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Original Article

Identification of putative biological targets of KIAA1456 in relation to its inhibition of ovarian cancer cell functions based on microarray profiling

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Abstract: Background: Substantial morbidity and mortality are associated with ovarian cancer. In our previous study, we confirmed that KIAA1456 inhibits ovarian cancer cell proliferation, invasion and metastasis; however, the mechanism and pathway underlying these activities are unknown. The aim of this study was to identify potential target genes of KIAA1456 in ovarian cancer cells. Methods: Microarray analysis was conducted to identify differentially expressed genes between HO8910/PM ovarian cancer cells (human ovarian cancer cells HO8910 possessing high metastatic ability) overexpressing KIAA1456 and control HO8910/PM cells lacking KIAA1456 overexpression. Based on results of gene chip-based screening, differences in gene expression between the two groups was validated through bioinformatic analyses, co-expression network construction, quantitative real-time PCR, and Western blotting. Results: Gene expression profiling identified 336 differentially expressed genes between the two groups, including 204 up-regulated genes and 132 down-regulated genes. These genes are primarily involved in gene expression and biopolymer biosynthesis. Further bioinformatic analyses indicated IQGAP1, TRIM29, UBE4A and SMARCA1 to be the most significantly differentially expressed target genes. The results of quantitative real-time PCR and Western blotting were consistent with the microarray findings. Conclusion: KIAA1456-induced IQGAP1, TRIM29, UBE4A and SMARCA1 up-regulation may be the mechanism underlying its inhibition of ovarian cancer cell proliferation, invasion and metastasis. Our study provides potential molecular targets for treatment of ovarian cancer.

Keywords: Ovarian cancer, microarray, KIAA1456, IQGAP1, TRIM29, UBE4A, SMARCA1

Introduction

Ovarian cancer is considered the most deadly gynecological malignancy [1]. Approximately three-fourths of epithelial ovarian cancer cases are detected at an advanced stage [2]. The current standard of care for late-stage ovarian cancer is cytoreductive surgery followed by 6-8 cycles of combination chemotherapy with a platinum-containing agent such as carboplatin [3]. However, the prognosis of ovarian cancer, especially epithelial ovarian cancer, remains poor [4]. To date, various approaches, including functional genomics, systems biology, and proteomics, have been applied in the development of different methods for the early and specific detection of ovarian cancer [2, 5]. In our previous study, we confirmed that KIAA1456 (human tRNA methyltransferase 9-like (hTRM9L)) inhibits the proliferation, invasion and metastasis of ovarian cancer cells.

Modification of tRNA bases, one process of gene regulation, has been shown to regulate the levels of specific proteins [6]. Several studies report that NSUN6 is a human RNA methyltransferase that catalyzes m^5C72 formation in specific tRNAs [7], and the tRNA methyltransferase Dnmt2 is required for accurate polypeptide synthesis during hematopoiesis [8]. TRNA-modifying enzymes may function as regulators of cancer progression. Indeed, emerging evidence indicates that the mRNA encoding human tRNA methyltransferase 9-like protein (KIAA1456, also known as hTRM9L) is down-regulated in human tumors, such as breast, bladder, cervical and testicular carcinomas, due to epigenetic gene silencing. *KIAA1456*, a candidate for the putative 8p colorectal cancer tumor-suppressor gene [9], is located at chromosome 8p22-p23 of the human genome. Recently, it has been demonstrated that loss of

heterozygosity (LOH) of 8p22-p23 occurs at high frequencies in many tumor types and is associated with metastasis and prognosis. For instance, overexpression of ZDHHC2 inhibits the proliferation, migration, and invasion of a hepatocellular carcinoma (HCC) cell line [10]. In addition, our previous study confirmed that KIAA1456 inhibits ovarian cancer cell proliferation, invasion and metastasis, though the mechanism and pathway underling these events are unknown.

In the current study, we employed microarray analysis to identify differentially expressed genes between human ovarian cancer cells HO8910/PM overexpressing KIAA1456 and control cells. Differentially expressed mRNAs between the two groups were selected, followed by identification of target genes based on bioinformatic methods and co-expression network construction. A total of 336 mRNAs were found to be differentially expressed between the two groups, including 204 up-regulated and 132 down-regulated genes. KIAA1456-specific effects on expression of IQGAP1, TRIM29, UBE4A and SMARCA1 genes were confirmed using real-time PCR and Western blot analyses. Taken together, our results reveal a novel link between KIAA1456 and these biological targets with regard to the regulation of cell proliferation, invasiveness and migration. The findings provide an experimental basis for the development of KIAA1456 as a new therapeutic target for ovarian cancer.

Materials and methods

Cell culture

The HO8910/PM cell line and the packaged retrovirus containing the KIAA1456 expression plasmid used in this study were generated in our previous work. Cells were cultured at 37°C in an atmosphere of 5% CO_2 in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin.

RNA extraction

Total RNA was extracted from the two groups of cells using the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA quantity and quality were measured using a NanoDrop spectrophotometer, and RNA integrity was assessed via standard denaturing agarose gel electrophoresis. The 28S/18S ratio was approximately 2.0. Final RNA preparations were resuspended in RNase-free water and stored at -80°C.

Microarray and bioinformatic analyses

Affymetrix GeneChip Human Transcriptome Array 2.0 was employed for detecting differentially expressed genes between the two groups of samples, this array can be used to detect lncRNAs and mRNAs simultaneously. Briefly, double-stranded complementary DNA (cDNA) was synthesized using RNA from each sample and then labeled, hybridized and imaged [11, 12]. The main bioinformatic analyses were as follows. (1) Cluster analysis. Hierarchical clustering was performed using R software. This method explicitly accounts for the dynamic nature of temporal gene expression profiles during clustering and identifies several distinct clusters [13, 14]. (2) Gene Ontology (GO) analysis. Functional GO categories enriched among the differentially expressed genes were determined using DAVID [15]. (3) Pathway analysis. Pathway analysis was used to determine significant pathways related to the differentially expressed genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and Reactome databases [14, 16]. (4) Gene co-expression network construction. By building an mRNA-lncRNA network according to their interactions and the differential expression results, we identified additional associations between mRNAs and established lncRNAs that regulate the expression of target mRNAs [14, 17].

After significance and false discovery rate (FDR) analyses, differentially expressed genes were selected according to a threshold p value. Genes significantly differentially expressed between HO8910/PM cells overexpressing KIAA1456 and non-transfected cells were selected based on a fold change in expression > 1.2 and $P < 0.05$.

Validation of differentially expressed genes via quantitative real-time PCR

Quantitative real-time PCR was used to verify differential expression of IQGAP1, TRIM29, UBE4A and SMARCA1. Total RNA was extracted from HO8910/PM cells overexpressing

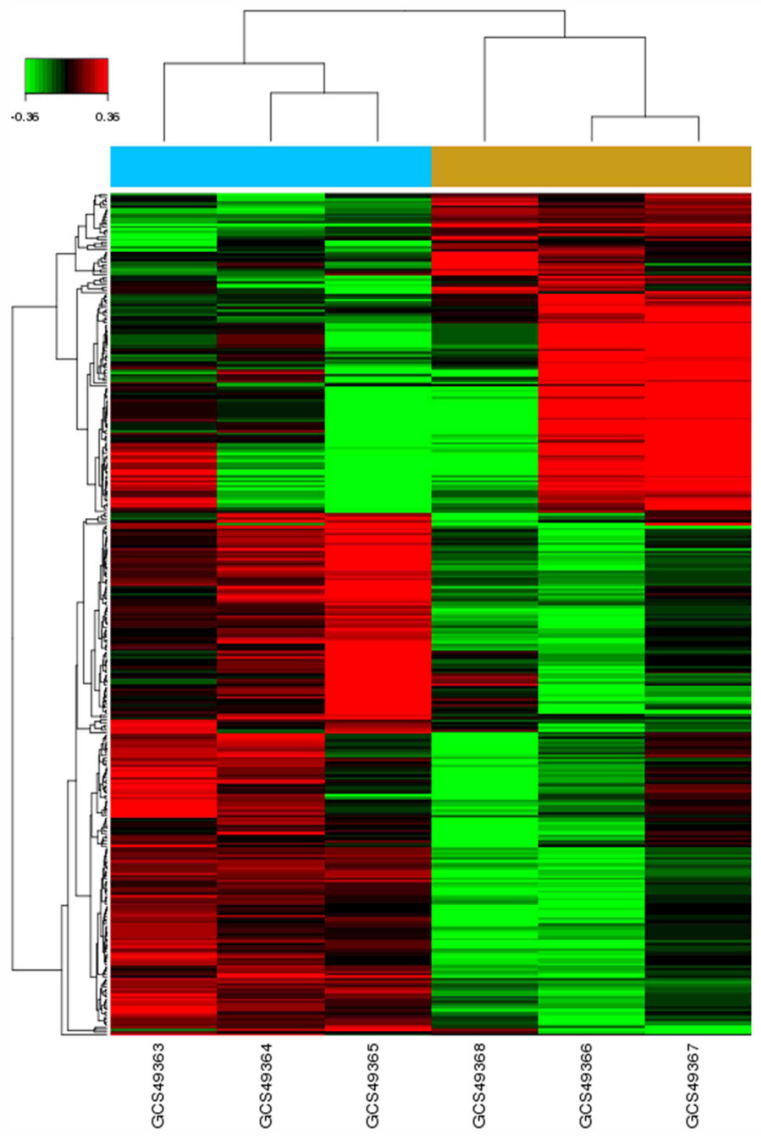

Figure 1. Hierarchical clustering analysis shows distinct mRNA expression profiles between KIAA1456-overexpressing ovarian cancer cells and control cells. Red represents up-regulation, and green represents down-regulation.

KIAA1456 and control cells as described above. RNA was reverse-transcribed into cDNA using a Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR analyses were performed using Power SYBR Green (Takara, Dalian, China). The results were normalized to the expression level of GAPDH. All reactions were performed in triplicate. Differences in gene expression levels between groups were compared using Student's t-test. A p value < 0.05 was considered to indicate a significant difference. Primers were synthesized by Shanghai Sangon Biological Engineering Technology.

Validation of differentially expressed genes by Western blotting

Cellular protein lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes using a standard protocol. The membranes were blocked with 5% nonfat milk in PBST at room temperature for 1 h and incubated with the indicated primary antibodies (mouse polyclonal antibodies against KIAA1456 and GAPDH and rabbit polyclonal antibodies against IQGAP1, TRIM29, UBE-4A and SMARCA1, 1:500 dilution) at 4°C overnight. The membranes were then incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at 37°C. Protein bands were detected using an enhanced chemiluminescence (ECL) system followed by exposure to X-ray film. GAPDH was used as a loading control. Digital images were quantified using Quantity-One software (Bio-Rad, USA). All experiments were repeated three times [1, 18].

Statistical analysis

All experiments were performed at least three times, and the results are shown as the means ± SD. A paired t-test was used for statistical analyses between two groups using SPSS 22.0 software. A p value < 0.05 was considered to indicate a statistically significant difference.

Results

Microarray data analysis

To elucidate the mechanism by which KIAA1456 inhibits ovarian cancer cell proliferation, migration and invasion, Affymetrix GeneChip Human Transcriptome Array 2.0 was used to detect differentially expressed genes between the two

Table 1. The most highly differentially expressed mRNAs between KIAA1456-overexpressing and control ovarian cancer cells

Gene Symbol	Accession Number	Gene Description	Fold Change	*P*-value	Gene Feature
IQGAP1	NM_003870	"Homo sapiens IQ motif containing GTPase activating protein 1 (IQGAP1), mRNA".	1.702313	0.000584	Up
UBE4A	NM_001204077	"Homo sapiens ubiquitination factor E4A (UBE4A), transcript variant 2, mRNA".	1.419223	0.00054	Up
PSMD1	NM_001191037	"Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 1 (PSMD1), transcript variant 2, mRNA".	1.365103	0.000545	Up
KIAA1456	NM_001099677	"Homo sapiens KIAA1456 (KIAA1456), transcript variant 2, mRNA".	1.314254	0.000115	Up
SMARCA1	NM_003069	"Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 (SMARCA1), transcript variant 1, mRNA".	1.286039	0.000653	Up
TRIM29	NM_012101	"Homo sapiens tripartite motif containing 29 (TRIM29), mRNA".	1.222872	0.000775	Up
CYP1A1	NM_000499	"Homo sapiens cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), mRNA".	-2.26197	0.013938	Down
IGHD2-21	ENST00000390572	Cdna: known chromosome: GRCh37: 14: 106354409: 106354436: -1 gene: ENSG00000211912 gene_biotype: IG_D_ gene transcript_biotype: IG_D_gene	-1.5694	0.026841	Down
IER3	NM_003897	"Homo sapiens immediate early response 3 (IER3), mRNA".	-1.50082	0.020279	Down
HMOX1	NM_002133	"Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA".	-1.47563	0.003978	Down

groups of cells. Hierarchical clustering analysis revealed distinct mRNA expression profiles between the experimental and control groups (**Figure 1**). Compared to the control group, 336 differentially expressed mRNAs were identified in the experimental group (fold change in expression ≥ 2.0, $P < 0.05$), including 204 up-regulated genes (e.g., TRIM29, UBE4A IQGAP1, SMARCA1) and 132 down-regulated genes (e.g., DMKA, VAV1, SSX5) (**Table 1**).

GO and pathway analyses

GO and pathway analyses were performed to analyze the main functions and important pathways related to the genes found to be differentially expressed. Highly enriched GO terms included gene expression, RNA metabolic process, cell cycle, and cell proliferation (**Table 2**). Based on the most recent version of the KEGG database, 42 signaling pathways, including RNA transport, proteoglycans in cancer, and pathways in cancer, are related to the genes observed as being differentially expressed (**Table 3**). Most of these GO functional and pathway terms are associated with cancer processes.

Construction of the mRNA and lncRNA co-expression network

According to previous studies, there is only limited understanding of the functions of KIAA1456. However, construction of a regulatory network of mRNAs and lncRNAs could illustrate potential connections between mRNAs and lncRNAs, potentially revealing the functions of KIAA1456 via this alternative approach. The co-expression network showed that KIAA1456, TRIM29, UBE4A and SMARCA1 are associated with each other, which may constitute a KIAA1456-SMARCA1-UBE4A-TRIM29 signaling pathway. Nonetheless, the existence of this pathway needs to be confirmed (**Figure 2**).

Quantitative real-time PCR measurement of IQGAP1, TRIM29, UBE4A and SMARCA1 mRNA expression

Based on the results of microarray and bioinformatic analyses of differentially expressed mRNAs, we selected IQGAP1, TRIM29, UBE4A and SMARCA1 for further verification. Quan-

Table 2. The top thirteen enriched Gene Ontology (GO) functional terms enriched among differentially expressed genes

GO Term	Diff Gene Counts in GO	Enrichment Score	*P*-value	FDR	Gene Symbols
Gene expression	35	7.250869	1.77E-19	2.19E-16	|TNPO1|PSMB7|EIF2S3|...
Small molecule metabolic process	39	3.959745	6.83E-13	4.24E-10	|PSMD1|ALAS1|IQGAP1|...
Extracellular matrix organization	15	9.884858	9.68E-11	4.01E-08	|MFAP5|FBN1|EFEMP1|...
RNA metabolic process	15	7.953334	2.01E-09	6.24E-07	|XRN2|PDCD4|PSMB1|...
S phase of mitotic cell cycle	11	12.17815	4.21E-09	1.05E-06	|PSMD1|RAD21|PSMB1|...
Viral reproduction	16	6.474293	1.07E-08	2.22E-06	|NUP205|PSMD1|SUPT16H|...
Blood coagulation	18	5.356955	2.26E-08	3.84E-06	|ITGAV|FN1|PLAUR|...
Mitotic cell cycle	16	6.099747	2.47E-08	3.84E-06	|PSMB5|PSMD1|PSMB1|...
Mitotic anaphase	11	9.282123	7.26E-08	9.75E-06	|PSMB5|PSMD5|CENPF|...
Virus-host interaction	14	6.270007	1.45E-07	1.64E-05	|POLA1|TFRC|UBE3A|...

Table 3. KEGG pathway analysis of differentially expressed genes

Pathway	Diff Gene Counts in Pathway	Enrichment Score	*P*-value	FDR	Gene Symbols
RNA transport	16	13.41944	1.95E-13	3.23E-11	|EIF2B2|EIF3A|GEMIN5|...
Protein processing in endoplasmic reticulum	12	9.944049	7.95E-09	6.56E-07	|SEC24A|CANX|UBE2G1|...
Lysosome	9	10.20895	5.72E-07	3.15E-05	|CTSL1|AP3M1|DNASE2|...
Proteoglycans in cancer	11	6.706027	1.91E-06	7.89E-05	|ITGAV|FN1|IQGAP1|...
Pathways in cancer	12	5.078459	1.11E-05	0.000366	|MET|HSP90AA1|ITGAV|...
Proteasome	5	15.72591	3.29E-05	0.000905	|PSMB7|PSME2|PSMD1|...
Antigen processing and presentation	6	10.12595	6.00E-05	0.001413	|HSPA4|CTSB|HSP90AA1|...
Purine metabolism	7	5.599515	0.000564	0.011625	|PRPS2|POLR2B|PNP|...
Ubiquitin mediated proteolysis	6	6.01687	0.00105	0.01575	|UBE4A|HERC3|UBE3A|...
Metabolic pathways	20	2.327805	0.001258	0.0173	|AMD1|PNP|EPRS|...

titative reverse transcription PCR (RT-PCR) was used to confirm mRNA expression of these genes, all of which were statistically significantly increased ($P < 0.05$) in KIAA1456-overexpressing cells compared to control cells. High consistency was observed between the quantitative RT-PCR results and microarray data (**Figure 3A**).

Confirmation of the protein levels of IQGAP1, TRIM29, UBE4A and SMARCA1 by Western blotting

We next performed Western blot analysis to examine the levels of protein expression in the two groups of cells. The expression levels of IQGAP1, TRIM29, UBE4A and SMARCA1 were significantly elevated in KIAA1456-overexpressing cells compared to control cells ($P < 0.05$). The Western blot results were largely consistent with the microarray data (**Figure 3B**).

Discussion

Ovarian cancer is one of the most common and aggressive tumor types in humans. The molecular mechanisms of ovarian cancer have been extensively studied to date. In our previous study, we found and confirmed that KIAA1456 inhibits ovarian cancer cell proliferation, invasion and metastasis. However, the mechanisms underlying these activities of KIAA1456 have remained unclear. Gene chip methods have the advantage of generating massive amounts of information, and in the present study, we applied bioinformatic analysis to predict the potential mRNA targets of KIAA1456 in ovarian cancer cells. Our results suggested that 204 mRNAs are up-regulated and 132 mRNAs down-regulated in KIAA1456-overexpressing HO8910/PM cells compared to control cells. Based on hierarchical clustering, GO and pathway analyses and mRNA-lncRNA network construction, we identified that IQGAP1,

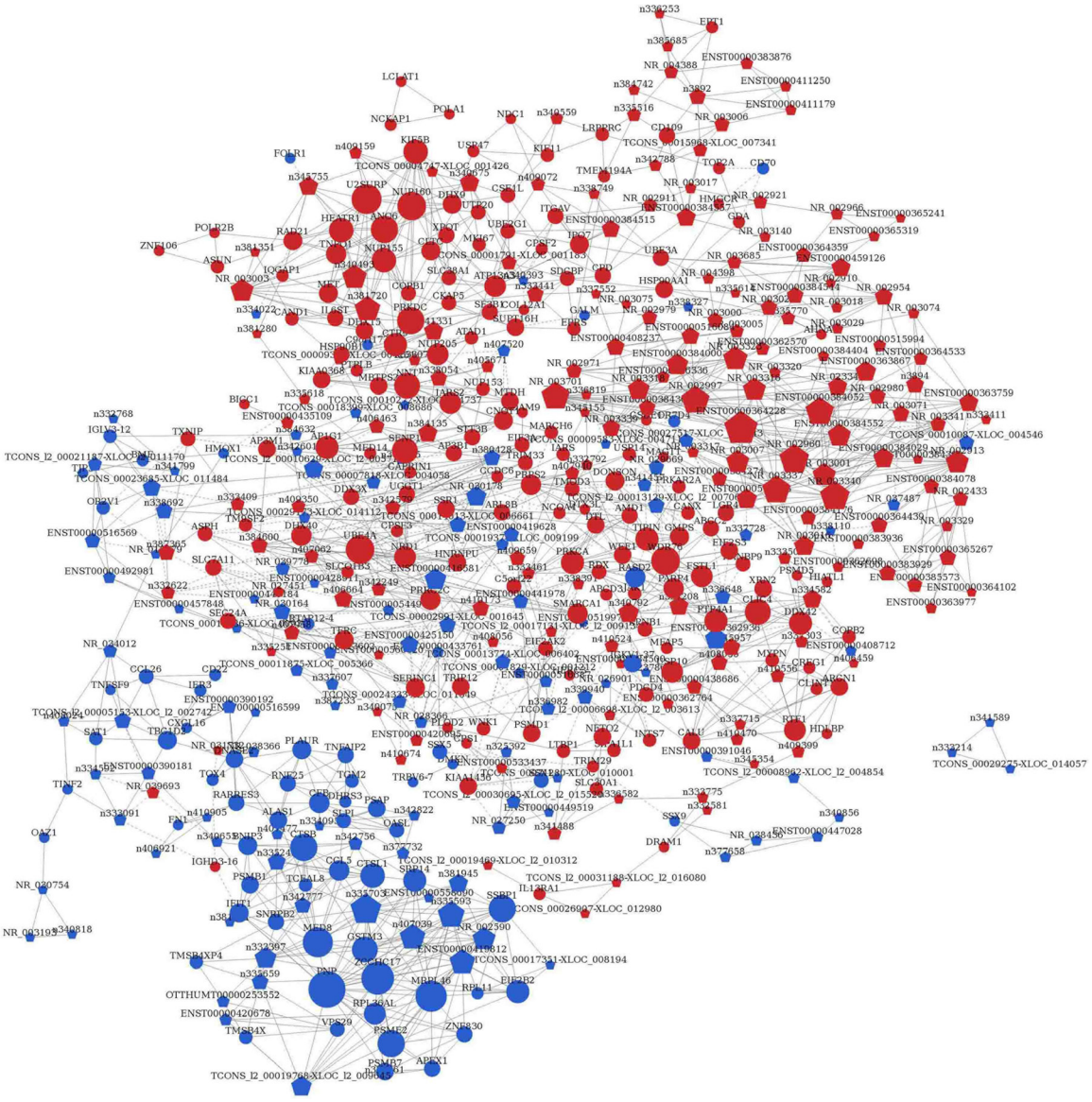

Figure 2. The mRNA-lncRNA association network was constructed according to interactions between mRNAs and lncRNAs. Circles represent target genes, and pentagons represent lncRNAs. Red represents up-regulation, and blue represents down-regulation. Solid lines indicate positive correlations, and dotted lines indicate negative correlations. The larger the area of the circle or pentagon, the greater the importance of the mRNA or lncRNA, respectively.

TRIM29, UBE4A and SMARCA1 play a central role in the mechanism by which KIAA1456 inhibits ovarian cancer cell proliferation, invasion and metastasis. Most of the identified GO functional and pathway categories are related to RNA transport and cancer processes. By applying quantitative RT-PCR and Western blotting, we confirmed that expression levels of QGAP1, TRIM29, UBE4A and SMARCA1 are increased in KIAA1456-overexpressing HO8-910/PM cells compared to control cells.

IQ-domain GTPase-activating protein (IQGAP)1, a member of the IQGAP family, is a scaffold protein that regulates distinct cellular processes, including cell adhesion, cell migration, extracellular signaling, and tumor progression, by interacting with numerous proteins [19, 20]. Abnormal expression of IQGAP1 is widely observed in many cancer types. IQGAP1 overexpression and interactions with β-catenin contribute to HCC progression by promoting cell proliferation and migration. IQGAP1 also has a

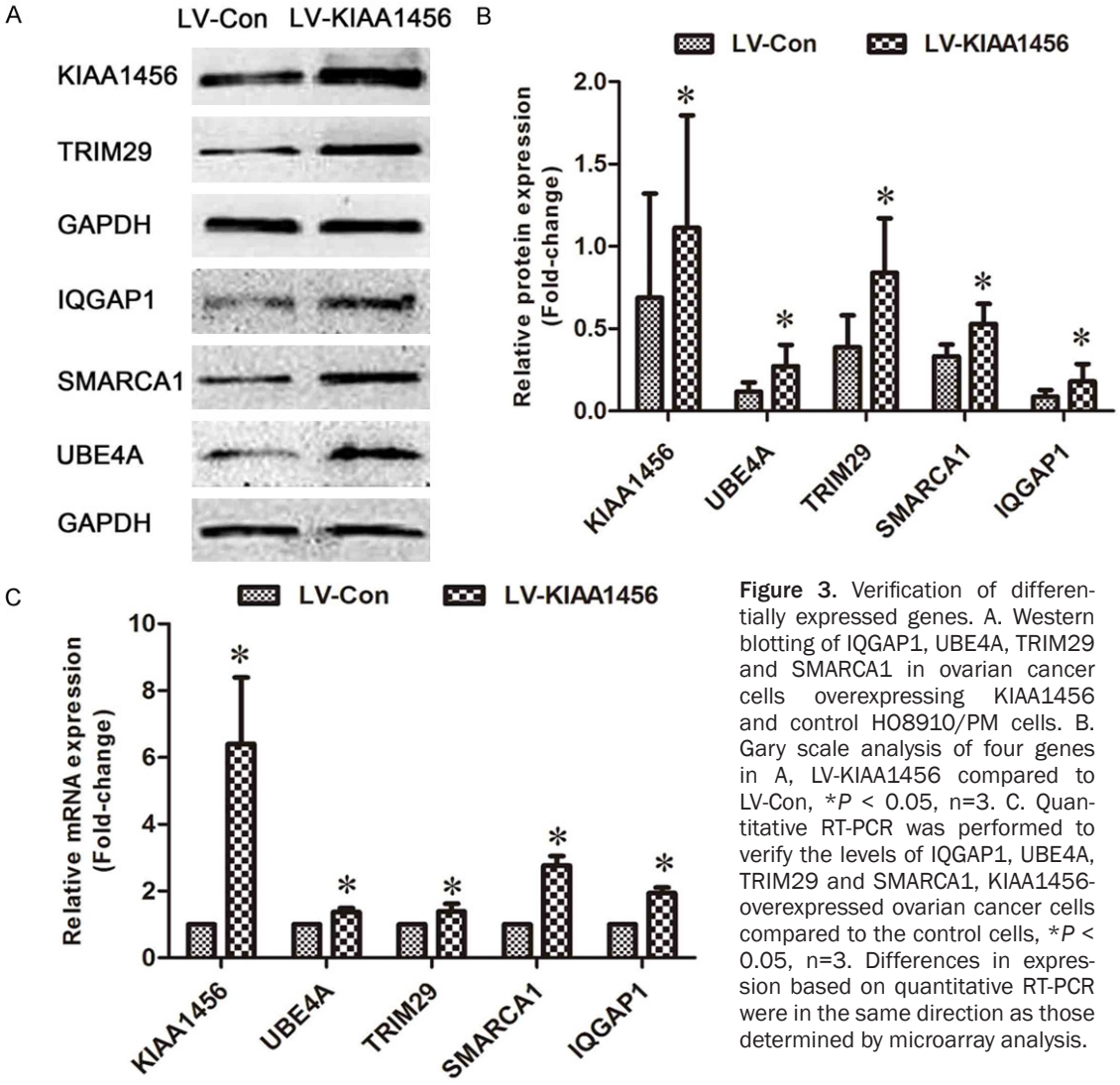

Figure 3. Verification of differentially expressed genes. A. Western blotting of IQGAP1, UBE4A, TRIM29 and SMARCA1 in ovarian cancer cells overexpressing KIAA1456 and control HO8910/PM cells. B. Gary scale analysis of four genes in A, LV-KIAA1456 compared to LV-Con, $*P < 0.05$, n=3. C. Quantitative RT-PCR was performed to verify the levels of IQGAP1, UBE4A, TRIM29 and SMARCA1, KIAA1456-overexpressed ovarian cancer cells compared to the control cells, $*P < 0.05$, n=3. Differences in expression based on quantitative RT-PCR were in the same direction as those determined by microarray analysis.

strong signaling relationship with Ras genes in the setting of HCC induction [21, 22], and IQGAP3 promotes EGFR-ERK signaling and the growth and metastasis of lung cancer cells [23]. Furthermore, IQGAP1 is involved in the enhanced aggressiveness of epithelial ovarian cancer stem cell-like cells during differentiation [24]. Our study showed that KIAA1456 inhibits ovarian cancer cell proliferation, invasion and metastasis by regulating IQGAP1, and GO and pathway analyses identified that IQGAP1 regulates the small molecule metabolic process and participates in the "proteoglycans in cancer" pathway.

The ubiquitination factor E4A (UBE4A) gene encodes a U-box-type ubiquitin ligase originally described as an E4 ubiquitination factor. UBE4A, which has been mapped to the 11q23.3 critical region, is a mammalian homolog of *Saccharomyces cerevisiae* Ufd2 [25]. In addition to ubiquitination, UBE4A expression in different tissues might play a specific role in various biochemical processes, including growth and differentiation. For instance, UBE4A contributes to neuroblastoma [26], and UBE4A expression is clearly enhanced in ovarian cancer [27]. UBE4A was also recently reported to constitute a new serological biomarker of inflammatory bowel disease [28]. In eukaryotes, protein ubiquitination is a key biochemical mechanism involved in multiple cellular processes, ranging from its main role in the control of protein quality and protein levels to regulation of gene expression [29]. In our study, KIAA1456 inhibited ovarian cancer cell prolif-

eration, invasion and metastasis by up-regulating UBE4A. Nonetheless, further research is needed to explore the specific pathway involved in these effects of KIAA1456.

Tripartite motif-containing (TRIM) 29, also known as ataxia-telangiectasia group D-associated protein (ATDC), is a member of the TRIM protein family, which is composed of multi-domain ubiquitin E3 ligases with a characteristic N-terminal tripartite motif (RING, B-box, and coiled coil domains). The TRIM family of proteins has been implicated in a variety of physiological processes, such as development, oncogenesis, apoptosis and antiviral defense [30-32]. There is increasing evidence that TRIM29 may function as an oncogene or a tumor suppressor depending on the type of tumor. For example, TRIM29 functions as an oncogene in gastric cancer and is regulated by miR-185 [33], and it could be useful as an auxiliary target of prostate-specific antigen (PSA) for early diagnosis of prostate cancer [34]. TRIM29 is highly expressed in pancreatic ductal adenocarcinoma and plays a critical role in DNA damage signaling and radioresistance in pancreatic cancer [35]. Furthermore, TRIM29 regulates the p63 pathway and the behavior of cervical cancer cells [36] and acts as a tumor suppressor in breast cancer by inhibiting TWIST1 and suppressing the epithelial-mesenchymal transition (EMT) [37]. Another study found that abnormal expression of ATDC might promote ovarian carcinoma invasion and metastasis [38]. TRIM29 is located at chromosome 11q23.3, the same region as UBE4A, and both proteins act as ubiquitin ligases. Taken together, our results show that TRIM29 and UBE4A participate in similar regulatory pathways and play vital roles in KIAA1456-induced inhibition of ovarian cancer cell proliferation, invasion and metastasis.

The role of the switch/sucrose nonfermenting (SWI/SNF) complex in chromatin remodeling has been the focus of several recent seminal studies describing the surprisingly high frequency of mutations in different subunits of this crucially important complex [39]. Modification of chromatin structure is an important regulatory mechanism for many processes such as DNA replication, transcription and repair [40-42], and emerging evidence reveals the importance of the SWI/SNF complex in the initiation and progression of cancer. For example, SMARCA5 overexpression was observed in human breast cancer cases and correlated with poor prognosis [43]. Additionally, a novel variant of endometrial carcinoma involved a predominant SMARCB1-positive but SMARCA4-deficient undifferentiated rhabdoid tumor component [39]. SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 1 (SMARCA1), also known as SNF2L, encodes an ATP-dependent chromatin remodeling protein of the SWI/SNF family [44, 45]. Although the current understanding of SMARCA1, especially with regard to cancer, is limited, our study identifies SMARCA1 as a tumor inhibitor regulated by KIAA1456 in ovarian cancer. However, further study is needed to reveal the specific mechanism underlying this effect of KIAA1456.

In summary, our microarray-based bioinformatic analysis provides new insight into the mechanism of ovarian cancer. Our findings show that KIAA1456 inhibits ovarian cancer cell proliferation, invasion and metastasis by up-regulating TRIM29, UBE4A, IQGAP1 and SMARCA1. The main signaling pathways enriched among these differentially expressed genes include RNA transport, proteoglycans in cancer, and pathways in cancer. Moreover, our co-expression network suggests that KIAA1456-SMARCA1-UBE4A-TRIM29 is a potential signaling pathway for KIAA1456. These factors may be useful biomarkers for predicting tumor metastasis and valuable therapeutic targets for the treatment of ovarian cancer in the clinical setting. However, further studies are needed to reveal their functions and interactions in ovarian cancer.

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Disclosure of conflict of interest

None.

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