Genome-wide ChIP-seq data with a transcriptome analysis reveals the groups of genes regulated by histone demethylase LSD1 inhibition in esophageal squamous cell carcinoma cells

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Abstract. Expression of genes is controlled by histone modification, histone acetylation and methylation, but abnormalities of these modifications have been observed in carcinogenesis and cancer development. The effect of the lysine-specific histone demethylase 1 (LSD1) inhibitor, a demethylating enzyme of histones, is thought to be caused by controlling the expression of genes. The aim of the present study is to elucidate the efficacies of the LSD1 inhibitor on the gene expression of esophageal cancer cell lines using chromatin immunoprecipitation (ChIP)-Seq. A comprehensive analysis of gene expression changes in esophageal squamous cell carcinoma (ESCC) cell lines induced by the LSD1 inhibitor NCL1 was clarified via analysis using microarray. In addition, ChIP-seq analysis was conducted using a SimpleChIP plus Enzymatic Chromatin IP kit. NCL1 strongly suppressed the proliferation of T.Tn and TE2 cells, which are ESCC cell lines, and further induced apoptosis. According to the combinatory analysis of ChIP-seq and microarray, 17 genes were upregulated, and 16 genes were downregulated in both cell lines. The comprehensive gene expression study performed in the present study is considered to be useful for analyzing the mechanism of the antitumor effect of the LSD1 inhibitor in patients with ESCC.

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Introduction

In the whole world, esophageal cancer is the sixth cause of death among various cancer types. Esophageal cancers are mainly classified into two histological types, esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (1). Then, ESCC is thought to be the main histological type which accounts for more than 90% in Asian countries including Japan, Korea and China (2), and it is known that ESCC is a highly malignant malignancy among many cancer types (3-5). Esophagectomy remains the mainstay potential curative treatment for ESCC (6). However, esophagectomy is still a highly invasive surgical procedure with high morbidity and mortality (7). Although remarkable advances have been made in chemotherapy and chemotherapy with radiotherapy as cancer therapies, These therapeutic effects can be said to be extremely limited as curative treatment (8,9). Therefore, understanding the characteristics of ESCC and developing new therapeutic tools are urgently required.

Both genetic mechanisms and epigenetic alterations are thought to be closely involved in the development and progression of ESCC (10). Several epigenetic abnormalities have been reported, including DNA methylation, histone modifications and non-coding RNAs (11,12). In our studies, epigenetic modifications play crucial roles in the regulation of gene expression in ESCC (12-19). In particular, the methylation of lysine residues on histone proteins in the chromatin structure has received attention due to their potential regulatory ability on DNA-based nuclear processes such as transcription, replication and repair (20). The methylation of histone lysine residues was first reported in the 1960s and was considered an irreversible posttranslational modification (21). In 2004, however, a lysine demethylase was discovered, and the methylation of histone lysine residues is now regarded as a dynamic modulation (22).

Abnormalities in histone lysine methylation are frequently observed in various cancers (23-26). Lysine-specific histone demethylase 1 (LSD 1), a histone demethylase, is an amine oxidase that removes monomethyl and dimethyl moieties from Lys 4 of histone H 3 and produces a demethylated H3 tail (27). Identifying the key points of regulation in the histone methylation network for cancer development and progression can provide innovative targets for cancer therapies.

In the present study, we focused on the mechanisms underlying how demethylated Lys4 of H3 influences the gene expressions in ESCC cells. We investigated microarray and chromatin immunoprecipitation sequencing (ChIP-seq) in order to explore the effect of demethylated Lys4 of H3 on the transcriptional state of ESCC cells and identified genes affecting cancer growth.

Materials and methods

Cell culture and chemicals. The human esophageal cell lines T.Tn and TE2 were cultured in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum. T.Tn cells were acquired from the Japanese Cancer Research Resources Bank (Tsukuba, Japan), and TE2 cells were obtained from Tohoku University (Sendai, Japan). NCL1, an LSD1 inhibitor, was provided by Kyoto Prefectural University of Medical Science (Graduate School of Medicine) (Kyoto, Japan) in cooperation. NCL1 showed higher inhibitory activity than the known LSD1 inhibitor, trans-2-phenylcyclopropylamine. Moreover, in the presence of NCL1, the methylation activity of H3K4 is observed and cell proliferation is inhibited in experiments using cancer cells (28-30). NCL1 was dissolved in dimethyl sulfoxide and used for *in vitro* studies.

Messenger RNA preparation and a cDNA microarray analysis. T.Tn or TE2 cells were seeded into a 225-cm^2 flask, incubated for 48 h, treated with or without an IC_{80} concentration of LSD1 inhibitor and harvested at 24 h. Subsequently, the cells were washed with phosphate-buffered saline (PBS; cat. no: 14190-250, Invitrogen, Carlsbad, CA, USA) and total RNA was extracted using RNeasy Plus Mini kit (Qiagen, Inc., Chatsworth, CA, USA). Changes in gene expression were compared between 5.5 tor of total RNA extracted from cells cultured by exposure to NCL 1 and 5.5 tor of total RNA extracted from cells cultured in a control culture using an Affymetrix Human Exon 1.0ST array (Affymetrix, Santa Clara, CA, USA). Hybridization signals were detected with a GeneChip scanner 3000 7 G (Affymetrix), and the scanned images were analyzed using the GeneChip command console software (AGCC). All the processes were basically carried out according to the previous report (31). All experiments were done in duplicate and the averaged data were subjected to statistical analysis.

ChIP-seq analyses. ChIP-seq analyses were performed using the SimpleChIP plus enzymatic chromatin IP kit (Magnetic Beads; Cell Signaling Technology, Danvers, MA, USA). T.Tn or TE2 cells were cultured for 48 h in a 225-cm^2 flask, then incubated under the condition with or without an IC_{80} concentration of LSD1 inhibitor and harvested at 24 h. The Cells were crosslinked with 1% formaldehyde for 10 min at room temperature, then washed twice with PBS containing 0.5 mM EDTA and collected. The cell pellet was lysed with 0.3 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS, and protease inhibitor) and incubated on ice for 10 min. Lysates of the cells were sonicated to obtain DNA fragments of 150 to 900 base pair (bp) in size. About 50 μg of cross-linked sheared chromatin solution was then used for immunoprecipitation. The solution with the Anti-Histone H3 (di methyl K4) antibody-ChIP Grade (Abcam, Inc., Cambridge, UK; cat. no: ab7766) was incubated overnight at 4˚C on a rotating shaker for immunoprecipitation. Magnetic beads were added to the solution, incubated at 4˚C for 1 h, and then washed with washing buffer. The cross-linking was reversed by adding NaCl at a final concentration of 200 mM and heating at 65˚C for 30 min. The DNA fragments were purified using a spin column. A sequencing library was prepared and massively parallel high throughput sequencing was performed with the Illumina HiSeq 2000 system (Illumina, Inc., San Diego, Calif., USA) and a 50-bp reads were aligned against the reference genome on a Burrows-Wheeler transform, and a minimum mapping quality filter 20 was applied (32). Enriched regions for each condition were detected and analyzed with MACS v1.4.0 (model-based analysis for ChIP-Seq) (33) and CEAS v1.0.2 (cis-regulatory element annotation system) (34,35). Peaks with overlaps in both cell lines were merged into a broad peak domain using BEDTools (36). All of the count data from the ChIP-Seq assays were analyzed with DESeq to normalize the peak signal (37).

The reverse transcription-quantitative PCR (RT-qPCR) for measuring the LDHB and AEG-1/MTDH mRNA expression. The mRNA expression of DUSP5, BHLHE40 and MXRA5 were examined by a RT-qPCR. T.Tn or TE2 cells were seeded into a 225-cm^2 flask, incubated for 48 h, treated with or without an IC_{80} concentration of LSD1 inhibitor and harvested at 24 h. Subsequently, the cells were washed with phosphate-buffered saline (PBS) and total RNA was extracted using an RNeasy Plus Mini kit (Qiagen, Inc., Chatsworth, CA, USA). The cDNA templates for the qPCR were synthesized from 1 μg of total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems).The Actin alpha 1 (ACTA1) gene served as an internal control. The PCR reaction consisted of Sso Fast Eva Green Supermix (BioRad; containing dNTPs, Sso7d fusion polymerase, MgCl2 , EvaGreen dye, stabilizers), the primers (each 1 μM) and cDNA. All reactions were run in duplicate on the MyiQ2 Two-Color Real-Time PCR detection system (BioRad). The PCR processes were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 55˚C for 10 sec. The following primer sequences were used: DUSP5; 5'-CCTGCTAAAACT GGGATGGA-3' and 5'-ACCTACCCTGAGGTCCGTCT-3': BHLHE40; 5'-GGCATAGCACGGTAGTGGTT-3' and 5'-TCA GACCTTGGTTTGGTTCC-3': MXRA5; 5'-CTGTCCAGT CCTCAGGAAGC-3' and 5'-TCCTGTGGAAACCTTTGT CC-3': ACTA1; 5'-CCTTCATCGGTATGGAGTC-3' and 5'-GTTGGCATACAGGTCCTT-3'.

The comparative quantitative cycle (C_q) method was applied to quantify the expression levels of mRNAs. The relative amount of DUSP5, BHLHE40 and MXRA5 to ACTA1mRNA was calculated using the following equation: $2_q^{-\Delta C}$, where $\Delta C_q=(C_{q\ \text{DUSP5, BHLHE40 or MXRA5 or AEG-1/MTDH-}}C_{q\ \text{ACTA1}})$.

Table I. List of genes up- or downregulated (>2-fold change) in T.Tn and TE2 cells.

A, Upregulated (>2-fold changes)

Genbank accession no.	Gene symbol	Maximum fold change		Gene description
		T.Tn	TE2	
BC005008	*CEACAM6*	2.84	3.62	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)
BC012172	*ACSS2*	1.10	1.24	acyl-CoA synthetase short-chain family member 2
BC008723	*ASNS*	1.92	1.96	asparagine synthetase (glutamine-hydrolyzing)
L19501	*CBS*	1.15	1.18	cystathionine-β-synthase
M29540	*CEACAM5*	2.45	2.90	carcinoembryonic antigen-related cell adhesion molecule 5
BC019625	*CHAC1*	1.34	2.11	ChaC, cation transport regulator homolog 1 (E. coli)
U03688	*CYP1B1*	1.49	1.67	cytochrome P450, family 1, subfamily B, polypeptide 1
BC007333	*ETV5*	1.73	1.81	ets variant 5
AF110400	*FGF19*	1.17	1.26	fibroblast growth factor 19
EF152283	*GCNT3*	1.17	1.66	glucosaminyl (N-acetyl) transferase 3, mucin type
AF019770	*GDF15*	2.01	1.75	growth differentiation factor 15
BC033089	*LCN2*	1.62	2.78	lipocalin 2
BC004863	*PSAT1*	1.34	2.87	phosphoserine aminotransferase 1
AF539739	*S100P*	1.26	2.06	S100 calcium binding protein P
AF097514	*SCD*	1.25	2.38	stearoyl-CoA desaturase (Δ-9-desaturase)
BC000658	*STC2*	1.95	1.59	stanniocalcin 2
BC011703	*TMPRSS4*	1.03	1.24	transmembrane protease, serine 4
AF022375	*VEGFA*	1.62	1.50	vascular endothelial growth factor A

B, Downregulated (>2-fold changes)

Genbank accession no.	Gene symbol	Maximum fold change		Gene description
		T.Tn	TE2	
J04948	*ALPPL2*	-1.01	-1.69	alkaline phosphatase, placental-like 2
BC098561	*EFEMP1*	-1.59	-1.76	EGF-containing fibulin-like extracellular matrix protein 1
AB031548	*GPR87*	-1.11	-1.06	G protein-coupled receptor 87
M19154	*TGFB2*	-1.72	-1.11	transforming growth factor, β2
BC142678	*PHLDB2*	-1.14	-1.04	pleckstrin homology-like domain, family B, member 2
BC146868	*COL12A1*	-1.13	-1.22	collagen, type XII, α1

Table I. Continued.

B, Downregulated (>2-fold changes)

Genbank accession no.	Gene symbol	Maximum fold change		Gene description
		T.Tn	TE2	
BC017782	*WISP2*	-1.12	-1.31	WNT1 inducible signaling pathway protein 2
AF098807	*LHFP*	-1.21	-1.26	lipoma HMGIC fusion partner
AK123348	*C3orf57*	-1.14	-1.72	chromosome 3 open reading frame 57

Table II. List of numbers of genes up- or down-regulated in the chromatin immunoprecipitation-seq analysis.

Cell line	Upregulated peaks, n	Downregulated peaks, n
T.Tn	468	532
TE2	814	612

Statistical analyses. The Student's t-test was performed to compare the differences in the mRNA expression levels. $P<0.05$ was considered to indicate a statistically significant difference. The SPSS v.16.0 (SPSS, Inc., Chicago, IL, USA) software program were used for the analyses.

Results

Effects of NCL1 on the expression of various genes in microarray analyses. We used a cDNA microarray to identify genes induced by LSD1 exposure in ESCC. We extracted genes with expression levels more than two-fold or greater compared to control, whether decreased or increased, as significant. In both T. Tn and TE2 cell lines, expression of 18 genes was increased, while expression of 9 genes was decreased (Table I).

ChIP-seq analyses. To assess the functional significance of demethylated Lys4 of H3 in ESCC cells, we also analyzed the genome-wide modified targets of demethylation Lys4 of H3 using deep sequencing based on chromatin immunoprecipitation (ChIP-seq). When we compared the findings with control cells (without LSD1 inhibitor), we identified up-regulated peaks in 468 and 814 demethylated Lys4 of H3-specific modification sites in T.Tn and TE2 cells, respectively (Fig. 1). We also identified down-regulated peaks in 532 and 612 demethylated Lys4 of H3-specific modification sites in T.Tn and TE2 cells, respectively (Fig. 1).

Identifying the relationship between histone modification states and the gene expression in ESCC cells. To clarify the gene expression change by the state of histone modification, the genes with up- or down-regulated expression were investigated using microarray data, and that the promoter region of these genes may be the targets of histone modification. The expression of some of these genes whose promoters were detected as candidates for targets of demethylated Lys4 of H3 were markedly changed according to the microarray data (Table II). The results showed that 17 genes were commonly up-regulated, while 16 genes were commonly down-regulated (Table III). These identified genes were categorized based on their function, referring to GENE ONTOLOGY™, and classified into 7 groups: Apoptosis, cell cycles, defense and immunity, metabolism, signal transduction and transcription, structural protein, and unclassified. The frequencies of these functionally classified genes in each cluster are shown in Table IV.

Validation of the gene expression changes induced by NCL1. Among the mRNAs that showed altered expression levels in both microRNA and ChIP-seq experiments, changes in the

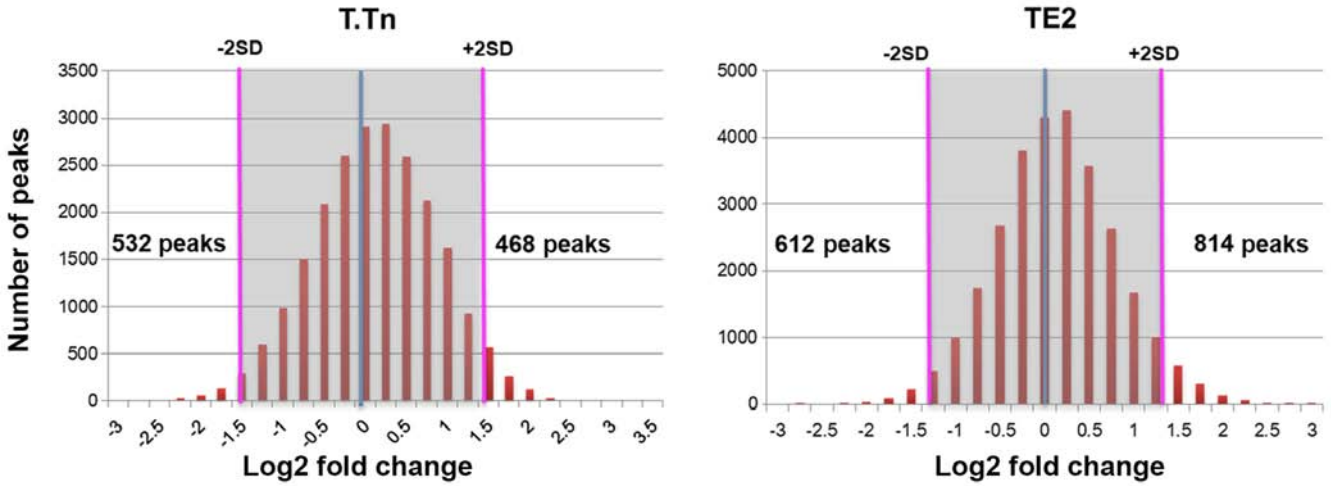

Figure 1. Extraction peaks with a log2 fold change in each cell line. For both cell lines, peaks outside the ± 2 SD range were extracted. SD, standard deviation.

expression levels of DUSP5, BHLHE40 and MXRA5 were confirmed by a RT-qPCR (Fig. 2). As expected, the expression of DUSP5 and BHLHE40 increased and the expression of MXRA5 decreased.

Discussion

In this study, we tried to clarify the changes in the gene expression due to histone demethylase LSD1 inhibitor using a microarray and ChIP-Seq analyses. Some LSD1 inhibitors have shown potent anti-cancer effects, and their pharmacological mechanisms have been elucidated (38,39). ORY-1001 is an LSD1 inhibitor that was shown to selectively inhibit KDM1A in clinical trials and is currently being assessed for its utility in treating patients with leukemia and solid tumors (40). Although clinical trials of LSD1 inhibitors are being conducted around the world, very few describe the mechanisms in detail (41,42).

We have already elucidated the anti-tumor effect of LSD1 inhibitors on ESCC, and this effect was shown to be caused by changes in the gene expression induced by the agent, with PHLDB2 reported to demonstrate a particularly enormous change in expression (19). In the present study, in addition to changes in the gene expression, genome-wide CHIP-Seq analyses were performed, and the histone methylation that occurred was evaluated.

DUSP5 is one of the nuclear localization members of the MKP/DUSP family and it is induced in response to the activation of ERK, specifically dephosphorylated, and has the function of anchoring the ERK in the nucleus (43). Furthermore, DUSP5 has been reported to increase RAF, MEK and ERK activities in the cytoplasm, in addition to its role in ERK nuclear inactivation. This activity has been shown to be caused by alleviation of upstream kinase inhibition and depends on its ability to sequester DUSP5 turnover rate and inactive ERK in the nucleus (44). Also, the expression of BRAFV 600 E oncoprotein, which has mutations in BRAF that are not sensitive to feedback inhibition, changes the function of DUSP 5 to become an inhibitor of the entire cell of ERK, and that the cell avoids hyperactivation and aging of ERK. These analysis results explain that DUSP5 functions as a tumor suppressor or a tumor promoter (45).

BHLHE 40 is an up-regulated gene and is a basic helix-loop-helix type transcription factor that has been shown to be involved in epithelial-mesenchymal transition (EMT). According to Asanoma *et al* (46), BHLHE 40 inhibited tumor cell invasion by suppressing the transcription of the EMT factors SNAI 1, SNAI 2 and TWIST 1. In addition, they showed an association between the transcription factor SP1 and the basal transcriptional activity of TWIST1 and BHLHE40 and competes with SP1 to regulate DNA transcription and control gene transcription. Therefore, BHLHE 40 is thought to function as a tumor suppressor.

It is thought that p53 reactivation and mass apoptosis induction (PRIMA-1), a low-molecular compound, restores the function of mutant TP53 to the function of wild-type TP53 and induces p53-mediated apoptosis (47). PRIMA-1 and its methylated form PRIMA-1 Met (APR-246) are thought to have antitumor effects and its effects are evident in several types of cancers such as osteosarcoma, multiple myeloma, lung cancer, breast cancer and colon cancer (48-52). Furthermore, several clinical trials using APR-246 have been performed, indicating its tolerability and clinical effects in hematologic malignancies and prostate cancer (53). Also in ESCC, Furukawa *et al* (47) reported that PRIMA-1 may restore the function of mutant TP 53 in ESCC with a TP 50 missense mutation, due to the enhanced expression of Noxa. Tissue inhibitor of metalloproteinase-3 (TIMP 3) which is one of the four members of the protein family is initially classified according to their function of inhibiting matrix metalloproteinases (MMP) (54-56). TIMP3 is thought to induce apoptosis in malignant cells, such as melanoma (57) human colon carcinoma (58), cervical carcinoma cells and breast cancer cells (59). The death domain of TIMP3, a region that inhibits the function of MMP, is localized at its N-terminus (60). TIMP3 has been reported in colon cancer cells and melanoma cells to increase susceptibility to apoptosis via stabilization of the TNF-α receptor on the cell surface (58,61). In ESCC, expression of TIMP-3 protein is correlated with depth of tumor infiltration, number of lymph node metastasis and stage of disease as a result of immunohistochemical analysis using clinical specimens (54). TIMP-3 protein was localizes in a shallow region of the tumor, and even in the same tumor, its expression was decreased in the

Table III. List of gene symbols commonly up- or downregulated in both the microarray and ChIP-seq assay.

	Microarray	
ChIP-seq	Upregulated	Downregulated
Upregulated	DUSP5, BHLHE40, TMC5 GNE, PMAIP1, TIMP3 C6orf223, PHLDA1,ERRFI1 MID1IP1, ULBP1, FGF19 GCNT3, HMOX1, TRIB3 VEGFA, CEACAM6	DIO2, RBMS3, LHFP PLK2, CP, TOM1L2 MXRA5, DKK1, EPHA4 EGLN3, CCDC80, MID1 SLC16A7, RAPGEF4, TOX VCL, MAP7D2, RASAL2 HAS2, TNS3, FLNA NEDD4L, KIAA1217, PSAPL1 SEMA3A, GPR126, EGFR
Downregulated	GDF15, CLGN	RHOB, KLHL13, ARID5B DIO2, MXRA5, THBS1 ALDH1A1, DKK1, C1orf116 SOX2, CACNG4, LHFP FGFR2, EPHA4, EFEMP1, PALMD

ChIP-seq, chromatin immunoprecipitation-seq.

Table IV. Categorization of genes regulated by the LSD1 inhibitor based on their functions, referring to GENE ONTOLOGY™, and classified into 7 groups.

	Microarray and ChIP-seq analysis	
Function	Upregulated	Downregulated
Apoptosis	PMAIP1,TIMP3, PHLDA1, FGF19, TRIB3, CEACAM6	RHOB, FGFR2
Cell cycles	HMOX1, VEGFA	KLHL13, THBS1
Defense and immunity	GCNT3	-
Metabolism	MID1IP1	ALDH1A1
Signal transduction and transcription	DUSP5, BHLHE40, GNE, ERRFL1, ULBP1	ARID5B, DKK1, SOX2, EPH!4, EFEMP1
Structural protein	TMC5,	DIO2, MXRA5, C1orf116, CACNG4, LHFP, PALMD
Unclassified	C6orf223	-

ChIP-seq, chromatin immunoprecipitation-seq.

deep part. Furthermore, the prognosis of cancer patients who lost TIMP-3 expression was significantly worse than that of TIMP-3-positive cancer patients.

PHLDA1 is a cell death mediator that induces cells into apoptosis and exerts antiproliferative activity (62-65). The overexpression of PHLDA1 inhibits cell proliferation and induces cell death in various malignant tumors, including breast cancer, melanoma and cervical carcinoma cells (66-68). Low expression of PHLDA1 mRNA and protein is strongly related to breast cancer and melanoma progression (63,64). Conversely, it has been found that in oral squamous cell carcinoma, high expression of PHLDA1 is associated with more advanced stage. Therefore, the function of PHLDA1 is still controversial, but from these results, it is possible that PHLDA1 functions as a tumor suppressor in ESCC.

The genes shown to be down-regulated on either the microarray analysis or ChIP-seq analysis in our study may have a potential oncogenic function in ESCC.

Rho protein belongs to the Ras superfamily and is a small molecule that functions as a binary switch in a wide range of signaling pathways (69,70). Rho proteins are a family of 20 intracellular signaling molecules, including RhoA, RhoB, RhoC, RhoG, RhoE, Rac1, Rac2, Cdc42Hs and TC10 (71). RhoB has the function of molecular switch, and it circulates between inactive GDP-bonded type and active GTP-bound type (72). RhoB was reported as a molecule that induces Ras-induced fibroblast transformation. New evidence suggests a potential role of RhoB in supporting the tumorigenic function. As an example, it is reported that RhoB protein expression is higher in T-acute lymphoblastic

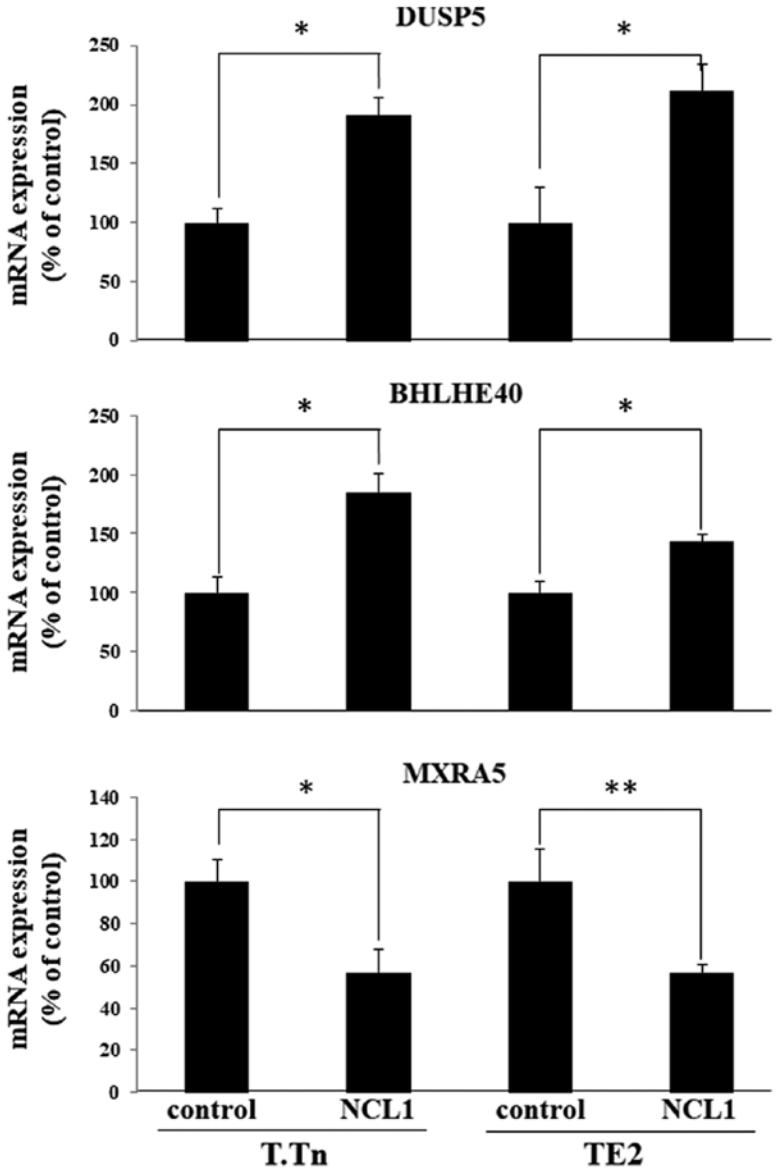

Figure 2. Among the mRNAs that exhibited altered expression levels in both microRNA and chromatin immunoprecipitation-seq experiments, changes in the expression of DUSP5, BHLHE40 and MXRA5 were confirmed by a reverse transcription-quantitative PCR. *P<0.05 and **P<0.01, as indicated. DUSP5, dual specificity phosphatase 5; BHLHE40, basic helix-loop-helix family member E40; MXRA5, matrix remodeling associated 5.

leukemia (T-All) cells compared to normal T cells, and it is shown to be significantly associated with leukemia cells (73). In the present study, the inhibition of RhoB increased cell apoptosis by ≥300%.

Matrix remodeling-associated protein 5 (MXRA5), also known as adhesion protein with leucine-rich repeats and immunoglobulin domains related to perlecan (Adlican), is one of matrix remodeling related proteins (74). The MXRA5 gene encodes a protein with a molecular weight of 312 kDa. The MXRA family contains three genes (MXRA5, MXRA7 and MXRA8), both of which are thought to be involved in cell adhesion and matrix remodeling (75). The increased expression of MXRA5 has been reported in many kinds of tumors, such as colorectal cancer, ovarian cancer and esophageal cancer (76,77). Furthermore, somatic mutation of MXRA5 has been reported, and this mutation has been confirmed in various malignant tumors such as lung, skin, brain, ovary and wall pleura (78).

Among thrombospondin, a family of extracellular matrix proteins, thrombospondin-1 (THBS 1) is the first member identified and its major roles are platelet aggregation, angiogenesis, and tumorigenesis (79). Also in ESCC, THBS 1 can activate the TGF-β signaling pathway, leading to the transcription of Cyr 61 and CTGF (78-80). In addition, its overexpression is thought to be significantly associated with TNM progression (P=0.029) and lymph node metastasis (P=0.026) in clinicopathologic studies. In the analysis of prognosis, it was shown that overexpression of THBS protein is a prognostic predictor in ESCC patients (P=0.042) (80).

The results in this study suggest that the large number of genes affected by demethylation of H3 in ESCC Lys4 may be greatly implicated in the development of ESCC cancer. The correlation between these gene groups and carcinogenesis and progression of ESCC needs to be verified in further studies, but the present results will be helpful for clarifying the mechanism.

The authors declare that they have no competing interests.

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Availability of data and materials

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

Authors' contributions

IH designed, analyzed and conducted all of the experiments and wrote the manuscript. MT, AK and II performed and analyzed the results of the ChIP-seq experiments. YA, KM, YM, HS, NS, TS and HM contributed to the study conception and design, and the acquisition, analysis and interpretation of data. FI and YI contributed to the acquisition, analysis and interpretation of data, drafted the manuscript and revised it critically for important intellectual content. KI performed RT-qPCR experiments. 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.

References

1. Liang H, Fan JH and Qiao YL: Epidemiology, etiology, and prevention of esophageal squamous cell carcinoma in China. Cancer Biol Med 14: 33-41, 2017.
2. Zhang Y: Epidemiology of esophageal cancer. World J Gastroenterol 19: 5598-5606, 2013.
3. Pennathur A, Gibson MK, Jobe BA and Luketich JD: Oesophageal carcinoma. Lancet 381: 400-412, 2013.
4. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
5. Enzinger PC and Mayer RJ: Esophageal cancer. N Engl J Med 349: 2241-2252, 2003.
6. Meng J, Zhang J, Xiu Y, Jin Y, Xiang J, Nie Y, Fu S and Zhao K: Prognostic value of an immunohistochemical signature in patients with esophageal squamous cell carcinoma undergoing radical esophagectomy. Mol Oncol 12: 196-207, 2018.
7. Scheepers JJ, van der Peet DL, Veenhof AA, Heijnen B and Cuesta MA: Systematic approach of postoperative gastric conduit complications after esophageal resection. Dis Esophagus 23: 117-121, 2010.
8. Baba Y, Saeki H, Nakashima Y, Oki E, Shigaki H, Yoshida N, Watanabe M, Maehara Y and Baba H: Review of chemotherapeutic approaches for operable and inoperable esophageal squamous cell carcinoma. Dis Esophagus 30: 1-7, 2017.
9. Smyth EC, Lagergren J, Fitzgerald RC, Lordick F, Shah MA, Lagergren P and Cunningham D: Oesophageal cancer. Nat Rev Dis Primers 3: 17048, 2017.
10. Lin DC, Wang MR and Koeffler HP: Genomic and epigenomic aberrations in esophageal squamous cell carcinoma and implications for patients. Gastroenterology 154: 374-389, 2018.
11. Hamm CA and Costa FF: Epigenomes as therapeutic targets. Pharmacol Ther 151: 72-86, 2015.
12. Hoshino I, Matsubara H, Hanari N, Mori M, Nishimori T, Yoneyama Y, Akutsu Y, Sakata H, Matsushita K, Seki N and Ochiai T: Histone deacetylase inhibitor FK228 activates tumor suppressor Prdx1 with apoptosis induction in esophageal cancer cells. Clin Cancer Res 11: 7945-7952, 2005.
13. Hoshino I, Matsubara H, Akutsu Y, Nishimori T, Yoneyama Y, Murakami K, Komatsu A, Sakata H, Matsushita K and Ochiai T: Gene expression profiling induced by histone deacetylase inhibitor, FK228, in human esophageal squamous cancer cells. Oncol Rep 18: 585-592, 2007.
14. Kano M, Seki N, Kikkawa N, Fujimura L, Hoshino I, Akutsu Y, Chiyomaru T, Enokida H, Nakagawa M and Matsubara H: miR-145, miR-133a and miR-133b: Tumor-suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. Int J Cancer 127: 2804-2814, 2010.
15. Isozaki Y, Hoshino I, Nohata N, Kinoshita T, Akutsu Y, Hanari N, Mori M, Yoneyama Y, Akanuma N, Takeshita N, *et al*: Identification of novel molecular targets regulated by tumor suppressive miR-375 induced by histone acetylation in esophageal squamous cell carcinoma. Int J Oncol 41: 985-994, 2012.
16. Takeshita N, Mori M, Kano M, Hoshino I, Akutsu Y, Hanari N, Yoneyama Y, Ikeda N, Isozaki Y, Maruyama T, *et al*: miR-203 inhibits the migration and invasion of esophageal squamous cell carcinoma by regulating LASP1. Int J Oncol 41: 1653-1661, 2012.
17. Takeshita N, Hoshino I, Mori M, Akutsu Y, Hanari N, Yoneyama Y, Ikeda N, Isozaki Y, Maruyama T, Akanuma N, *et al*: Serum microRNA expression profile: miR-1246 as a novel diagnostic and prognostic biomarker for oesophageal squamous cell carcinoma. Br J Cancer 108: 644-652, 2013.
18. Akanuma N, Hoshino I, Akutsu Y, Murakami K, Isozaki Y, Maruyama T, Yusup G, Qin W, Toyozumi T, Takahashi M, *et al*: MicroRNA-133a regulates the mRNAs of two invadopodia-related proteins, FSCN1 and MMP14, in esophageal cancer. Br J Cancer 110: 189-198, 2014.
19. Hoshino I, Akutsu Y, Murakami K, Akanuma N, Isozaki Y, Maruyama T, Toyozumi T, Matsumoto Y, Suito H, Takahashi M, *et al*: Histone demethylase LSD1 inhibitors prevent cell growth by regulating gene expression in esophageal squamous cell carcinoma cells. Ann Surg Oncol 23: 312-320, 2016.
20. McGrath J and Trojer P: Targeting histone lysine methylation in cancer. Pharmacol Ther 150: 1-22, 2015.
21. Allfrey VG and Mirsky AE: Structural modifications of histones and their possible role in the regulation of RNA synthesis. Science 144: 559, 1964.
22. Shi Y, Lan F, Matson C. Mulligan P, Whetstine JR, Cole PA, Casero RA and Shi Y: Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119: 941-953, 2004.
23. Lv T, Yuan D, Miao X, Lv Y, Zhan P, Shen X and Song Y: Over-expression of LSD1 promotes proliferation, migration and invasion in non-small cell lung cancer. PLoS One 7: e35065, 2012.
24. Kashyap V, Ahmad S, Nilsson EM, Helczynski L, Kenna S, Persson JL, Gudas LJ and Mongan NP: The lysine specific demethylase-1 (LSD1/KDM1A) regulates VEGF-A expression in prostate cancer. Mol Oncol 7: 555-566, 2013.
25. Zhao ZK, Yu HF, Wang DR, Dong P, Chen L, Wu WG, Ding WJ and Liu YB: Overexpression of lysine specific demethylase 1 predicts worse prognosis in primary hepatocellular carcinoma patients. World J Gastroenterol 18: 6651-6656, 2012.
26. Lim S, Janzer A, Becker A, Zimmer A, Schüle R, Buettner R and Kirfel J: Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. Carcinogenesis 31: 512-520, 2010.
27. Kerenyi MA, Shao Z, Hsu YJ, Guo G, Luc S, O'Brien K, Fujiwara Y, Peng C, Nguyen M and Orkin SH: Histone demethylase Lsd1 represses hematopoietic stem and progenitor cell signatures during blood cell maturation. Elife 2: e00633, 2013.
28. Ueda R, Suzuki T, Mino K, Tsumoto H, Nakagawa H, Hasegawa M, Sasaki R, Mizukami T and Miyata N: Identification of cell-active lysine specific demethylase 1-selective inhibitors. J Am Chem Soc 131: 17536-17537, 2009.
29. Hamada S, Suzuki T, Mino K, Koseki K, Oehme F, Flamme I, Ozasa H, Itoh Y, Ogasawara D, Komaarashi H, *et al*: Design, synthesis, enzyme-inhibitory activity, and effect on human cancer cells of a novel series of jumonji domain-containing protein 2 histone demethylase inhibitors. J Med Chem 53: 5629-5638, 2010.

30. Etani T, Suzuki T, Naiki T, Naiki-Ito A, Ando R, Iida K, Kawai N, Tozawa K, Miyata N, Kohri K and Takahashi S: NCL1, a highly selective lysine-specific demethylase 1 inhibitor, suppresses prostate cancer without adverse effect. Oncotarget 6: 2865-2878, 2015.
31. Pitroda SP, Wakim BT, Sood RF, Beveridge MG, Beckett MA, MacDermed DM, Weichselbaum RR and Khodarev NN: STAT1-dependent expression of energy metabolic pathways links tumour growth and radioresistance to the Warburg effect. BMC Med 7: 68, 2009.
32. Li H and Durbin R: Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754-1760, 2009.
33. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C, Myers RM, Brown M, Li W and Liu XS: Model-based analysis of ChIP-Seq (MACS). Genome Biol 9: R137, 2008.
34. Ji X, Li W, Song J, Wei L and Liu XS: CEAS: Cis-regulatory element annotation system. Nucleic Acids Res 34 (Web Server Issue): W551-W554, 2006.
35. Shin H, Liu T, Manrai AK and Liu XS: CEAS: Cis-regulatory element annotation system. Bioinformatics 25: 2605-2606, 2009.
36. Quinlan AR and Hall IM: BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26: 841-842, 2010.
37. Anders S and Huber W: Differential expression analysis for sequence count data. Genome Biol 11: R106, 2010.
38. Zheng YC, Yu B, Jiang GZ, Feng XJ, He PX, Chu XY, Zhao W and Liu HM: Irreversible lsd1 inhibitors: Application of tranylcypromine and its derivatives in cancer treatment. Curr Top Med Chem 16: 2179-2188, 2016.
39. Theisen ER, Gajiwala S, Bearss J, Sorna V, Sharma S and Janat-Amsbury M: Reversible inhibition of lysine specific demethylase 1 is a novel anti-tumor strategy for poorly differentiated endometrial carcinoma. BMC Cancer 14: 752, 2014.
40. Maes T, Mascaró C, Tirapu I, Estiarte A, Ciceri F, Lunardi S, Guibourt N, Perdones A, Lufino MMP, Somervaille TCP, *et al*: ORY-1001, a potent and selective covalent KDM1A inhibitor, for the treatment of acute leukemia. Cancer Cell 33: 495-511.e12, 2018.
41. Maes T, Carceller E, Salas J, Ortega A and Buesa C: Advances in the development of histone lysine demethylase inhibitors. Curr Opin Pharmacol 23: 52-60, 2015.
42. Maiques-Diaz A and Somervaille TC: LSD1: Biologic roles and therapeutic targeting. Epigenomics 8: 1103-1116, 2016.
43. Kidger AM, Rushworth LK, Stellzig J, Davidson J, Bryant CJ, Bayley C, Caddye E, Rogers T, Keyse SM and Caunt CJ: Dual-specificity phosphatase 5 controls the localized inhibition, propagation, and transforming potential of ERK signaling. Proc Natl Acad Sci USA 114: E317-E326, 2017.
44. Yan X, Liu L, Li H, Huang L, Yin M, Pan C, Qin H and Jin Z: Dual specificity phosphatase 5 is a novel prognostic indicator for patients with advanced colorectal cancer. Am J Cancer Res 6: 2323-2333, 2016.
45. Hwang JH, Joo JC, Kim DJ, Jo E, Yoo HS, Lee KB, Park SJ and Jang IS: Cordycepin promotes apoptosis by modulating the ERK-JNK signaling pathway via DUSP5 in renal cancer cells. Am J Cancer Res 6: 1758-1771, 2016.
46. Asanoma K, Liu G, Yamane T, Miyanari Y, Takao T, Yagi H, Ohgami T, Ichinoe A, Sonoda K, Wake N and Kato K: Regulation of the mechanism of TWIST1 transcription by BHLHE40 and BHLHE41 in cancer cells. Mol Cell Biol 35: 4096-4109, 2015.
47. Furukawa H, Makino T, Yamasaki M, Tanaka K, Miyazaki Y, Takahashi T, Kurokawa Y, Nakajima K, Takiguchi S, Mori M and Doki Y: PRIMA-1 induces p53-mediated apoptosis by upregulating Noxa in esophageal squamous cell carcinoma with TP53 missense mutation. Cancer Sci 109: 412-421, 2018.
48. Saha MN, Jiang H, Yang Y, Reece D and Chang H: PRIMA-1Met/APR-246 displays high antitumor activity in multiple myeloma by induction of p73 and Noxa. Mol Cancer Ther 12: 2331-2341, 2013.
49. Zandi R, Selivanova G, Christensen CL, Gerds TA, Willumsen BM and Poulsen HS: PRIMA-1Met/APR-246 induces apoptosis and tumor growth delay in small cell lung cancer expressing mutant p53. Clin Cancer Res 17: 2830-2841, 2011.
50. Liang Y, Besch-Williford C and Hyder SM: PRIMA-1 inhibits growth of breast cancer cells by re-activating mutant p53 protein. Int J Oncol 35: 1015-1023, 2009.
51. Li XL, Zhou J, Chan ZL, Chooi JY, Chen ZR and Chng WJ: PRIMA-1met (APR-246) inhibits growth of colorectal cancer cells with different p53 status through distinct mechanisms. Oncotarget 6: 36689-36699, 2015.
52. Lu T, Zou Y, Xu G, Potter JA, Taylor GL, Duan Q, Yang Q, Xiong H, Qiu H, Ye D, *et al*: PRIMA-1Met suppresses colorectal cancer independent of p53 by targeting MEK. Oncotarget 7: 83017-83030, 2016.
53. Lehmann S, Bykov VJ, Ali D, Andrén O, Cherif H, Tidefelt U, Uggla B, Yachnin J, Juliusson G, Moshfegh A, *et al*: Targeting p53 in vivo: A first-in-human study with p53-targeting compound APR-246 in refractory hematologic malignancies and prostate cancer. J Clin Oncol 30: 3633-3639, 2012.
54. Miyazaki T, Kato H, Nakajima M, Faried A, Takita J, Sohda M, Fukai Y, Yamaguchi S, Masuda N, Manda R, *et al*: An immunohistochemical study of TIMP-3 expression in oesophageal squamous cell carcinoma. Br J Cancer 91: 1556-1560, 2004.
55. Apte SS, Olsen BR and Murphy G: The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem 270: 14313-14318, 1995.
56. Visse R and Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res 92: 827-839, 2003.
57. Ahonen M, Baker AH and Kahari VM: Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. Cancer Res 58: 2310-2315, 1998.
58. Smith MR, Kung H, Durum SK, Colburn NH and Sun Y: TIMP-3 induces cell death by stabilizing TNF-alpha receptors on the surface of human colon carcinoma cells. Cytokine 9: 770-780, 1997.
59. Baker AH, George SJ, Zaltsman AB, Murphy G and Newby AC: Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. Br J Cancer 79: 1347-1355, 1999.
60. Bond M, Murphy G, Bennett MR, Amour A, Knauper V, Newby AC and Baker AH: Localization of the death domain of tissue inhibitor of metalloproteinase-3 to the N terminus. Metalloproteinase inhibition is associated with proapoptotic activity. J Biol Chem 275: 41358-41363, 2000.
61. Ahonen M, Poukkula M, Baker AH, Kashiwagi M, Nagase H, Eriksson JE and Kähäri VM: Tissue inhibitor of metalloproteinases-3 induces apoptosis in melanoma cells by stabilization of death receptors. Oncogene 22: 2121-2134, 2003.
62. Coutinho-Camillo CM, Lourenço SV, Nonogaki S, Vartanian JG, Nagai MA, Kowalski LP and Soares FA: Expression of PAR-4 and PHLDA1 is prognostic for overall and disease-free survival in oral squamous cell carcinomas. Virchows Arch 463: 31-39, 2013.
63. Neef R, Kuske MA, Pröls E and Johnson JP: Identification of the human PHLDA1/TDAG51 gene: down-regulation in metastatic melanoma contributes to apoptosis resistance and growth deregulation. Cancer Res 62: 5920-5929, 2002.
64. Nagai MA, Fregnani JH, Netto MM, Brentani MM and Soares FA: Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. Breast Cancer Res Treat 106: 49-56, 2007.
65. Oberst MD, Beberman SJ, Zhao L, Yin JJ, Ward Y and Kelly K: TDAG51 is an ERK signaling target that opposes ERK-mediated HME16C mammary epithelial cell transformation. BMC Cancer 8: 189, 2008.
66. Park CG, Lee SY, Kandala G, Lee SY and Choi Y: A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. Immunity 4: 583-591, 1996.
67. Gomes I, Xiong W, Miki T and Rosner MR: A proline- and glutamine-rich protein promotes apoptosis in neuronal cells. J Neurochem 73: 612-622, 1999.
68. Hossain GS, van Thienen JV, Werstuck GH, Zhou J, Sood SK, Dickhout JG, de Koning AB, Tang D, Wu D, Falk E, *et al*: TDAG51 is induced by homocysteine, promotes detachment-mediated programmed cell death, and contributes to the cevelopment of atherosclerosis in hyperhomocysteinemia. J Biol Chem 278: 30317-30327, 2003.
69. Ju JA and Gilkes DM: RhoB: Team oncogene or team tumor suppressor? Genes (Basel) 9: pii: E67, 2018.
70. Parri M and Chiarugi P: Rac and Rho GTPases in cancer cell motility control. Cell Commun Signal 8: 23, 2010.
71. Vega FM and Ridley AJ: Rho GTPases in cancer cell biology. FEBS Lett 582: 2093-2101, 2008.
72. Porter AP, Papaioannou A and Malliri A: Deregulation of Rho GTPases in cancer. Small GTPases 7: 123-138, 2016.
73. Bhavsar PJ, Infante E, Khwaja A and Ridley AJ: Analysis of Rho GTPase expression in T-ALL identifies RhoU as a target for Notch involved in T-ALL cell migration. Oncogene 32: 198-208, 2013.

74. Poveda J, Sanz AB, Fernandez-Fernandez B, Carrasco S, Ruiz-Ortega M, Cannata-Ortiz P, Ortiz A and Sanchez-Niño MD: MXRA5 is a TGF-β1-regulated human protein with anti-inflammatory and anti-fibrotic properties. J Cell Mol Med 21: 154-164, 2017.
75. He Y, Chen X, Liu H, Xiao H, Kwapong WR and Mei J: Matrix-remodeling associated 5 as a novel tissue biomarker predicts poor prognosis in non-small cell lung cancers. Cancer Biomark 15: 645-651, 2015.
76. Wang GH, Yao L, Xu HW, Tang WT, Fu JH, Hu XF, Cui L and Xu XM: Identification of MXRA5 as a novel biomarker in colorectal cancer. Oncol Lett 5: 544-548, 2013.
77. Buckanovich RJ, Sasaroli D, O'Brien-Jenkins A, Botbyl J, Hammond R, Katsaros D, Sandaltzopoulos R, Liotta LA, Gimotty PA and Coukos G: Tumor vascular proteins as biomarkers in ovarian cancer. J Clin Oncol 25: 852-861, 2007.
78. Xiong D, Li G, Li K, Xu Q, Pan Z, Ding F, Vedell P, Liu P, Cui P, Hua X, *et al*: Exome sequencing identifies MXRA5 as a novel cancer gene frequently mutated in non-small cell lung carcinoma from Chinese patients. Carcinogenesis 33: 1797-1805, 2012.
79. Huang T, Sun L, Yuan X and Qiu H: Thrombospondin-1 is a multifaceted player in tumor progression. Oncotarget 8: 84546-84558, 2017.
80. Zhou ZQ, Cao WH, Xie JJ, Lin J, Shen ZY, Zhang QY, Shen JH, Xu LY and Li EM: Expression and prognostic significance of THBS1, Cyr61 and CTGF in esophageal squamous cell carcinoma. BMC Cancer 9: 291, 2009.