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Original Article

Potential biomarkers for paclitaxel sensitivity in hypopharynx cancer cell

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Abstract: Paclitaxel has been proved to be active in treatment and larynx preservation of HNSCC, however, the fact that about 20-40% patients do not respond to paclitaxel makes it urgent to figure out the biomarkers for paclitaxel-based treatment in Hypopharynx cancer (HPC) patients to improve the therapy effect. In this work, Fadu cells, treated or untreated with low dose of paclitaxel for 24 h, were applied to DNA microarray chips. The differential expression in mRNAs and miRs was analyzed and the network between expression-altered mRNAs and miRs was constructed. Differentially expressed genes were mainly enriched in superpathway of cholesterol biosynthesis (ACAT2, MSMO1, LSS, FDFT1 and FDPS etc.), complement system (C3, C1R, C1S, CFR and CFB etc.), interferon signaling (IFIT1, IFIT3, IFITM1 and MX1 etc.), mTOR signaling (MRAS, PRKAA2, PLD1, RND3 and EIF4A1 etc.) and IGF1 signaling (MRAS, IGFBP7, JUN and FOS etc.), most of these pathways are implicated in tumorigenesis or chemotherapy resistance. The first three pathways were predicted to be suppressed, while the last two pathways were predicted to be induced by paclitaxel, suggesting the combination therapy with mTOR inhibition and paclitaxel might be better than single one. The dramatically expression-altered miRs were miR-112, miR-7, miR-1304, miR-222*, miR-29b-1* (these five miRs were upregulated) and miR-210 (downregulated). The 26 putative target genes mediated by the 6 miRs were figured out and the miR-gene network was constructed. Furthermore, immunoblotting assay showed that ERK signaling in Fadu cells was active by low dose of paclitaxel but repressed by high dose of paclitaxel. Collectively, our data would provide potential biomarkers and therapeutic targets for paclitaxel-based therapy in HPC patients.

Keywords: Paclitaxel, hypopharynx cancer, DNA microarray, mTOR signaling

Introduction

Hypopharynx cancer (HPC) is an uncommon type of squamous cell carcinomas of head and neck (HNSCC); approximately 2,500 new cases are diagnosed in the United States each year (American Cancer Society). Almost all HPCs are mucosal squamous cell carcinomas (SCCs) and clinically tend to be aggressive and are characterized by diffuse local spread, early metastasis, and a relatively high rate of distant spread [1, 2]. Approximately 80% of HPC patients were found to have stage III or stage IV disease, more than 50% of HPC patients have clinically positive cervical nodes and as many as 17% of HPCs may be associated with distant metastases when clinically diagnosed [3]. Surgery, radiotherapy, and chemotherapy are the main means for curative management of locally advanced HNSCC (i.e., stage III or IV), including HPC. With the advances in treatment, not only survival but also larynx preservation becomes important goals for treatment of HPC patients.

Growing evidences have suggested that chemotherapy is beneficial to larynx preservation. Induction chemotherapy or chemotherapy administered concomitantly with radiation for advanced HNSCC patients provides a survival advantage as well as a significantly increased rate of organ preservation when compared with radiation alone [4, 5], and these two treatment modalities have been proven to be equally efficacy for larynx preservation and overall survival [6]. The previously standard cisplatin/5-fluoruracil (5-FU) combination is being replaced by the triple combination of taxane/cisplatin/5-FU [7]. Randomized trials showed that increased activity with the triplet regimen resulted in improved long-term disease control and surviv-

al [8, 9]. Paclitaxel (Pac), one type of taxane, is a mitotic inhibitor used in cancer chemotherapy. Pac has proved its activity in treatment of various solid tumors, when used as monotherapy or combination with other drugs. When used alone for HNSCC treatment, the objective response rate (ORR) to Pac is 20-40% [10, 11]. When combined with carboplatin for advanced HNSCC, the ORR to Pac is 52% [12]. But when cisplatin plus Pac is used as first-line therapy for locally advanced HNSCC, the ORR is 78-83% [13, 14]. Furthermore, several reports suggest that Pac-based regimens provide an 81-84% 2-year organ preservation rate with acceptable toxicity in HNSCC patients, including patients with HPC, larynx and oropharynx cancer [5, 15]. Collectively, Pac-based regimens, including induction chemotherapy or concurrent chemoradiotherapy, have good effects on patients' survival and larynx preservation. However, there are still 20-40% of patients who do not respond to paclitaxel-based therapy. So, it is of great interest to find potential biomarkers for paclitaxel sensitivity evaluation to improve the therapy.

Several genes or microRNAs (miRs) have been implicated in paclitaxel sensitivity or resistance of various cancers. For example, expression of β-tubulin isotypes [16], γ-actin [17] and LIMK2 [18], and the extracellular matrix protein transforming growth factor-β induced (TGFBI) [19] was correlated with paclitaxel sensitivity in different cancers; paclitaxel sensitivity of various cancer cells was also associated to expression of miR-200c [20], miR-148a [21], miR-125b [22], miR-21 [23], miR-337-3p [24] and miR-34a [25]. However, there are few studies on biomarkers of HPC cells for paclitaxel.

In present study, to systematically understand the roles of genes or miRs in paclitaxel sensitivity, Fadu cells, untreated or treated with lower dose of paclitaxel for 24 h, were applied to DNA microarray chips for gene and miR expression profile analysis. The differentially expressed genes and miRs were identified and the relationships between significantly expression-altered miRs and genes were analyzed.

Materials and methods

Cell culture

Fadu cell line was purchased from ATCC (HTB-43) and maintained in DMEM medium supplemented with 10% FBS (Hyclone), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies). Cells in the exponential growth phase were used for all the experiments.

MTS assay for Fadu cell viability

Fadu cells (4×10^3) were cultured in 100 μl of DMEM medium each well in a 96-well plate. 24 h later, the cells were treated with paclitaxel (0, 2, 6.3, 20, 63, 200, 630, 2000 nmol/L, respectively) for 72 h. Every treatment was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 2 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's instructions. The IC50 calculation was performed with GraphPad Prism 5.0 software.

The concentration of paclitaxel at which Fadu cell viability was suppressed by 10% or so in 24 h was determined as follow: Fadu cells were treated with paclitaxel (0, 0.2, 0.63, 2.0, 6.3 and 20 nmol/L, respectively) for 24 h. Every treatment was triplicate in the same experiment. The cell viability was examined as above mentioned.

The time-course of paclitaxel treatment was carried out as follow: Fadu cells were left untreated or treated with paclitaxel (2 nmol/L) for 24, 48 and 72 h, respectively. Every treatment was triplicate in the same experiment. The cell viability was calculated relatively to the untreated cells at every time point.

Microarray analysis: gene and miR expression profile

Fadu cells (8×10^4) were grown in 2 ml of DMEM medium (10% FBS) each well in a 6-well plate. After 24 h, the cells were treated with paclitaxel (2 nmol/L) for 24 h or left untreated, respectively. Every treatment was duplicated in the same experiment. All the samples were homogenized with 1 ml Trizol (Invitrogen, Life Technologies) and total RNAs were extracted according to the manufacturer's instructions.

500 ng total RNA was used to synthesize double-strand cDNA and in vitro transcribed to cRNA, purified 10 μg cRNA was used to synthesize 2nd-cycle cDNA and then hydrolyzed by RNase H and purified. Above steps were per-

Table 1. The significantly expression-altered genes following paclitaxel treatment in Fadu cells

Gene	Probe ID	Fold change	*P* value	Gene	Probe ID	Fold change	*P* value
FOS	7975779	4.47	0.0002	ADAMTS1	8069676	1.73	0.005
CPA4	8136200	4.06	0.0001	AFAP1L2	7936439	1.72	0.03
DUSP6	7965335	3.70	0.001	IL6	8131803	1.72	0.03
DUSP1	8115831	3.34	0.0001	ODC1	8050240	1.70	0.0004
NT5E	8120967	2.99	0.001	SERPINB8	8021653	1.69	0.02
CTGF	8129562	2.93	0.0001	SPRY4	8114797	1.69	0.01
JUN	7916609	2.75	0.01	RRM2	8040223	1.69	0.03
EGR1	8108370	2.67	0.0002	CDH5	7996264	1.69	0.002
SNORD102	7968232	2.59	0.03	CCNE2	8151871	1.68	0.03
IL1RL1	8044021	2.42	0.0004	IER2	8026163	1.67	0.02
SNORD51	8047778	2.38	0.01	ROR1	7901969	1.67	0.01
PPP1R15A	8030128	2.28	0.005	GJB3	7899932	1.66	0.003
IER3	8178435	2.27	0.001	IL1R2	8043981	1.65	0.04
CYR61	7902687	2.24	0.002	DOCK10	8059413	1.64	0.02
IER3	8179704	2.16	0.001	SERPINE1	8135069	1.64	0.01
IER3	8124848	2.16	0.001	THBS1	7982597	1.64	0.002
SERPINB2	8021635	2.15	0.02	PHLDA1	7965040	1.63	0.01
EFNB2	7972713	2.10	0.01	EPHA2	7912706	1.63	0.0003
FOSB	8029693	2.08	0.05	ARRDC4	7986350	1.62	0.002
PTGS2	7922976	1.99	0.02	BRMS1	7949603	1.61	0.02
LOC100127886	8066275	1.99	0.04	INPP4B	8102950	1.61	0.04
IL24	7909271	1.99	0.01	SLC5A6	8051030	1.60	0.01
FST	8105302	1.98	0.03	CSRNP1	8086330	1.60	0.02
FOSL1	7949532	1.93	0.01	TRAM2	8127051	1.60	0.001
DUSP4	8150076	1.92	0.01	SNORA67	8004508	1.60	0.02
DHRS2	7973433	1.86	0.004	CCDC86	7940349	1.58	0.01
ARL4C	8059854	1.86	0.02	RRP15	7909782	1.58	0.04
TRNAP24P	7998927	1.83	0.005	FAM111B	7940147	1.58	0.01
FJX1	7939365	1.80	0.01	SLIT2	8094301	1.57	0.02
CTNNAL1	8163063	1.80	0.01	CDH2	8022674	1.57	0.02
MB21D2	8092765	1.80	0.01	MRPL1	8095894	1.57	0.05
BAG1	8160647	1.79	0.02	GADD45A	7902227	1.57	0.01
GPR39	8045336	1.79	0.00	DUSP10	7924450	1.56	0.02
SNORD101	8122142	1.79	0.05	RPL22L1	8092067	1.56	0.02
AKAP12	8122807	1.77	0.02	ID2	8040103	1.56	0.005
SNORA13	8107326	1.77	0.04	VGLL1	8170179	1.56	0.02
DUSP5	7930413	1.75	0.00	LANCL2	8132897	1.56	0.0005
DNER	8059580	1.74	0.04	GDPD3	8000799	1.56	0.01
TRIB1	8148304	1.74	0.004	NAV3	7957298	1.56	0.05
SNORA62	8078918	1.73	0.003	MIR22HG	8011193	1.55	0.02
CYB5D1	8004694	1.55	0.002	C1R	7960744	0.47	0.002
C8orf33	8148955	1.55	0.01	MUC1	7920642	0.47	0.02
EPB41L4A-AS1	8107321	1.55	0.001	CFB	8178115	0.47	0.01
VEGFC	8103822	1.55	0.02	C3	8033257	0.47	0.003
PLK3	7901054	1.54	0.01	RARRES3	7940775	0.47	0.03
TIPIN	7989915	1.54	0.01	CLDN1	8092726	0.47	0.001
HAS2	8152617	1.54	0.03	FADS1	7948612	0.47	0.001

ZFP36	8028652	1.54	0.02	GLDC	8160024	0.46	0.04
METTL1	7964548	1.54	0.01	COX20	7925561	0.46	0.0001
ETS1	7952601	1.53	0.002	MSMO1	8098195	0.46	0.01
OTUB2	7976425	1.53	0.03	PCSK9	7901696	0.46	0.01
KLF6	7931810	1.53	0.002	PLCG2	7997453	0.46	0.001
SAMD15	7975971	1.53	0.01	TRIM22	7938035	0.46	0.01
SURF2	8159008	1.53	0.01	GBP4	7917561	0.45	0.02
STAG1	8090898	1.52	0.03	ALDH3B2	7949882	0.45	0.02
ATP6V1D	7979698	1.52	0.04	SERPINB13	8021603	0.45	0.01
TOE1	7901091	1.52	0.002	ZMAT1	8174119	0.44	0.01
MRTO4	7898549	1.52	0.003	TCP11L2	7958262	0.44	0.03
RND3	8055688	1.52	0.02	LOC440173	8162183	0.44	0.01
SPHK1	8010061	1.51	0.04	BTBD8	7902965	0.44	0.02
ENC1	8112615	1.51	0.04	C4orf34	8099912	0.44	0.002
GPATCH4	7921076	1.51	0.002	ASS1	8158671	0.43	0.003
TMCO1	7921987	1.51	0.01	TMEM154	8103226	0.43	0.02
FAM171A1	7932243	1.51	0.002	HSD17B7P2	7927082	0.43	0.01
ERRFI1	7912157	1.51	0.01	KIAA1107	7902977	0.42	0.002
CDCA5	7949364	1.51	0.01	OAS2	7958913	0.42	0.004
GPAM	7936322	1.50	0.01	CFI	8102328	0.41	0.04
AEN	7985767	1.50	0.01	PLD1	8092134	0.40	0.01
RUNX1	8070194	1.50	0.002	HERC5	8096361	0.40	0.004
MME	8083494	0.50	0.02	METTL7A	7955441	0.39	0.002
RAET1E	8130151	0.50	0.02	CCL5	8014316	0.39	0.01
PLA2R1	8056151	0.50	0.003	SEPP1	8111915	0.38	0.01
ALDOC	8013660	0.49	0.001	OASL	7967117	0.38	0.001
A4GALT	8076497	0.49	0.01	CASP1	7951397	0.38	0.03
GRAMD1C	8081758	0.49	0.02	DDIT4	7928308	0.37	0.002
HMGCS1	8111941	0.48	0.002	TNFSF10	8092169	0.37	0.01
LSS	8070961	0.48	0.0002	IFI44L	7902541	0.37	0.01
SREBF2	8073522	0.48	0.01	DDX60	8103563	0.35	0.01
CFB	8118345	0.47	0.01	ACSS2	8062041	0.34	0.0005
KYNU	8045539	0.47	0.002	C1S	7953603	0.34	0.01
SCNN1A	7960529	0.34	0.0003				
STARD4	8113491	0.32	0.01				
INSIG1	8137526	0.30	0.001				
SERPINB3	8023688	0.29	0.04				
SCD	7929816	0.26	0.001				
SLCO4C1	8113369	0.21	0.0001				
CP	8091385	0.11	0.001				

formed with Ambion WT Expression Kit. 5.5 μg 2nd-cycle cDNA was fragmented and the single-stranded cDNA was labeled with GeneChip2 WT Terminal Labeling Kit and Controls Kit (Affymetrix, PN 702880). About 700 ng fragmented and labeled single-stranded cDNA were hybridized to an Affymetrix GeneChip Human Gene 1.0 ST array, which was washed and stained with GeneChip2 Hybridization, Wash and Stain kit (Affymetrix).

Total RNA from Fadu cells, untreated or treated with lower dose of paclitaxel for 24 h, was processed and hybridized to Affymetrix GeneChip® miRNA 2.0 Array, which recognizes 1,105 separate human miRs in accordance with the Sanger

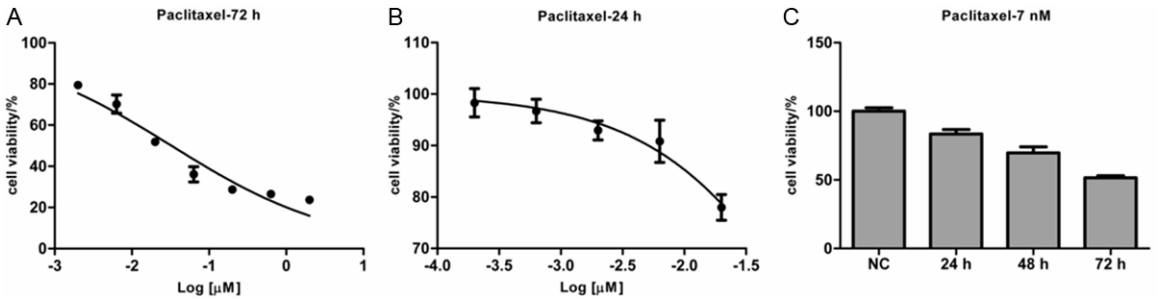


Figure 1. Fadu cells are moderately sensitive to paclitaxel. A: Fadu cells were treated with paclitaxel (0, 2, 6.3, 20, 63, 200, 630, 2000 nmol/L, respectively) for 72 h and then the cell viability was detected by the MTS assay. B: Fadu cells were treated with paclitaxel (0, 0.2, 0.63, 2.0, 6.3 and 20 nmol/L, respectively) for 24 h. The cell viability was detected by the MTS assay and plotted. C: The time-course effect of paclitaxel on Fadu cells. Fadu cells were left untreated or treated with paclitaxel (2 nmol/L) for 24, 48 and 72 h, respectively. Every treatment was triplicate in the same experiment. The cell viability was calculated relatively to the untreated cells at every time point.

Institute miRBase version 15. Each sample was duplicate for miR expression profile.

Microarray data analysis was done using Significance Analysis of Microarrays (SAM) method, as described before [26]. Gene set enrichment analysis (GSEA) was performed to the differential expression genes with Ingenuity Pathway Analysis (IPA) online software.

Quantitative real-time PCR (qPCR)

Total RNA was synthesized to cDNA using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with mixture of oligo-dT and Random Primer (9 mer). The primers used for qPCR validation were list in Supplementary Table 1. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in untreated control cells.

miR target prediction and miRNA target correlation

miR target prediction was performed with miRWalk online software. The comparative analysis was done by 5 prediction programs: miRanda, miRDB, miRWalk, RNA22 and TargetScan. 6 miRs induced or repressed by paclitaxel were selected to perform miR target prediction. Genes predicted by greater than or equal to 3 programs were selected as putative downstream target of some miR. The putative downstream genes were done intersection with genes that expression level altered significantly (FDR<10%) following paclitaxel treatment. The overlapped genes were selected to construct miR-gene networks with the aid of Cytoscape 2.8 software.

Protein isolation and western blotting

Fadu cells were treated with 2, 5 or 10 nmol/L of paclitaxel for 24 h or left untreated. Cell pellets were resuspended in 1×SDS loading buffer (1 mmol/L Na_3VO_4, 10 mmol/L NaF, 1 mmol/L PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), ERK (Abclonal, A0228) and p-ERK (Cell signaling, #9106S, pT202/204), were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

Results

Fadu cells are moderate sensitive to paclitaxel

To determine chemosensitivity of Fadu cells to paclitaxel, Fadu cells were treated with paclitaxel at different concentrations for 72 h, cell viability was examined by MTS assay and IC50 dose to paclitaxel was calculated. IC50 dose of Fadu to paclitaxel at 72 h is 0.032 μmol/L (R^2=0.94) (**Figure 1A**). According to data reported in DTP Data Search, the mean IC50 of NCI-60 cell panel to paclitxel is 0.009-0.035 μmol/L. So, Fadu cell line is moderate sensitive to paclitaxel.

To find a suitable dose to inhibit Fadu cells growth by 10% or so, we used a narrower range of paclitaxel concentrations to treat Fadu cells

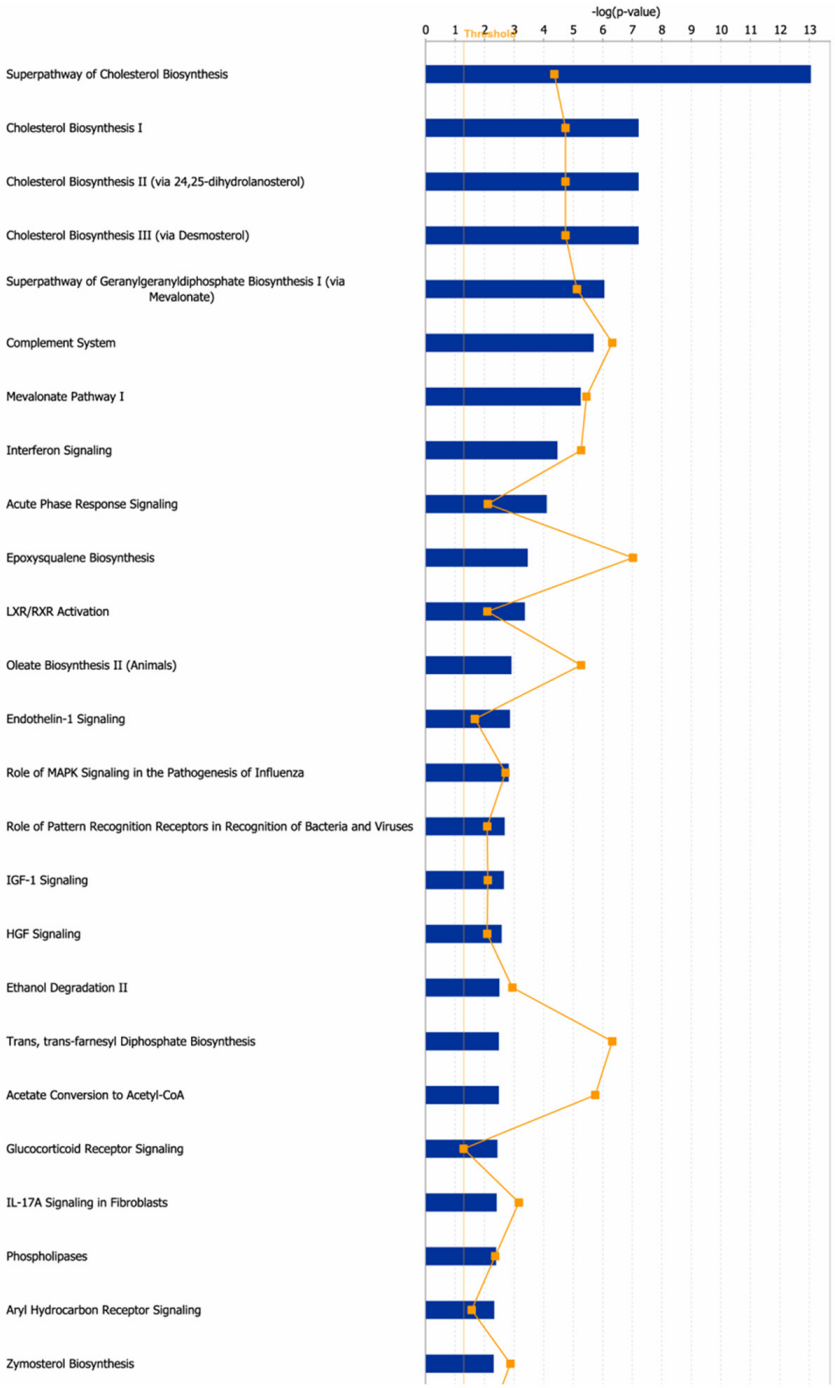


Figure 2. The canonical pathways for the differentially expressed genes in Fadu cells following paclitaxel treatment. 167 genes whose expression altered by higher than 2-fold after paclitaxel treatment were applied to IPA. The canonical pathways ($p<0.05$) were shown in this figure. The thresh line represents $p=0.05$. The ratio means the proportion that the amounts of genes involved in some pathway account for all the genes involved in this pathway.

for 24 h. The IC50 of Fadu to paclitaxel at 24 h is 0.147 μmol/L ($R^2=0.97$). As Fadu cells viability was repressed by 7.1% at the concentration of 2 nmol/L (**Figure 1B**), this concentration is suitable for study on paclitaxel sensitivity. The concentration was far lower than the corresponding IC50 dose.

And then, Fadu cells were treated with 2 nmol/L of paclitaxel for 24, 48 and 72 h or left untreated. The time-course effect of paclitaxel treatment on proliferation of Fadu cells was present in **Figure 1C**. The results showed that the proliferation inhibition was apparently time-dependent under this condition. Furthermore, when treated with the lower concentration of paclitaxel for 24 h, Fadu cell viability was suppressed by 17% or so.

Gene expression analysis

Fadu cells, treated with a lower dose of paclitaxel for 24 h or left untreated, were applied to gene expression chips. The results of bioinformatics analysis showed that when cells were treated with this moderate condition, expression of 109 genes was significantly ($p<0.05$) increased by higher than 50%, and that of 58 genes was significantly decreased by higher than 50% following paclitaxel treatment (**Table 1**). The most markedly expression-altered genes were FOS (FBJ murine osteosarcoma viral oncogene homolog, up to 4.47-fold), JUN (jun proto-oncogene, up to 2.75-fold), SCD (stearoyl-CoA desaturase, down

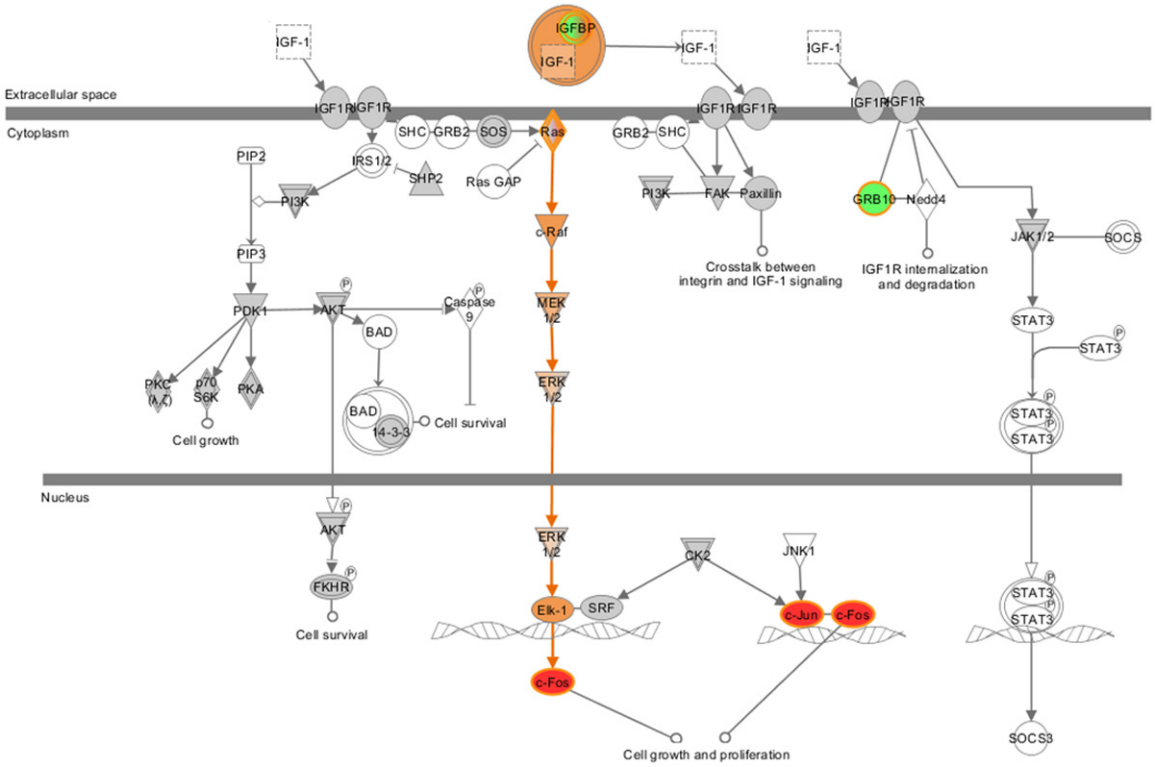
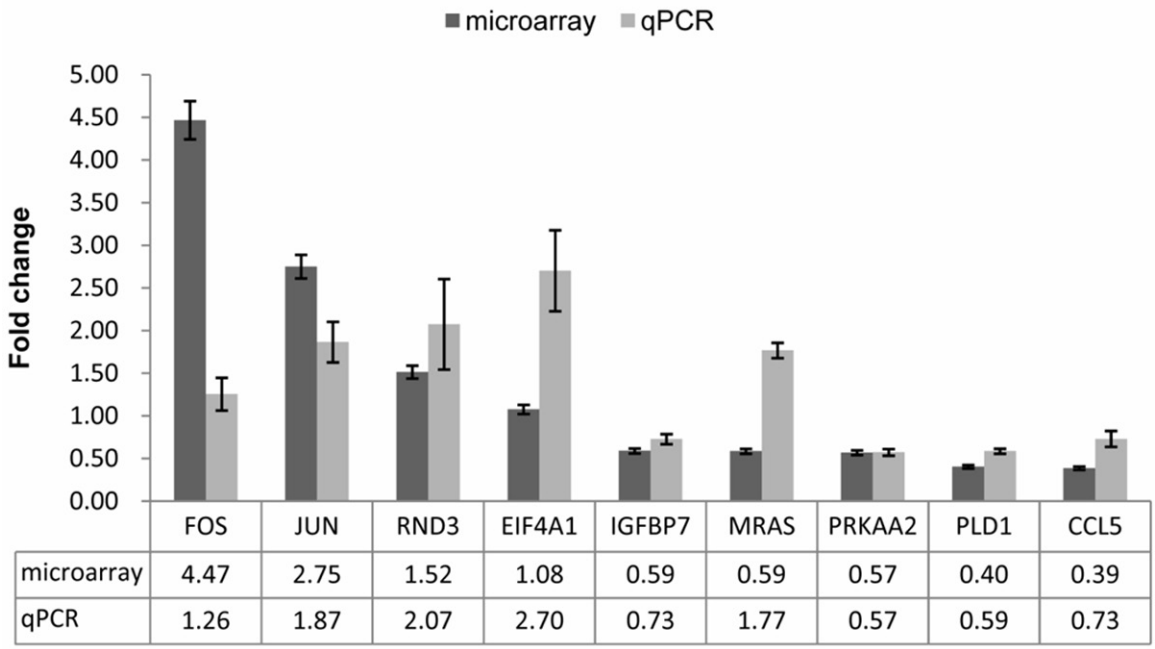


Figure 3. IGF1 signaling was induced by paclitaxel in Fadu cells. This figure was derived from IPA. The colored molecules were genes whose expression altered significantly in Fadu cells following paclitaxel treatment. The red color represented up-regulated genes, while the green color represented down-regulated genes, the orange color represented genes whose expression was predicted to be increased.



	FOS	JUN	RND3	EIF4A1	IGFBP7	MRAS	PRKAA2	PLD1	CCL5
microarray	4.47	2.75	1.52	1.08	0.59	0.59	0.57	0.40	0.39
qPCR	1.26	1.87	2.07	2.70	0.73	1.77	0.57	0.59	0.73

Figure 4. qPCR validation for microarray data. 9 genes were selected to perform qPCR. The expression was calculated relative to expression in untreated cells. The change folds determined by qPCR and microarray were plotted, respectively. Bars represent the standard errors.

Table 2. The significantly expression-altered miRs following paclitaxel treatment in Fadu cells

Probe Set ID	Fold change	*p* value
hsa-miR-122	5.52	0.03
hsa-miR-7	3.26	0.03
hsa-miR-1304	3.10	0.03
hsa-miR-222-star	2.71	0.02
hsa-miR-29b-1-star	2.05	0.04
hsa-miR-210	0.38	0.01

to 0.26-fold), PLD1 (phospholipase D1, phosphatidylcholine-specific, down to 0.40-fold), MRAS (muscle RAS oncogene homolog, down to 0.59-fold) and CP (ceruloplasmin, down to 0.11-fold). IPA results showed that these 167 genes (109 upregulated genes and 58 down-regulated genes following paclitaxel treatment) are involved in cell death and survival, lipid metabolism and small molecule biochemistry. Those significantly altered pathways were list in **Figure 2**. IPA analysis showed these 167 genes were mainly enriched in Superpathway of cholesterol biosynthesis (ACAT2, MSMO1, LSS, FDFT1 and FDPS etc.), complement system (C3, C1R, C1S, CFR and CFB etc.), interferon signaling (IFIT1, IFIT3, IFITM1 and MX1 etc.), mTOR signaling (MRAS, PRKAA2, PLD1, RND3 and EIF4A1 etc.) and IGF1 signaling (MRAS, IGFBP7, JUN and FOS etc.). The former three pathways were predicted to be inactivated, while the latter two pathways were predicted to be induced (**Figure 3**).

qPCR validation of gene expression

Then 9 genes were selected for further validating the fold change determined by microarray. Within these genes, expression of 3 genes (FOS, JUN and RND3) was up-regulated and that of 5 genes (IGFBP7, MRAS, PRKAA2, PLD1 and CCL5) was down-regulated, while one gene (EIF4A1) expression was not changed markedly in microarray data following paclitaxel treatment. As showed in **Figure 4**, the expression trends of 7 genes were consistence between microarray data and qPCR results following paclitaxel treatment, although expression fold change varied to some extents. For EIF4A1 and MRAS, qPCR data showed their expression was increased, which was in contrast to microarray data. In terms of expression trends, 7 of 9 (78%) genes induced or repressed following paclitaxel treatment were positively validated by qPCR. These data suggested that microarray data were mostly reliable whereas expression of some genes needs to be validated by qPCR. FOS, JUN, RND3, EIF4A1 and MRAS were significantly upregulated, while IGFBP7, PRKAA2, PLD1 and CCL5 were markedly downregulated.

miR expression analysis

Bioinformatics analysis showed that expression of only tens of miRs was markedly altered after paclitaxel treatment. Expression of 5 miRs (Has-miR-112, 7, 1304, 222*, 29b-1*) was significantly increased by higher than 100% and that of one miR (Has-miR-210) was markedly decreased by higher than 100% (**Table 2**).

MiRs-genes network construction

To construct the network between significantly expression-altered miRs and genes, 6 miRs whose expression altered the most markedly were selected to perform miR target prediction and miRNA target correlation. For example, miR-112 was predicted to mediate expression of 2226 genes (predicted by ≥3 programs) with the aid of miRWalk online software. Then this 2226 genes set was done intersection with the down-regulated 58 genes and the overlapped genes were FADS1, ALDOC, TNFSF10, CCL5, GBP4, MUC1, ALDH3B2, SERPINB3. Thus the downregulated expression of these 8 genes was putatively mediated by the upregulated expression of miR-122 following paclitaxel treatment. For the other 5 miRs, we did the prediction and target correlation according to the same procedure and found that there were 26 genes whose expression was putatively been mediated by these 6 miRs, the network was constructed with the aid of Cytoscape 2.8 software (**Figure 5**). Expression-increased miR-29b-1* putatively mediated the decreased expression of SCD, PLD1 and INSIG1, expression-decreased miR-210 putatively mediated the upregulation of ARRDC4, EPHA2, IER3, OTUB2 and TRIB1, while the down-regulated TNFSF10 was putatively mediated by expression-increased miR-122, miR-1304 and miR-222*.

Signaling pathway detection at protein level

To investigate the underlying mechanism by which paclitaxel exerts its cytotoxic effect on

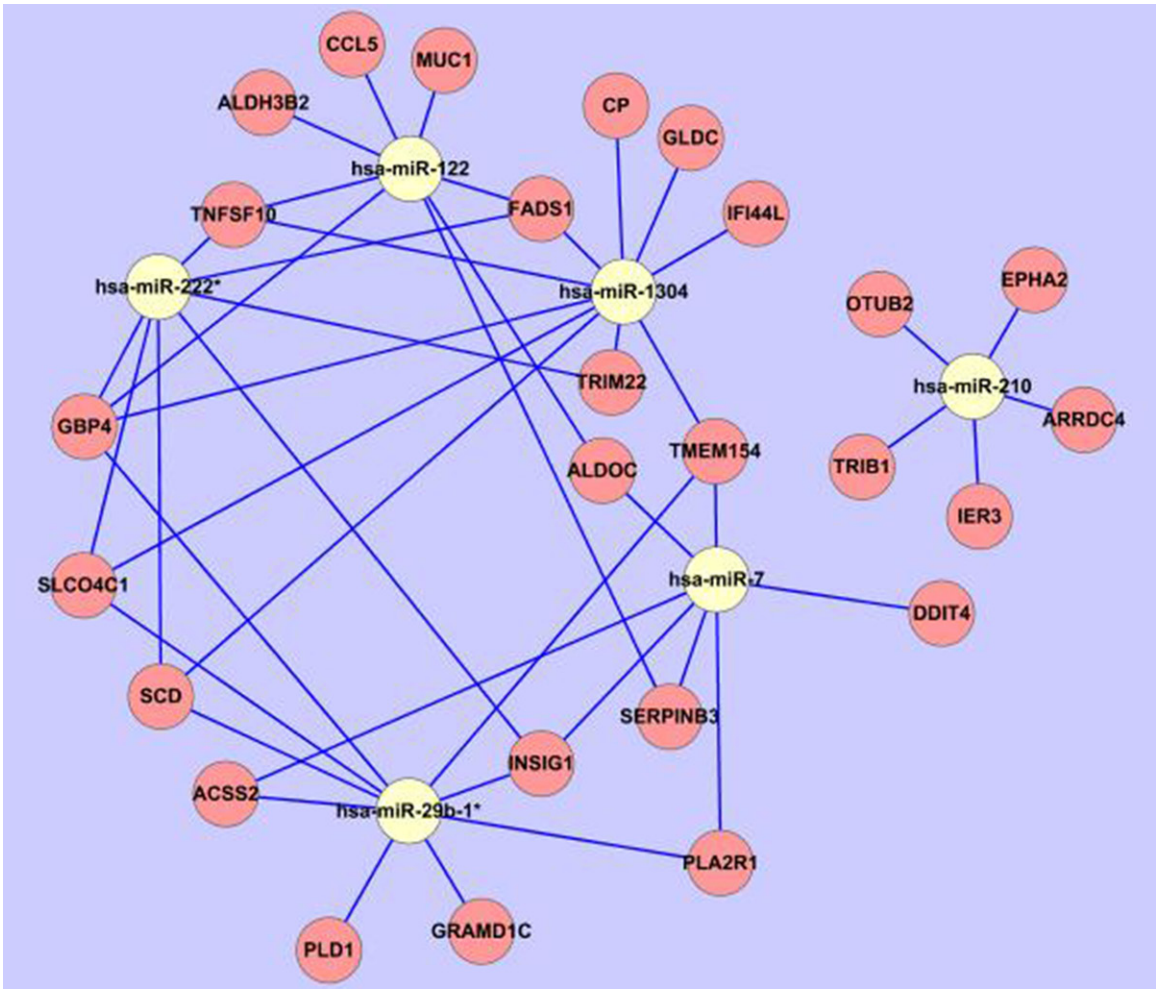


Figure 5. MiR-gene network. The most significantly expression-altered 6 miRs were respectively applied to miRWalk online software to predict the downstream putative target genes. And then the targets were done intersection with the expression-altered genes (109 upregulated genes or 58 downregulated genes, respectively) determined by microarray. The overlapped genes were considered as potential downstream targets and used to construct the miRs-genes network with the aid of Cytoscape 2.8 software. The white circle represented the miR, while the pink circle represented the genes; the blue line represented the regulation from miR to gene.

cancer cells, immunoblotting experiments were done with ERK/p-ERK antibodies for Fadu cells treated with three different doses of paclitaxel or untreated. The results showed that when treated with the lowest dose (2 nmol/L) of paclitaxel, the phosphorylated ERK (p-ERK) of Fadu cells was moderately upregulated (**Figure 6**), which was consistent with the predicted results by IPA (**Figure 3**). However, when the concentration of paclitaxel was increased stepwise, the p-ERK was dramatically downregulated (**Figure 6**).

Discussion

Hypopharynx cancer (HPC), a rare type of HNSCC, generally has a poor prognosis and is difficult to treat. Paclitaxel has been proved to be active in treatment and larynx preservation of HNSCC, however, the clinical data that only a half of patients responding to paclitaxel makes it urgent to figure out the biomarkers for paclitaxel-based treatment in HPC patients to improve the therapy effect.

In this work, Fadu cells, untreated or treated with low dose of paclitaxel for 24 h, were applied to DNA microarray chips. The differential expression in mRNAs and miRs was analyzed and the network between expression-altered mRNAs and miRs was constructed.

Fadu cells were moderate sensitive to paclitaxel. Following low dose (2 nmol/L) of paclitaxel

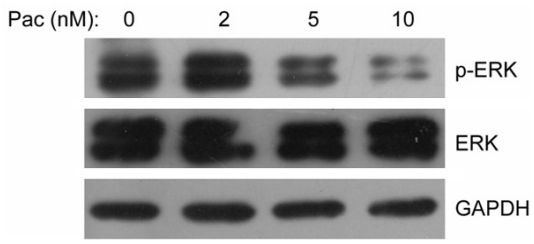


Figure 6. ERK signaling was induced by low dose of paclitaxel but repressed by high dose of paclitaxel. Fadu cells were left untreated or treated with three distinct dose of paclitaxel (2, 5 and 10 nmol/L), and then the samples were collected for western blotting. The ERK and p-ERK protein were detected as described in Materials and Methods.

treatment, expression of 167 genes (109 upregulated genes and 58 downregulated genes) was significantly altered. IPA results showed that these genes are involved in cell death and survival, lipid metabolism and small molecule biochemistry. The mainly altered pathways were superpathway of cholesterol biosynthesis, complement system, interferon signaling, mTOR signaling and IGF1 signaling.

Deregulated energy metabolism in cancer cells may play a role in supporting the large-scale biosynthetic programs that are required for active cell proliferation, and therefore have been proposed to be an emerging hallmark of cancer [27]. Recent evidences have identified substantial overlap between metabolic and oncogenic biochemical pathways, suggesting novel approaches to cancer intervention. For example, cholesterol lowering statins act as chemopreventive agents in prostate, glioma and other cancers [28-30]. In our data, cholesterol biosynthesis pathway was predicted to be inactivated following paclitaxel treatment, suggesting that downregulation of cholesterol biosynthesis caused by paclitaxel may be one underlying mechanism by which paclitaxel exert its cytotoxic effect on cancer cells. Therefore, cholesterol biosynthesis may be a potential therapeutic target for HPC patients.

MRAS is a member of the Ras family of small GTPases. These membrane-associated proteins function as signal transducers in multiple processes including cell growth and differentiation, and dysregulation of MRAS signaling has been associated with many types of cancer [31, 32]. In our microarray data, MARS was downregulated following paclitaxel treatment; however, subsequent validation by qPCR showed MRAS was induced by paclitaxel. Therefore, the IPA results were modified with qPCR data and the mTOR signaling and IGF1 signaling were predicted to be activated.

The mammalian target of rapamycin (mTOR) signaling pathway integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation and survival. Faried et al reported in 2007 that inhibition of mTOR by rapamycin increases chemosensitivity of CaSki (cervical cancer) cells to paclitaxel [33]. Abrams et al suggested that combinations of signal transduction inhibitors (such as mTOR inhibition) and chemotherapy (such as paclitaxel) enhance therapeutic efficacy in non-oncogene addicted cells [34]. Our data showed that paclitaxel induced mTOR signaling at lower concentration (2 nmol/L), suggesting the initiation of resistance to paclitaxel mediated by mTOR signaling in a very short time (24 h).

Furthermore, IGF1 signaling was predicted to be activated by paclitaxel in our data. IGF1 has been suggested to play an important role in tumor progression [35, 36]. It is proposed that IGF1-induced cell proliferation was mediated by the PI3K/Akt/mTOR signaling pathway [37]. Since paclitaxel induced the mTOR signaling and IGF1 signaling in our data, these results together indicate the combination of mTOR inhibition and paclitaxel may be one possible solution for HPC patients resistant to paclitaxel and this hypothesis deserves further validation in more cancer cells and mouse models and patient samples.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Primers for qPCR validation

Gene	Forward	Reverse
FOS	ACTACCACTCACCCGCAGAC	CCAGGTCCGTGCAGAAGT
JUN	TGCGGCCCCGAAACT	GGCGTTGAGGGCATCGT
RND3	GCGCTGCTCCATGTCTTC	GCCGTGTAATTCTCAAACACTG
EIF4A1	AGCCCGAAGGCGTCATC	TCCGAGAGGTTCATGTCATCAA
IGFBP7	GGCCCAGAAAAGCATGAAGTAA	TGGCACTCATATTCTCCAGCAT
MRAS	ACAAGGTCGATTTGATGCACT	GCACTGGTTTCTATGTACGGAAT
PRKAA2	CGGCTCTTTCAGCAGATTCTG	TCAGGTCTCGATGAACAACCAT
PLD1	GGAAGGTGGGACGACAATGA	CCAGAGACGGTCCTGAAGTGA
CCL5	CTCTGCGCTCCTGCATCTG	AGTGGGCGGGCAATGTAG
Actb	GCATCCCCCAAAGTTCACAA	GGACTTCCTGTAACAACGCATCT