Regulation of $TPD52$ by antitumor microRNA-$218$ suppresses cancer cell migration and invasion in lung squamous cell carcinoma

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Abstract. The development of targeted molecular therapies has greatly benefited patients with lung adenocarcinomas. In contrast, these treatments have had little benefit in the management of lung squamous cell carcinoma (lung SCC). Therefore, new treatment options based on current genomic approaches are needed for lung SCC. Aberrant microRNA (miRNA) expression has been shown to promote lung cancer development and aggressiveness. Downregulation of microRNA-$218$ (miR-$218$) was frequently observed in our miRNA expression signatures of cancers, and previous studies have shown an antitumor function of miR-$218$ in several types of cancers. However, the impact of miR-$218$ on lung SCC is still ambiguous. The present study investigated the antitumor roles of miR-$218$ in lung SCC to identify the target genes regulated by this miRNA. Ectopic expression of miR-$218$ greatly inhibited cancer cell migration and invasion in the lung SCC cell lines EBC-1 and SK-MES-1. Through a combination of in silico analysis and gene expression data searching, tumor protein D52 ($TPD52$) was selected as a putative target of miR-$218$ regulation. Moreover, direct binding of miR-$218$ to the 3'-UTR of $TPD52$ was observed by dual luciferase reporter assay. Overexpression of $TPD52$ was observed in lung SCC clinical specimens, and knockdown of $TPD52$ significantly suppressed cancer cell migration and invasion in lung SCC cell lines. Furthermore, the downstream pathways mediated by $TPD52$ involved critical regulators of genomic stability and mitotic checkpoint genes. Taken together, our data showed that downregulation of miR-$218$ enhances overexpression of TPD52 in lung SCC cells, promoting cancer cell aggressiveness. Identification of tumor-suppressive miRNA-mediated RNA networks of lung SCC will provide new insights into the potential mechanisms of the molecular pathogenesis of the disease.

Introduction

Lung cancer remains the most frequent cause of cancer-related death in developed countries (1). Approximately 80% of lung cancers are categorized as non-small cell lung cancer (NSCLC), and NSCLC is divided into four major subtypes pathologically: adenocarcinoma, squamous cell carcinoma (SCC), large cell carcinoma and neuroendocrine cancer (2). Development of targeted molecular therapies has remarkably improved the overall survival of patients with adenocarcinoma (3-6). In contrast, targeted molecular therapies have offered few benefits in the management of lung SCC (7). Therefore, there is a need for effective treatment options based on current genomic approaches for lung SCC.

After the human genome sequencing era, the discovery of an extremely large number of non-coding RNAs conceptually transformed cancer research. MicroRNAs (miRNAs) are small non-coding RNAs involved in the repression or degradation of target RNA transcripts in a sequence-dependent manner (1,2). The ability of miRNAs is unique; a single miRNA may regulate a vast number of protein-coding or non-coding RNAs in human cells. Therefore, aberrantly expressed miRNAs may upset tightly regulated cellular RNA networks. This failing of the RNA network contributes to cancer development, aggressiveness and drug resistance (3-5). In fact, aberrantly expressed miRNAs have been reported in various types of human cancers, including lung SCC (6-8). Based on miRNA expression signatures, we identified tumor-suppressive miRNAs and the cancer RNA networks regulated by these miRNAs (9-11). We hypothesize that elucidation of tumor-suppressive miRNA-regulated oncogenic networks will provide new insights into the potential molecular mechanisms of lung SCC.

In our earlier studies determining miRNA expression signatures, we showed that miRNA-$218$ was significantly...
downregulated in several types of cancer tissues (12-15). Our previous studies also demonstrated that downregulation of miR-218 enhanced overexpression of extracellular matrix (ECM) protein components or actin-related proteins, and this promoted cancer cell migration and invasion (16-18). Tumor-suppressive roles of miR-218 were reported in several types of cancer. However, the impact of miR-218 on lung SCC remains ambiguous.

The aim of the present study was to investigate the functional significance of miR-218 in lung SCC and to identify molecular targets regulated by this miRNA. We found that restoration of miR-218 significantly suppressed cancer cell migration and invasion. Using luciferase reporter assay, tumor protein D52 (TPD52) was shown to be directly regulated by miR-218. Overexpression of TPD52 was observed in lung SCC clinical specimens and downregulation of the TPD52 gene significantly inhibited cancer cell aggressiveness. miR-218/TPD52-regulated RNA networks may provide new insights into the potential mechanisms of lung SCC pathogenesis.

Materials and methods

Clinical specimens, cancer cell lines and RNA extraction. In all, 31 lung SCC specimens and 24 non-cancerous lung specimens were obtained from patients who underwent thoracic surgery at Kagoshima University Hospital from 2010 to 2013. The present study was approved by the Institutional Review Board for Clinical Research of the Kagoshima University School of Medicine. Prior written informed consent and approval were obtained from each patient. Table I describes the patient background and clinical characteristics of collected specimens. Samples were staged using the TNM scheme according to the International Association for the Study of Lung Cancer and were graded histologically (19). These archival formalin-fixed paraffin-embedded (FFPE) samples were subsequently studied by qRT-PCR analysis and immunohistochemistry as previously described (9-11).

The human lung SCC cell lines EBC-1 and SK-MES-1 were acquired from the Japanese Cancer Research Resources Bank (Osaka, Japan) and the American Type Culture Collection ATCC; Manassas, VA, USA), respectively. Total RNA was isolated using Isogen II (code no: 311-07361; Nippon Gene, Co., Ltd., Tokyo, Japan) as directed by the manufacturer.

Quantitative reverse transcription PCR (qRT-PCR). PCR quantification was performed as previously described (9-11). The expression of miR-218 was determined using stem-loop RT-PCR as directed by the manufacturer (P/N: 000521; Applied Biosystems, Foster City, CA, USA). The TaqMan probe and TPD52 primers were from Assay-on-Demand™ Gene Expression products (P/N: Hs00893105_m1; Applied Biosystems). For quantification, miRNA and mRNA data were normalized against human RNU48 (P/N: 001006; Applied Biosystems) and STEO48 (P/N: Hs99999908_m1; Applied Biosystems), respectively.

Transfection of mature miRNA and small interfering RNA (siRNA). Pre-miRTM miRNA precursors for miR-218 (hsa-miR-218-5p, P/N: AM1 7100; Applied Biosystems) and negative control miRNA (P/N: AM 17111; Applied Biosystems), Stealth Select RNAi siRNA, si-TPD52 (P/N: HSS120730 and HSS120731; Invitrogen, Carlsbad, CA, USA), and negative control siRNA (P/N: 4390843; Invitrogen) were used in this study. EBC-1 and SK-MES-1 cells in Opti-MEM medium (cat. no. 31985070; Thermo Fisher Scientific, Waltham, MA, USA) were transfected with Lipofectamine RNAiMAX transfection reagent (P/N: 56532; Invitrogen) with 10 nM mature miRNA or siRNA.

Cell proliferation, migration and invasion assays. Cell proliferation was determined by XTT assay using Cell Proliferation kit (SKU: 20-300-1000; Biological Industries, Kibbutz Beit Haemek, Israel). Cell migration activity was analyzed by wound-healing assay, and cell invasion was analyzed using Corning BioCoat Matrigel Invasion chamber (cat. no. 354480; BD Biosciences, Bedford, MA, USA). The cell proliferation, migration, and invasion assays were carried out as previously described (9-11).

Identification of putative miR-218 target genes in lung SCC cells. Genome-wide gene expression analysis of miR-218-transfected EBC-1 cells was performed (accession number: GSE77790). Putative miR-218-regulated genes were identified by searching the TargetScan database (http://www.targetscan.org/). We then examined the expression levels

| Table I. Characteristics of the lung cancer and non-cancerous cases. |
|-----------------|-----------------|-----------------|
| **A. Characteristics of the lung cancer cases** |
| Lung cancer patients | n | (%) |
| Total no | 31 |
| Median age (range) | 70 (50-88) |
| Gender | | |
| Male | 29 | (93.5) |
| Female | 2 | (6.5) |
| Pathological stage | | |
| IA | 4 | (12.9) |
| IB | 9 | (29.0) |
| IIA | 4 | (12.9) |
| IIB | 5 | (16.1) |
| IIIA | 8 | (25.8) |
| IIIB | 1 | (3.2) |
| **B. Characteristics of the non-cancerous cases** |
| Non-cancerous tissues | n |
| Total number | 24 |
| Median age (range) | 69 (50-88) |
| Gender | | |
| Male | 24 |
| Female | 0 |
of putative miR-218 targets in lung SCC clinical expression data from the GEO database (accession number: GSE19188). Oligo-microarray procedures and data mining methods were conducted as previously described (20,21).

**Western blot analysis.** Cells were harvested 96 h after transfection, and proteins were extracted from lysed cells. Protein lysates (20 µg) were separated on NuPAGE 4-12% Bis-Tris gels (cat. no. NP0323BOX; Invitrogen) before transfer of proteins to a polyvinylidene fluoride membrane. Immunoblotting was performed using diluted primary anti-TDP52 antibodies (1:250 dilution; Human Protein Atlas no. HPA028427; Atlas Antibodies, Stockholm, Sweden) and anti-GAPDH antibodies (1:10,000 dilution; cat. no. MAB374; Chemicon International, Inc., Temecula, CA, USA). These assays were carried out as previously described (9-11).

**Plasmid construction and dual-luciferase reporter assay.** The procedure for the dual-luciferase reporter assay was previously described (9-11). A partial sequence of the wild-type TPD52 3’-UTR containing the miR-218 target site or the TPD52 3’-UTR partial sequence lacking the miR-218 target site was cloned into the psiDHECK-2 vector between the XhoI-PmeI restriction sites in the 3’-UTR of the hRluc gene (cat. no. C8021; Promega, Madison, WI, USA).

**Immunohistochemistry.** The expression status of TPD52 in lung SCC clinical specimens (BC04002; US Biomax, Inc., Rockville, MD, USA) was confirmed via immunohistochemistry using an UltraVision Detection system (cat. no. TP-015-HD, Thermo Fisher Scientific) according to the manufacturer’s protocol. Tissues were incubated with primary rabbit polyclonal anti-TPD52 antibodies (1:3,000 dilution; HPA028427) then treated with biotinylated goat anti-rabbit secondary antibodies. Antibodies were visualized using diaminobenzidine hydrogen peroxidase as the chromogen, and slides were counterstained with 0.5% hematoxylin.

**Identification of downstream targets regulated by TPD52 in lung SCC.** Gene expression analysis using si-TPD52-transfected EBC-1 cells identified molecular targets regulated by TPD52 in lung SCC cells. Microarray expression profiles of si-TPD52 transfectants were compiled and deposited into the GEO database (accession number: GSE82108).

**Statistical analysis.** RT-PCR results were analyzed using Mann-Whitney U tests to evaluate the relationships between the 2 groups, while Bonferroni-adjusted Mann-Whitney U tests were used to analyze the relationships among three or more variables. All analyses were performed using Expert StatView (version 5; SAS Institute Inc., Cary, NC, USA).

**Results**

**Expression of miR-218 in lung SCC tissues and cell lines.** To confirm the miRNA expression signatures of lung SCC cells, we evaluated the expression level of miR-218 in lung SCC tissues (n=31) and non-cancerous lung tissues (n=24). The patient clinicopathological features are summarized in Table I. The expression level of miR-218 was significantly downregulated in cancer tissues compared to non-cancerous tissues (P<0.0001; Fig. 1A). Expression of miR-218 in EBC-1 and SK-MES-1 cell lines was also lower than in non-cancerous lung tissues (Fig. 1A).

**Effects of miR-218 restoration on the proliferation, migration and invasion of EBC-1 and SK-MES-1 cells.** To investigate the antitumor activity of miR-218, we performed gain-of-function
assays using miRNA transfection into EBC-1 and SK-MES-1 cells. Cell proliferation assays showed that cancer cell growth was slightly inhibited in miR-218 transfectants compared with mock- or miR control-transfected EBC-1 and SK-MES-1 cells (Fig. 1B). Cancer cell migration and invasion activities were significantly inhibited in miR-218 transfectants compared with mock- or miR-control-transfectants (P<0.0001; Fig. 1C and D).

Identification of putative targets regulated by miR-218 in EBC-1 cells. To identify putative targets of miR-218, we employed a combination of in silico analysis, genome-wide gene expression analysis and analysis of gene expression in clinical specimens using the GEO database. First, we identified 513 genes downregulated by miR-218 transfection into EBC-1 cells (log₂ ratio <-1.0). Among these 513 genes, 163 genes have putative miR-218 binding sites in their 3'-UTR regions according to the TargetScan database. Next, we cross-referenced these genes with genes upregulated (fold change >1.5) in NSCLC clinical specimens from the GEO gene expression data set (accession number: GSE19188). A total of

![Figure 2](image-url) Figure 2. Flow chart illustrating the analysis strategy for miR-218 target genes in lung SCC cells.

![Figure 3](image-url) Figure 3. Direct regulation of TPD52 by miR-218 in lung SCC cells. (A) TPD52 mRNA expression was evaluated by qRT-PCR in EBC-1 and SK-MES-1 cells 72 h after transfection with miR-218. GUSB was used as an internal control. *P<0.0001. (B) TPD52 protein expression in EBC-1 and SK-MES-1 cells was evaluated using western blotting 72 h after transfection with miR-218. GAPDH was used as a loading control. (C) Putative miR-218 binding sites in the 3'-UTR of TPD52 mRNA. (D-F) Dual luciferase reporter assays using vectors encoding putative miR-218 target sites in the TPD52 3'-UTR for both wild-type and deleted regions. Normalized data were calculated as Renilla/firefly luciferase activity ratios. *P<0.0001.
36 candidate genes were identified as putative target genes of miR-218 regulation in lung SCC (Table II). Fig. 2 shows our strategy for selecting putative miR-218 target genes. In the present study, we focused on the TPD52 gene, investigating TPD52 function and downregulated TPD52-mediated pathways.

Table II. Downregulated genes in miR-218 transfectant.

| Entrez gene ID | Gene symbol | Description | miR-218 target site | EBC-1 miR-218 transfectant (log₂ ratio) | GSE: 19188 fold change |
|---------------|-------------|-------------|---------------------|----------------------------------------|-----------------------|
| 7163          | TPD52       | Tumor protein D52 | 4 2 2 | -1.33 | 2.66 |
| 5563          | PRKAA2      | Protein kinase, AMP-activated, α 2 catalytic subunit | 4 0 4 | -1.04 | 2.50 |
| 2673          | GFPT1       | Glutamine-fructose-6-phosphate transaminase 1 | 4 1 3 | -1.17 | 1.92 |
| 332           | BIRC5       | Baculoviral IAP repeat containing 5 | 2 0 2 | -2.05 | 12.42 |
| 56938         | ARNTL2      | Aryl hydrocarbon receptor nuclear translocator-like 2 | 2 0 2 | -1.09 | 7.85 |

| Entrez gene ID | Gene symbol | Description | miR-218 target site | EBC-1 miR-218 transfectant (log₂ ratio) | GSE: 19188 fold change |
|---------------|-------------|-------------|---------------------|----------------------------------------|-----------------------|
| 112399        | EGLN3       | Egl-9 family hypoxia-inducible factor 3 | 2 1 1 | -1.45 | 5.97 |
| 84181         | CHD6        | chromodomain helicase DNA binding protein 6 | 2 1 1 | -1.35 | 2.30 |
| 29927         | SEC61A1     | Sec61 α 1 subunit (S. cerevisiae) | 2 1 1 | -1.09 | 2.13 |
| 8776          | MTMR1       | Myotubularin related protein 1 | 2 1 1 | -1.06 | 1.96 |
| 131566        | DCBLD2      | Discoidin, CUB and LCCL domain containing 2 | 2 2 0 | -1.46 | 1.88 |
| 2820          | GPD2        | Glycerol-3-phosphate dehydrogenase 2 (mitochondrial) | 2 0 2 | -1.02 | 1.83 |
| 399694        | SHC4        | SHC (Src homology 2 domain containing) family, member 4 | 2 1 1 | -1.25 | 1.58 |
| 3486          | IGFBP3      | Insulin-like growth factor binding protein 3 | 1 0 1 | -2.09 | 4.12 |
| 144406        | WDR66       | WD repeat domain 66 | 1 0 1 | -1.11 | 2.98 |
| 25907         | TMEM158     | Transmembrane protein 158 (gene/pseudogene) | 1 0 1 | -1.12 | 2.96 |
| 10447         | FAM3C       | Family with sequence similarity 3, member C | 1 1 0 | -2.08 | 2.90 |
| 4017          | LOXL2       | Lysyl oxidase-like 2 | 1 0 1 | -1.96 | 2.56 |
| 9139          | CBFA2T2     | Core-binding factor, runt domain, α subunit 2; translocated to 2 | 1 0 1 | -1.11 | 2.42 |
| 3691          | ITGB4       | Integrin, β 4 | 1 0 1 | -1.12 | 2.20 |
| 6745          | SSR1        | Signal sequence receptor, α | 1 1 0 | -1.47 | 1.92 |
| 1457          | CSNK2A1     | Casein kinase 2, α 1 polypeptide | 1 1 0 | -1.30 | 1.90 |
| 157638        | FAM84B      | Family with sequence similarity 84, member B | 1 0 1 | -1.02 | 1.90 |
| 949           | SCARB1      | Scavenger receptor class B, member 1 | 1 0 1 | -1.54 | 1.84 |
| 2011          | MARK2       | MAP/microtubule affinity-regulating kinase 2 | 1 1 0 | -1.41 | 1.75 |
| 9289          | GPR56       | G protein-coupled receptor 56 | 1 0 1 | -1.53 | 1.70 |
| 55959         | SULF2       | Sulfatase 2 | 1 0 1 | -1.11 | 1.67 |
| 65985         | AACS        | Acetoacetyl-CoA synthetase | 1 0 1 | -1.10 | 1.63 |
| 160           | AP2A1       | Adaptor-related protein complex 2, α 1 subunit | 1 1 0 | -1.14 | 1.63 |
| 54928         | IMPAD1      | Inositol monophosphatase domain containing 1 | 1 1 0 | -1.01 | 1.62 |
| 1889          | ECE1        | Endothelin converting enzyme 1 | 1 0 1 | -1.55 | 1.61 |
| 55609         | ZNF280C     | Zinc finger protein 280C | 1 1 0 | -1.10 | 1.61 |
| 6533          | SLC6A6      | Solute carrier family 6 (neurotransmitter transporter), member 6 | 1 1 0 | -1.13 | 1.61 |
| 80000         | GREB1L      | Growth regulation by estrogen in breast cancer-like | 1 0 1 | -1.15 | 1.59 |
| 10776         | ARPP19      | cAMP-regulated phosphoprotein, 19 kDa | 1 1 0 | -1.33 | 1.52 |
| 83637         | ZMIZ2       | Zinc finger, MIZ-type containing 2 | 1 1 0 | -1.59 | 1.52 |
| 79139         | DERLI       | Derlin 1 | 1 0 1 | -1.16 | 1.51 |
TPD52 is directly targeted by mir-218 in lung SCC cells. First, we measured the expression of TPD52 in lung SCC cells. RT-PCR and western blotting showed that two siRNAs (si-TPD52-1 and si-TPD52-2) could effectively reduce the expression of TPD52 in SK-MES-1 cells (Fig. 4A and B). Next, we carried out functional assays using these two siRNAs. XTT assays demonstrated that cell proliferation was inhibited by si-TPD52 transfection only in SK-MES-1 cells (Fig. 4C). Cell migration and invasion activities were significantly inhibited by si-TPD52 transfection in both EBC-1 and SK-MES-1 cells in comparison with mock- or negative-control transfectants (Fig. 4D and E).

TPD52 was strongly expressed in clinical lung SCC specimens. To analyze whether TPD52 was upregulated in lung SCC clinical specimens, we carried out immunohistochemical staining of lung SCC and non-cancerous lung tissues. Thirty specimens were stained in this study (20 lung SCC specimens and 10 non-cancerous tissues); all lung SCC specimens stained moderately or strongly, whereas all non-cancerous specimens stained weakly or negatively for TPD52 (Table III and Fig. 5).

Identification of TPD52-mediated downstream pathways in lung SCC cells. To identify the downstream genes regulated by TPD52, genome-wide gene expression analysis and in silico
analysis were performed in lung SCC cells transfected with si-TPD52. A total of 2,278 genes were identified as downregulated in si-TPD52-transfected EBC-1 cells compared with the control (log2 ratio < -0.5). Among them, 215 genes were upregulated in NSCLC specimens in the GEO database (accession number: GSE19188). We categorized the 215 genes according to KEGG pathways, and 7 pathways were identified as significantly enriched pathways (Table IV). Fig. 6 shows our strategy for selecting TPD52-mediated downstream pathways. Among these pathways, we focused on the ‘Cell cycle pathway’, ‘DNA replication pathways’ and ‘p53 signaling pathway’. Genes involved in these pathways are listed in Table V.

Discussion

A single miRNA can regulate an extremely large number of protein-coding or non-coding RNAs. Thus, aberrant expression of miRNAs disrupts the RNA network in cancer cells. Identification of aberrantly expressed miRNAs and the novel cancer networks they regulate is a research trend of the post-genome-sequencing era. Recent evidence shows that disruption of normally-regulated RNA networks by aberrantly expressed miRNAs triggers cancer cell development, progression and metastasis (3,4). Our recent studies of miRNA expression signatures in various cancers indicated that miR-218 is frequently downregulated in cancers (9-11). Investigating
miR-218-regulated RNA networks in lung SCC is the first step in construction of a new treatment strategy for the disease. Our current data showed that expression of miR218 was significantly reduced in lung SCC specimens. The mature form of miR-218 is generated from two separate loci, miR-218-1 and miR-218-2, which are located on chromosomes 4p15.31 and 5q35.1 within the introns of SLIT2 and SLIT3, respectively (22). Several lines of evidence suggest that miR-218 is...
frequently downregulated in various cancers (17,18,23,24). In this study, ectopic expression of miR-218 significantly inhibited cancer cell migration and invasion, suggesting this miRNA suppresses metastasis-promoting genes. Our previous studies showed that antitumor effects of miR-218 explicitly contribute to migration and invasion in head and neck cancer, cervical cancer, renal cell carcinoma and prostate cancer (17,18,23,24). Interestingly, miR-218-regulated genes were involved in ECM-related components such as collagens, laminins and integrins (17,18,23,24). Accumulating evidence indicates that the interaction of cancer cells with their microenvironment influences the initiation, development and metastasis of cancer cells (25,26). Overexpression of ECM components and activation of ECM-integrin signaling were observed in several types of cancers and shown to activate cancer cell aggressiveness (27). The discovery of tumor-suppressive miR-218-regulated genes and pathways may provide important insights into the potential mechanisms of lung SCC metastasis.

To better understand lung SCC development and metastasis, we identified miR-218 target genes using a combination of in silico and genome-wide gene expression analyses. We have identified antitumor miRNA-regulated oncopgenes and novel cancer networks using this strategy (9-11). In the present study, a total of 36 putative target genes of miR-218 were identified. Among these genes, we demonstrated that LOXL2 (lysyl oxidase homolog 2) was directly regulated by miR-218 in head and neck cancer and prostate cancer (28). Another group showed direct regulation of BIRC5 (baculoviral IAP repeat containing 5) by miR-218 in cervical cancer (29). These findings show the effectiveness of our strategy in identifying target genes of miR-218-regulation in cancer cells.

In the present study, we focused on TPD52 and investigated the functional significance of this gene in lung SCC. TPD52 was initially cloned through differential screening using a breast cancer cDNA library (30). This cDNA clone had novel sequences and was expressed in breast and basal cell carcinomas (30). Interestingly, this gene is located on the human chromosome 8q21.13, a frequently observed region of chromosomal amplification in several types of cancers (31-33). In lung adenocarcinoma, chromosome 8q21.13, which includes TPD52, was one of the most notable amplified genomic regions (34). A vast number of studies showed that TPD52 is overexpressed both at the mRNA and protein levels in several cancers. Likewise, overexpression of TPD52 was reported in small cell lung cancer, lung adenocarcinoma and lung SCC (35-37). The influence of smoking is important in lung cancer pathogenesis. Expression levels of TPD52 were enhanced in airway epithelial cells in smokers with lung cancer compared to smokers without cancer (38). The functional roles of TPD52 were investigated using overexpression or knockdown analytical methods. Ectopic expression of mouse TPD52 in 3T3 fibroblasts resulted in a transformed phenotype that progressed to metastasis (39). Several studies have shown that increased expression of TPD52 enhances the proliferation of prostate cancer cell lines under both normal and androgen-resistant conditions (40,41). Other studies showed that TPD52 knockdown increases apoptotic cell death in ERBB2-amplified breast cancer cell lines (33). These findings suggest that overexpression of TPD52 enhances cancer cell aggressiveness and contributes to several oncogenic pathways.

Recently, several studies demonstrated that TPD52 expression is regulated by several miRNAs in cancer cells. Downregulation of TPD52 expression was observed by miR-107 and miR-185 transfection into non-small cell lung cancer cells and miR-34a transfection into colorectal cancer cells (42,43). Our previous data showed that the tumor-suppressive miR-224 directly regulates oncogenic TPD52 in prostate cancer cells, and silencing TPD52 results in significant reductions in cancer cell migration and invasion in prostate cancer cells (44). More recently, miR-218 was shown to directly regulate TPD52 in prostate cancer cells (45). These data agree with our present report in lung SCC and support our conclusions. Control of TPD52 expression by miRNAs is a novel molecular mechanism of cancer cells, and further investigation of the miRNA-TPD52 axis is needed.

In the present study, we identified TPD52-mediated cancer pathways using genome-wide gene expression analysis of si-TPD52-transfected lung SCC cells. Our data showed that several pathways were identified downstream of TPD52 pathways, such as the ‘Cell cycle pathway’, ‘DNA replication pathway’ and ‘p53 signaling pathway’. The genes involved in these pathways were critical regulators of genomic stability and were mitotic checkpoint genes (46,47). The functional insights obtained in the current studies indicated that several cell cycle kinases, such as TTK, BUB1 and PLK1, were multi-functional enzymes and contributed to cancer cell migration, invasion and metastasis (48-50). The protein kinase TTK promoted the cell proliferation and migration through activation of AKT-mTOR and MDM2-p53 signaling pathways in hepatocellular carcinoma cells (48). Using the siRNA screen of the human kimevo revealed that a serine/threonine kinase BUB1 acted as an essential mediator of TGFβ-dependent signaling (49). This study demonstrated that BUB1 interacted with both, TGFBR1 and TGFBR2 and promoted the TGFβ-dependent epithelial mesenchymal transition (EMT), cell migration and invasion (49). PLK1 is a serine/threonine kinase and a pivotal player of cell cycle regulator (50). Overexpression of PLK1
was observed in prostate cancer and involved in enhancing EMT and stimulation of cell migration and invasion (50). In bladder cancer, CCNB2 was overexpressed in cancer cells and knockdown of CCNB2 inhibited invasion and metastatic abilities (51). These findings have supported our present data of knockdown of TPD52 in lung cancer cells. Exploration of novel TPD52-mediated pathways may lead to the development of new treatment protocols for this disease.

In conclusion, expression of mir-218 was frequently downregulated in lung SCC clinical specimens and appeared to function in anti-migration and anti-invasion roles through targeting of TPD52. Elucidation of mir-218-regulated cancer networks should provide new information on potential therapeutic targets in the treatment of lung SCC development and metastasis.

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