
		GO:0044424	Intracellular part	382	1.08×10^{-9}
		GO:0005634	Nucleus	219	2.68×10^{-9}
		GO:0043227	Membrane-bounded organelle	320	5.19×10^{-9}
		GO:0043231	Intracellular membrane-bounded organelle	319	5.71×10^{-9}
	MF	GO:0003677	DNA binding	98	3.25×10^{-5}
		GO:0016791	Phosphatase activity	18	4.00×10^{-4}
		GO:0000988	Protein binding transcription factor activity	28	8.00×10^{-4}
		GO:0005515	Protein binding	244	11.00×10^{-3}
		GO:0005488	Binding	362	13.00×10^{-3}
Downregulated	BP	GO:0048729	Tissue morphogenesis	28	2.61×10^{-6}
		GO:0002009	Morphogenesis of an epithelium	24	3.72×10^{-6}
		GO:0044237	Cellular metabolic process	221	7.50×10^{-5}
		GO:0044238	Primary metabolic process	224	8.50×10^{-5}
		GO:0048736	Appendage development	12	8.77×10^{-5}
	CC	GO:0044424	Intracellular part	291	3.52×10^{-8}
		GO:0043231	Intracellular membrane-bounded organelle	243	1.83×10^{-7}
		GO:0005622	Intracellular	291	2.11×10^{-7}
		GO:0043227	Membrane-bounded organelle	243	2.69×10^{-7}
		GO:0043229	Intracellular organelle	257	2.01×10^{-6}
	MF	GO:0097159	Organic cyclic compound binding	154	2.31×10^{-6}
		GO:1901363	Heterocyclic compound binding	152	2.77×10^{-6}
		GO:0001191	RNA polymerase II transcription factor binding transcription factor activity involved in negative regulation of transcription	6	2.35×10^{-5}
		GO:0001106	RNA polymerase II transcription corepressor activity	5	3.99×10^{-5}
		GO:0003714	Transcription corepressor activity	14	8.22×10^{-5}

GO, gene ontology; CC, cellular component; BP, biological process; MF, molecular function.

Discussion

The worldwide incidence rate of lung cancer is high (26). It is important to develop novel, more effective treatment modalities for dealing with this disease. DAMTC is a potential inducer of apoptosis and an inhibitor of cell growth (1). Thus, identifying the molecular mechanism underlying the effects of DAMTC is imperative. In the present study, microarray analysis demonstrated that 500 upregulated DEGs and 389 downregulated DEGs were identified in DAMTC-treated A549 cells, compared with DAMTC-untreated controls. Among the DEGs, *IL6*, which was downregulated, was a hub protein with the highest degree in the PPI network. The upregulated DEGs *MDM2*, *CDC42* and *MAX*, which were also hub genes, had different functions and were involved in different pathways, including the p53 signaling and endocytosis pathways. Additionally, the apoptosis, tight junction, and endocytosis pathways were determined to cross-talk with small cell lung cancer and non-small cell lung cancer.

IL6 encodes a multifunctional cytokine, which can function in inflammation and the maturation of B cells (27). Hodge *et al* (28) demonstrated that the release of various survival factors, including IL6, serve to block apoptosis in cancer cells during the inflammatory process, keeping them alive in toxic environments. Additionally, studies have demonstrated that drugs designed to restore apoptosis exhibit potential to effectively treat numerous cancer types, including inhibitors of apoptosis, protein inhibitors and MDM2 antagonists (29,30). Furthermore, in keeping with the previous study, our study identified that IL6 was downregulated in DAMTC-treated A549 groups and was involved in the pathways in cancer (Table I). Thus, it was indicated that DAMTC may induce cancer cell apoptosis through targeting *IL6* in lung adenocarcinoma.

Table II. The enriched pathways of differentially-expressed genes in 7, 8-diacetoxy-4-methylcoumarin-treated lung cancer groups.

Expression changes	KEGG-ID	Name	n	P-value	Genes
Upregulated	04115	p53 signaling pathway	9	0.0001	APAF1, CCNG2, CYCS, GADD45A, MDM2, PPM1D, RPRM, SESN1 and SESN3
	00051	Fructose and mannose metabolism	5	0.0032	ALDOC, C12orf5, HK2, MTMR1 and PFKFB4
	05219	Bladder cancer	5	0.0062	FGFR3, MAP2K1, MDM2, PGF and RASSF1
	04722	Neurotrophin signaling pathway	9	0.0095	CDC42, GAB1, MAP2K1, MAP3K3, MAPK7, NFKBIE, NTF3, PDK1 and ZNF274
	05200	Pathways in cancer	17	0.0103	BMP2, CCNA1, CDC42, CYCS, EGLN1, FGF18, FGFR3, FZD1, FZD4, FZD7, MAP2K1, MAX, MDM2, PGF, PIAS3, RASSF1 and RASSF5
	04130	SNARE interactions in vesicular transport	4	0.0180	BNIP1, STX11, STX7 and VAMP1
	04144	Endocytosis	11	0.0265	CDC42, CXCR4, DNM2, FGFR3, GIT2, HLA-E, MDM2, RAB11FIP1, SH3GL2, SH3GL3 and VPS37B
	05211	Renal cell carcinoma	5	0.0472	CDC42, EGLN1, GAB1, MAP2K1 and PGF
Downregulated	05217	Basal cell carcinoma	6	0.0008	BMP4, FZD2, GLI2, STK36, TCF7L2 and WNT7B
	00130	Ubiquinone and other terpenoid-quinone biosynthesis	2	0.0079	COQ5 and COQ7
	01100	Metabolic pathways	33	0.0130	ACSS1, AK2, ATP6V1E2, B3GALT6, BAAT, BCAT1, COQ5, COQ7, CYP2R1, DPAGT1, GGPS1, GPT2, HKDC1, HLCS, OXSM, PAFAH2, PCK2, PFAS, PIGM, PIGV, PIGW, PIK3C2B, PLD1, PMM2, POLG2, POLR1B, POLR3H, PSPH, SEPHS2, SLC33A1, ST3GAL5, ST6GAL1 and SYNJ2
	00563	Glycosylphosphatidylinositol-anchor biosynthesis	3	0.0132	PIGM, PIGV and PIGW
	04120	Ubiquitin mediated proteolysis	7	0.0185	ANAPC13, BIRC6, FBXO4, ITCH, KLHL9, UBE2J1 and UBE2Q1
	04340	Hedgehog signaling pathway	4	0.0255	BMP4, GLI2, STK36 and WNT7B
	05200	Pathways in cancer	12	0.0300	BMP4, DAPK1, FZD2, GLI2, IL6, PLD1, RARB, STAT1, STK36, TCF7L2, TRAF5 and WNT7B

n, number of genes enriched in the corresponding pathway; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Additionally, the other upregulated DEG *MAX* was enriched in the pathways in cancer and identified to be a TAG in the present study. MAX is a member of the basic helix-loop-helix leucine zipper family of TFs and could interact with other family members, including v-myc avian myelocytomatosis viral oncogene homolog (MYC), MAX interactor 1, dimerization protein and mothers against decapentaplegic (MAD), to form homodimers or heterodimers (31). The study by Grandori *et al* (32) demonstrated that the MYC/MAX/MAD network was comprised of a group of TFs controlling cell cycle differentiation, progression and death. Additionally, Zhu *et al* (33) reported that levels of MAD1 rapidly decrease upon mitogen stimulation, and this degradation could be regulated by the MAPK pathway. Furthermore, Goel *et al* (34) reported that DAMTC could induce apoptosis through the mitochondrial pathway by modulating the MAPK pathway. Thus, it was indicated that DAMTC may induce cell apoptosis by upregulating the expression of *MAX* and modulating the MAPK pathway in lung adenocarcinoma.

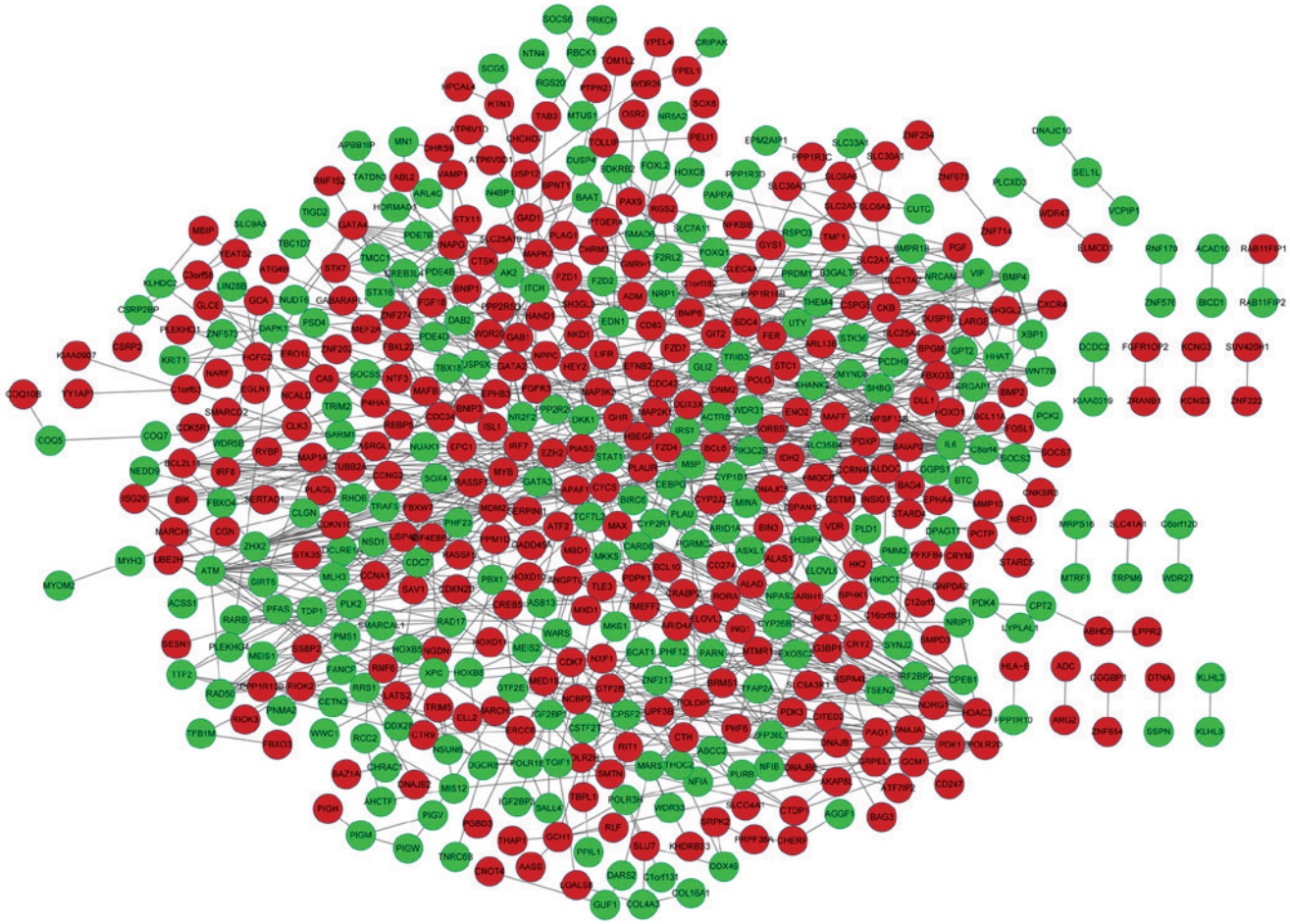

Figure 2. Protein-protein interaction network of differentially-expressed genes. The red nodes indicate upregulated genes and green nodes represent downregulated genes.

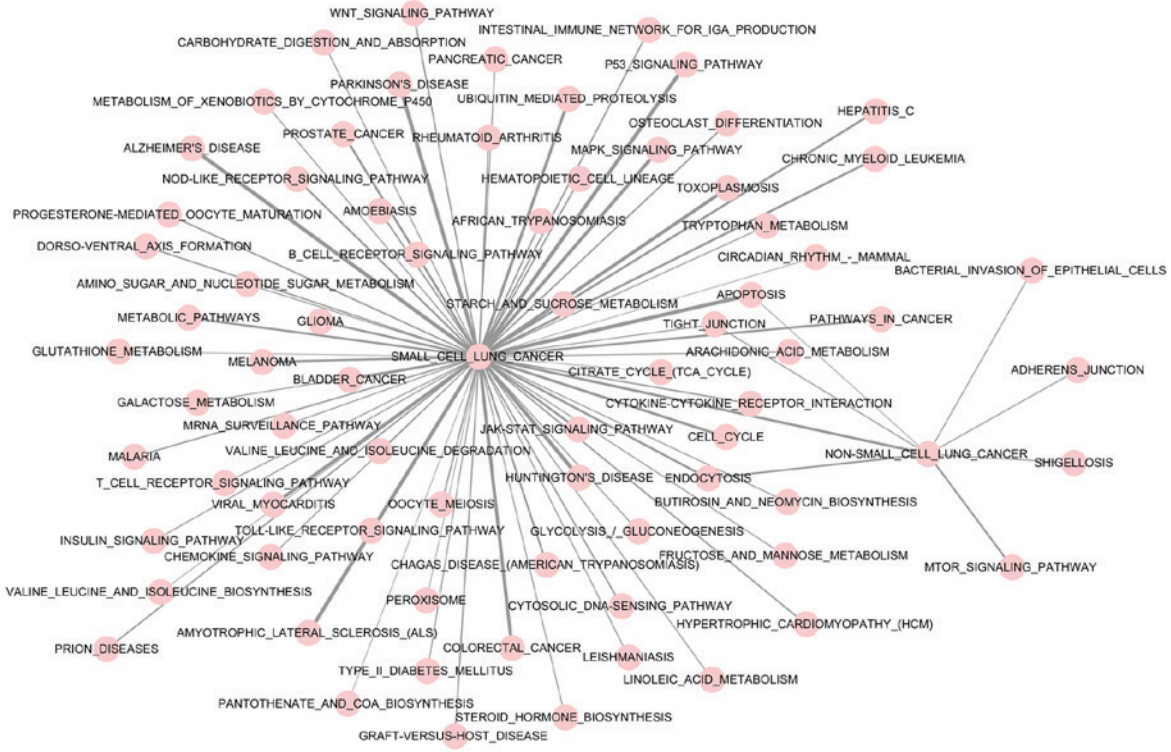

Figure 3. Pathway crosstalk analysis of 7, 8-diacetoxy-4-methylcoumarin-treated lung adenocarcinoma. The pink nodes represent the Kyoto Encyclopedia of Genes and Genomes pathway. The width of solid line indicates the strength of the crosstalk.

Table III. Functional annotation of differentially-expressed genes in 7, 8-diacetoxy-4-methylcoumarin-treated lung cancer groups.

Expression changes	TF	TSG	Other TAG
Upregulated	ATF2, BCL6, CDK7, ERCC6, EZH2, FOXD1, GATA2, GATA4, GMEB1, GTF2B, HAND1, HDAC3, HEY2, HOXD10, HOXD11, ING1, IRF7, IRF8, IRX5, ISL1, MAFB, MAFF, MAX, MEF2A, NFIL3, PAX9, PLAGL1, RFX2, RORA, SOX8, TMF1, VDR, ZNF10 and ZNF133	APAF1, ARID3B, BCL10, BIK, BMP2, BRMS1, CAMTA1, CDKN1C, CDKN2D, DDX3X, EGLN1, FBXW7, HDAC3, ING1, IRF8, LATS2, NDRG1, PHF6, PLAGL1, PLEKHO1, PPP1R3C, RASSF1, RASSF5, SLC9A3R1, TMEFF2 and TP53INP1	CHRM3, FZD7, LGALS8, MAFB, MAP1A, MAX, PIAS3 and RGS2
Downregulated	AKNA, CEBPG, FOXL2, FOXQ1, GATA3, GLI2, GTF2E1, HOXB5, HOXB8, HOXC8, MEIS1, MEIS2, MSC, NFIA, NFIB,NPAS2, NR2E3, NR2F2, NR5A2, PBX1, PRDM1, SMAD6, SOX4, STAT1, TBX18, TCF7L2 and XBP1	ABLIM3, ARID1A, ASXL1, CDH4, COL4A3, DAB2, DAPK1,DFNA5, DKK1, KCNRG, KRIT1, MN1, MTUS1, NRCAM, PLK2, PRDM1, RARB and TCF7L2	ATM, PMS1, RHOB, SSPN and TFAP2A

TF, transcription factor; TSG, tumor suppression genes; TAG, tumor-associated gene.

Furthermore, pathway crosstalk analysis in the present study demonstrated that apoptosis, tight junction and endocytosis had cross-talks with non-small and small lung cancer. Additionally, the present study also revealed that *MDM2* and *CDC42*, which were another two hub genes in the PPI network, were identified to be involved in different pathways, including the p53 signaling and endocytosis pathways. *MDM2* encodes a nuclear-localized E3 ubiquitin ligase that mediates ubiquitination of p53 (35). Oren (36) demonstrated that proteins encoded by one or more p53 target genes could serve an essential role in causing p53-mediated apoptosis. This was consistent with the observations by Goel *et al* (12), demonstrating that DAMTC could induce apoptosis by modulating the p53 pathway in A549 cells. For the other DEG, CDC42 is a small GTPase of the Rho-subfamily, which can regulate signaling pathways that control numerous cellular functions, including cell endocytosis, migration and cell cycle progression (37). Georgiou *et al* (38) reported that *CDC42* could act together with par-6 family cell polarity regulator and protein kinase C in the regulation of Arp2/3-mediated endocytosis to control local adherens junction stability, modulating actin filament dynamics (38). Additionally, Mosesson *et al* (39) revealed that aberrant endocytosis of transmembrane proteins could contribute to malignant transformation. Furthermore, Goel *et al* (34) demonstrated that DAMTC could inhibit cell motility in A549 cells. Collectively, it was confirmed that DAMTC could induce cancer cell apoptosis by modulating the p53 pathway. In addition, it was indicated that DAMTC may inhibit cell motility through targeting *CDC42* and affecting the endocytosis pathway in lung adenocarcinoma.

In conclusion, the present study determined a network of genes, including *IL6*, *MDM2*, *CDC42* and *MAX*, targeted by DAMTC that participated in different pathways were involved in the mechanism of DAMTC-treated lung adenocarcinoma. DAMTC may induce cell apoptosis by targeting *IL6* and *MAX*, which was involved in the MAPK pathway in lung adenocarcinoma. Additionally, DAMTC may inhibit cell motility through targeting *CDC42* and affecting the endocytosis pathway in lung adenocarcinoma. These activities may contribute to the anti-carcinogenic action of DAMTC. Due to the relatively small number of samples in the present study, further studies using a larger sample size to validate and determine the role of the potential molecular mechanism identified are required. Ongoing studies may emphasize the potential of DAMTC as an anticancer therapeutic.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available on reasonable request from the corresponding author.

Authors' contributions

BW conceived and designed the study. YC and YK acquired the data. XL and HF analyzed and interpreted the data. SZ and YK performed statistical analysis. BW drafted the manuscript. YC and TZ revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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