Exosomal IncRNA PVT1/VEGFA Axis Promotes Colon Cancer Metastasis and Stemness by Downregulation of Tumor Suppressor miR-152-3p

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Research

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Abstract

**Background:** Late-stage colon cancer remains a treatment challenge in clinical settings because of the development of drug resistance and distant metastasis. Nevertheless, the mechanisms through which colon cancer cells acquire the ability to metastasize are complicated and require more research.

**Methods:** Bioinformatic analysis was performed to determine gene associated with exosomal IncRNA PVT1/VEGFA axis of colon cancer patients. Biological importance of exosomal IncRNA PVT1/VEGFA axis was investigated *in vitro* (HCT116 and LoVo cell lines) and *in vivo* (PDX mouse model) through knockdown (siPVT1) and overexpression (add exosomes from sera of distant metastasis patients). PVT1/VEGFA axis related protein expression in and cell lines were investigated through RT-qPCR, immunoblotting, and immunohistochemistry analysis. Colony formation Assay, cell invasion, migration, and tumorsphere-formation assay were used to explore possible molecular mechanism.

**Results:** First, using public databases, we demonstrated that PVT1 overexpression is associated with poor prognosis and increased metastatic markers, such as vascular endothelial growth factor A (VEGFA) and epidermal growth factor receptor (EGFR). This finding was then validated in a small cohort of patients with colon cancer, where increased PVT1 expression was correlated with colon cancer incidence, disease recurrence, and distant metastasis. Notably, serum exosomes from patients with metastatic (M-exo) colon cancer were enriched with PVT1 and VEGFA and increased both migratory and invasive abilities in colon cancer cell lines when cocultured. This metastasis-promoting effect was accompanied by an increased expression of Twist1, Vimentin, and MMP2. Notably, M-exo promoted metastatic incidence in patient-derived xenograft mice. *In vitro* silencing of PVT1 led to decreased colon tumorigenic properties, including colony formation, tumorsphere formation, and metastatic potential. Further analysis revealed that miR-152-3p has multiple targets, including PVT1, VEGFA, and EGFR. Increased miR-152-3p resulted in decreased tumorigenesis, and the reverse was true when the miR-152-3p level was decreased.

**Conclusion:** In conclusion, we provided evidence regarding the role of exosomal PVT1 in promoting metastasis in colon cancer through its association with EGFR and VEGFA expression. PVT1 and VEGFA are both targets of miR-152-3p, and this regulatory pathway could be explored for drug and prognostic biomarker development.

**Highlights**

i. Serum exosomes enriched with IncRNA PVT1/VEGFA in colon cancer

ii. IncRNA PVT1/VEGFA promotes metastatic potential and stemness.

iii. IncRNA PVT1/VEGF is association with downregulation of miR-152-3p.

**Introduction**
Colon cancer is among the most prevalent malignancies globally and has consistently ranked as the top three leading causes of cancer-associated deaths [1]. Notably, one of the most challenging tasks in clinics is treating distant metastasis. The most frequently observed metastatic sites in patients with colon cancer are the liver (approximately 20–30%) and lungs [2]. Despite advancements being made over the past decade regarding the development of chemotherapeutic and targeted therapy agents, metastatic colon cancer treatment remains a challenge. Therefore, a better understanding of the molecular and signaling mechanisms involved in the metastatic progression in colon cancer, namely epithelial-to-mesenchymal transition (EMT), could be of value for designing and developing effective therapeutic agents.

Exosomes or extracellular vesicles are nanosized (30–150 nm) cargos produced by cells for intracellular communications. The fact that exosomes might contain proteins, lipids, and nucleic acids has been validated [3], and the functional attributes of exosomes in tumorigenesis have garnered attention. Accumulating evidence suggests that cancer cells secrete exosomes enriched with a variety of signaling molecules to promote tumor initiation, angiogenesis, distant metastasis, and the acquisition of drug resistance [3]. A recent study revealed that colon cancer cells secreted exosomes containing noncoding RNA, namely miR-193a, to promote progression [4]. Moreover, these exosomes could be detected in the serum, thereby making them ideal prognostic markers. Furthermore, it has become clear that noncoding RNA molecules, once thought to be evolutionary remnants, play instrumental roles in virtually every aspect of cellular activities by providing another level of regulatory function and specificity in gene expression. In this study, we examined PVT1, a long noncoding RNA locus, which has been identified as a candidate oncogene in several tumors, including colon cancer [5]. However, its role and regulatory mechanisms in promoting distant metastasis in colon cancer remain unexplored.

Epithelial-mesenchymal transition (EMT) drives distant metastasis and generates cancer stemness [6, 7]. Therefore, characterizing the key molecular players involved in EMT could provide insights into this process and its mechanisms. Notably, a numerous EMT markers has been proposed, and they all converged toward angiogenesis, with vascular endothelial growth factor (VEGF)-associated signaling playing a crucial role, enabling cancer cells to translocate from their primary niche to a secondary site [8]. In addition, the EMT ability is associated with the emergence of cancer stemness, the ability to initiate tumor, and generate drug-resistant clones [9–10]. Hence, identifying mechanisms or agents capable of inhibiting the members of the VEGF-associated pathway could provide an opportunity to decrease the incidence of distant metastasis. The microarray data in the public data set classifies more than 400 other tumors. This allows us to analyze the clinical characteristics of colorectal cancer (such as survival time) and identify important prognostic markers [11].

In this study, we first collected tissues from both healthy individuals and patients with distant metastatic colon cancer and identified that PVT1 expression was significantly increased in patients with metastatic colon cancer. In addition, by searching public databases, we determined that exosomes from patients with metastatic colon cancer and patients with disease recurrence contained increased PVT1. Furthermore, the potential promoter function of PVT1 was examined both in vitro and in vivo. First, we
demonstrated that exosomes from patients with metastases significantly enhanced the migratory, invasive abilities of human colon cancer cell lines, HCT116 and LoVo, in association with the increased expression of vascular endothelial growth factor A (VEGFA), Vimentin, and MMP2. Notably, exosomes isolated from metastatic colon cancer promoted metastasis in patient-derived xenograft mice (with the nonmetastatic tumor). In vitro evidence indicated that PVT1-silenced HCT116 and LoVo cells exhibited significantly reduced tumorigenic properties, including colony- and tumorsphere-forming abilities as well as migratory and invasive potential. Finally, we explored the possible regulatory mechanism of PVT1 and observed that a tumor suppressor miR-152-3p had binding sites at the 3′UTR of PVT1 and VEGFA. By upregulating and downregulating miR-152-3p, we provided evidence for the regulatory machinery miR-152-3p/PVT1/VEGFA as part of the mechanistic explanation of metastatic colon cancer. Therefore, targeting this signaling axis could help in developing future effective therapeutic agents for metastatic colon cancer.

**Materials And Methods**

**Clinical sample collection and preparation**

Written informed consents for the collection of clinical samples were given to all the participants and IRB was approved by the medical ethics committee of Tri-Service General Hospital (TSGH-2019-00168) (Taipei, Taiwan). Serum samples were collected from healthy individuals, patients with primary colon cancer and metastatic based on the histological examinations (Supplementary Table 1). Fresh tissues (tumor, adjacent nontumor, and lung metastasis) obtained from patients with colon cancer were processed within 20 min after resection. The samples were then analyzed and confirmed by pathologists. Venous blood samples from patients were collected, and cell-free serum was isolated using a previously established protocol, which consisted of centrifugation of 1,600 g for 10 min, followed by another 16,000 g for 10 min at 4 °C. Samples either underwent exosome isolation immediately or were stored at −80 °C.

**Cell culture**

Human colon cancer cell lines HCT116 and LoVo were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells had been recently authenticated through short tandem repeat profiling by the Shanghai Biowing Applied Biotechnology Co. Ltd. (http://www.biowing.com.cn). Both cell lines were maintained and passaged in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO) in a 5% CO₂ humidified incubator at 37 °C until 90% confluence. Supernatants were collected, centrifuged for 1,600 g for 10 min, followed by centrifugation at 16,000 g for 10 min at 4 °C, and then stored at −80 °C until exosome extraction.

**Isolation of Exosomes**

Serum samples and culture medium were first filtered through a 0.45-μm pore polyvinylidene fluoride filter (Millipore, Darmstadt, Germany). ExoQuick solution (System Biosciences, Palo Alto, CA) was added to the serum samples and incubated at room temperature for 30 min, whereas ExoQuick-TC solution was added
to the culture medium samples and incubated at 4 °C for 12 h. Exosomes were sedimented and collected using centrifugation (1,500 g, 30 min). Resultant exosome pellets were resuspended in 25 μL of phosphate-buffered saline (PBS).

**Transmission electron microscopy**

Exosomes were diluted to a final concentration of 0.5 mg/mL using PBS. Exosomes were spotted onto a glow-discharged copper grid and dried. Samples were stained with a drop of 1% phosphotungstic acid for 5 min and dried. The morphological analysis of exosomes was performed using transmission electron microscopy (FEI TECNAI, Hillsboro, Oregon) at 200 keV.

**RNA preparations**

Total RNA was isolated from samples (tumor chunks and cell lines) using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and quantified using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). RNA from exosomes was extracted using a miRNeasy Micro Kit (QIAGEN, Hilden, Germany). In brief, the exosome suspension (20 μL) was mixed with QIAzol lysis buffer (700 μL) and processed per the vendor's protocol. RNA samples were subsequently eluted with 25 μL of RNase-free water (repeated twice with the same 25 μL of RNase-free water to concentrate the samples). The RNA concentration in the samples was again determined using NanoDrop.

**Real-time quantitative reverse transcription polymerase chain reaction**

Reverse transcription of miRNAs and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)-based quantification of miRNA levels were performed using miDETECT A Track miRNA qRT-PCR Starter Kit (RIBOBIO, Tokyo, Japan). For PVT1 gene expression analysis, the first strand of cDNA was generated using a PrimeScript first-strand cDNA synthesis kit (TaKaRa, Tokyo, Japan). The qRT-PCR was performed using the SYBR Premix Ex Taq II kit (Takara, Tokyo, Japan) on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA). The qPCR primers used in this study are listed in Supplementary Table 2.

**Migration and invasion assays**

Colon cancer cells were seeded and cultured in six-well plates for 24 h. The cells were incubated with mitomycin (10 μg/mL) for 1 h. A linear scratch was created through the cell monolayer using a 200-μL pipette tip. Cellular debris was removed, and the cells were allowed to migrate for 24–48 h. Gap healing was determined from micrographs taken before and after the wound was created under a microscope (Nikon, Japan). Migration distance was measured from images (three random fields) taken at indicated time points. The gap size was subsequently analyzed using ImageJ software. Invasion assay was performed according to a previously established protocol. In brief, 3 × 10⁵ colon cancer cells were seeded onto Matrigel (BD Biosciences, San Jose, CA, USA) in culture plate inserts (pore size: 8 μm, Corning) in a serum-free medium. Three independent and random fields per well were photographed, and the number
of cells per field was counted. An average of the three determinations was obtained for each chamber. Each invasion assay was performed a minimum of three times.

**Tumor spheroid formation assay**

Colon cancer cells were transferred to serum-free low-adhesion culture plates containing Dulbecco's modified Eagle's medium (DMEM)/F-12 with N2 supplement (Invitrogen), 20 ng/ml EGF and 20 ng/ml basic-FGF (stem-cell medium; PeproTech, Rocky Hill, NJ, USA) for 2 weeks to allow tumor sphere formation. The spheres were counted under microscope. The tumor ball formation efficiency was calculated as the ratio of the number of balls to the number of implanted cells.

**Cell Transfections**

Colon cancer cells (cell lines or clinical samples) were cultured and maintained, as described in the previous sections. Gene manipulation experiments, namely PVT1 silencing experiments, were performed using the siRNA technique (RIBOBIO, Guangzhou, China) according to the vendor's experimental instructions. Reagents for miR-152-3p mimic and inhibitor molecules and scramble negative controls were purchased from RIBOBIO (Guangzhou, China), and the transfection procedures were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

**Western blotting**

Total cellular protein lysates from colon cancer cells were extracted using RIPA buffer (Millipore, Darmstadt, Germany), and the lysate concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Thirty micrograms of protein were dissolved in SDS-PAGE and transferred onto PVDF membrane (Millipore, Darmstadt, Germany). After being blocked at 37 °C for 1 h, the membranes were immunoblotted with different antibodies at 4 °C overnight. All antibodies were purchased from Cell Signaling Technology (Danvers, MA) unless otherwise specified. Epidermal growth factor receptor (EGFR) (#2232, 1:400), MMP2 (#13667, 1:300), Vimentin (#5741, 1:500), Twist1 (#46702, 1:400), GAPDH (#D16H11, 1:2000); VEGFA (ab52917, 1:500, abcam, USA); exosomal markers CD9 (5G6, 1:200) and MCT-1 (P14612, 1:500, Invitrogen, USA) were purchased from Novus Biologicals (Centennial, CO, USA).

**In vivo mouse studies**

Mice were obtained from Laboratory Animal Center of Tri-Service General Hospital (TSGH) (Taipei, Taiwan). All experiments involved with mice were carried out according to the protocols approved by the Experimental Animal welfare committee of our institute (approval number: TSGH-2019-00168). For the patient-derived xenograft experiment, pieces of tumor mass (approximately 0.05 cm³ each, from patient diagnosed with colon cancer, non-metastatic) were subcutaneously implanted into NSG mice. Tumor-bearing mice were allowed for the establishment of tumor growth (until tumor became palpable), and exosomes isolated from patients with lung metastasis (M-exo) or primary colon cancer (non-metastatic, P-exo) were systematically injected through the lateral tail vein (20 µg/mouse, 3 times/week, for 4 weeks),
each group contained 5 mice. Tumor growth was monitored and measured using a standard caliper on a weekly basis. The tumor volume was determined using the following formula. Tumor volume = \( \text{width}^2 \times \text{length}/2 \). Animals were humanely sacrificed post experiments and tumor samples and tissues were collected for further analyses.

**Statistical analysis**

The statistical package for the Social Sciences (version 13.0; SPSS Inc., Chicago, IL) was used to perform all statistical analysis. Each experiment was performed three times. All data in the figures are expressed as mean ± standard deviation (SD). Comparison between two groups was performed using the t test. All statistical tests were two-sided, and \( p < 0.05 \) was considered significant. Continuous data were analyzed using paired t-test or Wilcoxon rank test. Categorical data were analyzed using \( \chi^2 \) or Fisher’s exact test. Survival analysis was estimated by Kaplan-Meier method accompanying using log-rank test to calculate differences between the curves.

**Results**

**Increased expression of PVT1 in colon cancer and influences survival rate**

Recent studies have revealed PVT1 to be an oncogenic marker for multiple cancer types. Using public, a database, figure 1A shows the expression of PVT1 in Pan-Cancer analysis from the Cancer Genome Atlas (TCGA) database, and PVT1 has a higher level of expression in colorectal cancer. We found that colon cancer patients (GSE17537) with a higher PVT1 expression had a significantly shorter survival time (Fig. 1B). Further analysis showed that the expression of PVT1 in advanced colorectal cancer is more significant and statistically significant (Fig. 1C). Since advanced colorectal cancer can be defined as colorectal cancer that metastasizes when it appears or recurs, we then analyze the difference between PVT1 in primary and metastasis tumor. Therefore, we analyzed the performance of PVT1 between primary and metastatic tumors from the GSE49355 dataset. The results show that PVT1 has a higher level of expression in metastatic tumors (Fig. 1D). Finally, we performed the correlation analysis between PVT1 and vascular endothelial growth factor A (VEGFA) from the Cancer Genome Atlas (TCGA) database. The results showed that PVT1 is positively correlated with VEGFA and the exosome biomarker MCT-1 (Fig. 1E). The above results link the possible relationship between exosomal IncRNA PVT1/VEGFA axis and metastatic colorectal cancer.

**Serum exosomes from patients with metastatic colon cancer patient promoted metastatic potential in non-metastatic colon cancer cells**

Once establishing that an increased level of PVT1 in either tissue samples or sera of colon cancer patients, we further analyzed the serum exosomes from patients. The representative micrographs are shown P-exo and M-exo denote the exosomes from primary (non-metastatic) and metastatic samples. The average size of the exosomes ranges from 100-200 nm. We also detected the expression levels of the two biomarkers CD9 and MCT-1 in exosomes. Western blots demonstrated increased CD9 and MCT-1
(exosome markers) in the sera collected from patients with metastatic colon cancer (M, M-exo) as compared to ones collected from patients with primary tumor (P, P-exo) (Fig. 2A). We cultured human colon cancer cells, HCT116 and LoVo with P-exo and M-exo under serum-deprived conditions. Subsequently, qPCR analysis showed that M-exo contained a significantly higher level of PVT1 and VEGFA as compared to that of P-exo (Fig. 2B). In addition, we found that both cell lines exhibited an increased ability to form tumor spheres with the presence of M-exo as compared to that with P-exo (Fig. 2C). In addition, the migratory and invasive ability of HCT116 and LoVo was significantly increased under the influence of M-exo but not P-exo (Fig. 2D). Western blots of both HCT116 and LoVo cells cultured with M-exo demonstrated the increased in EMT markers including Twist1, Vimentin and MMP2 as well as stemness marker, Sox2, as compared to their P-exo cultured counterparts (Fig. 2E).

**M-exo promoted distant metastasis in patient-derived xenograft mouse model**

Patient-derived xenograft (PDX) mouse models were established using NSG mice bearing patient samples from primary (non-metastatic) colon cancer. Serum exosomes, M-exo and P-exo were injected intravenously one-week post tumor implantation. Injections were given 3 times a week for 4 weeks. PDX mice received M-exo injections showed a significantly higher tumor and tissue growth (Fig. 3A), Mice received M-exo also exhibited more distant lesions in the lungs as compared to other groups (Fig. 3B); a similar observation was made in the lymph node metastasis (Fig. 3C). We also compared the ability to generate tumor spheres among the groups and found that tumor cells harvested from M-exo mice exhibited an enhanced ability to form tumor spheres, in both numbers and size (Fig. 3D); the number of spheres formed from P-exo injected mice group did not show significant difference when compared with the control group.

**PVT1-silencing led to reduced colon tumorigenic properties**

We intended to demonstrate the functional roles of PVT1 in colon cancer by silencing PVT1 through the siRNA technique. PVT1-silenced HCT116 and LoVo cells had a significantly reduced colony-forming ability (Fig. 4A). For example, PVT1-silenced LoVo cells formed approximately 70% fewer colonies than their counterparts (Fig. 4A). HCT116 and LoVo cells transfected with si-PVT1 were significantly less potent in forming tumorspheres compared with their parental cells (Fig. 4B). Our bioinformatics research was supported by the fact that PVT1-silenced HCT116 and LoVo cells contained a significantly lower mRNA (left panels, Fig. 4C) and protein (right panels, Fig. 4C) expression levels of metastatic markers, including VEGFA, MMP2, Twist1, and oncogenic marker EGFR. This observation was reflected by the markedly suppressed migratory (Fig. 4D) and invasive abilities (Fig. 4E). The downregulation of PVT1 suppressed the migratory ability in HCT116 cells approximately by 50% and 55% in LoVo cells; the invasive ability was reduced by at least 50% in both HCT116 and LoVo cells post PVT1 silencing.

**Tumor suppressor miR-152-3p inhibits the expression of PVT1 and metastatic potential in colon cancer**

Furthermore, through different online algorithms, miR-152-3p was identified as a potential inhibitor of PVT1 and VEGFA. The potential binding sequences are indicated in Figure 5A. On the basis of this
finding, we employed miR-152-3p mimic and inhibitor molecules to determine their effects on colon tumorigenesis. An increased level of miR-152-3p in both HCT116 and LoVo cells led to a significantly lower expression of PVT1, VEGFA, and EGFR (Fig. 5B). Moreover, the overexpression of miR-152-3p significantly reduced the tumorsphere-forming ability in both cell lines (Fig. 5C). However, the subsequent addition of miR-152-3p inhibitor led to a partial restoration of the ability to form tumorspheres (Fig. 5C). In addition, a similar observation was made regarding invasive ability. The invasive ability of HCT116 and LoVo cells were significantly reduced when the cells were transfected with miR-152-3p mimic molecules (Fig. 5D), with invasiveness restored by the subsequent addition of miR-152-3p inhibitor (Fig. 5D). These findings were supported by the Western blots, which revealed decreased levels of metastasis-associated markers such as VEGFA, Vimentin, and oncogenic marker EGFR in the mimic-transfected cells (Lane M, Fig. 5E) that were rescued by the addition of miR-152-3p inhibitor (Lane I, Fig. 5E). Additional support came from the analysis of the TCGA database [12], comprising 450 patients with colon cancer, which revealed a negative correlation between the expression of PVT1 and miR-152-3p (coefficient $R$ value = -0.149 and $P$ value = 1.58E-03) (Fig. 5F).

Discussion

Exosome is a small vesicle secreted by cells, which is a type of extracellular vesicles (EVs). It can be found in various body fluids, and it plays the role of communication between cells. In recent years, researchers have discovered that the protein and related RNA contained in Exosome can be analyzed as biological indicators for cancer diagnosis and prognosis assessment. PVT1 is an oncogenic IncRNA and is associated with many cancer types, including colorectal cancer and gastric cancer. PVT1 participates in many aspects of cancer biology through a complex signal network, such as tumor growth, metastasis, and response to chemotherapy and radiotherapy. This complex signal network involves interactions with DNA, RNA, and proteins. This study identified PVT1 as a prognostic biomarker in patients with colon cancer. Notably, patients with higher PVT1 expression had a poor survival ratio compared with patients with a lower PVT1 level. In addition, a higher PVT1 level was noted in the serum of patients with recurrent disease. Notably, serum exosomes isolated from patients with and without metastasis revealed a comparatively increased PVT1 levels in the exosomes of metastatic samples. Moreover, cell line experiments exhibited elevated PVT1 level in HT29 colon spheres compared with their parental counterparts, along with increased VEGFA and EGFR—the two major metastatic or oncogenic markers in colon cancer. These observations suggested that PVT1 in serum exosomes might play a crucial role in promoting metastasis in colon cancer.

Furthermore, our comparative experiments by using M-exo and P-exo revealed that M-exo not only contained a significantly higher PVT1 level compared with that in P-exo but also promoted migration and invasion in human colon cancer cell lines HCT116 and LoVo. This finding indicated that exosomes enriched with PVT1 could be one of the venues for colon cancer cells with metastatic ability to transform the neighboring cancer cells. Moreover, this phenomenon is observed in non–small cell lung cancer, wherein a long noncoding RNA, MALAT1, was observed to be protected by exosomes and involved in the promotion of distant metastasis [13]. Concordantly, we noted that the serum exosomes from patients
with metastatic colon cancer contained a higher level of PVT1 compared with those from patients with primary, nonmetastatic colon cancer. Therefore, PVT1-enriched serum exosomes could be used as a potential new prognostic marker for metastatic colon cancer.

Notably, another phenomenon observed in our study was the association between increased PVT1 expression and the generation of colon tumorspheres. In addition, the PVT1 level was significantly higher in HT29 tumorspheres (based on our public database analysis). Furthermore, our in-house in vitro experiments revealed that the ability to form tumorspheres was severely compromised when PVT1 was silenced in both HCT116 and LoVo cells. Moreover, M-exo promoted the formation of tumorspheres in our PDX mouse model. This finding provides an indirect link to the concept of EMT-promoted stemness hypothesis first proposed by Weinberg [14]. In the case of colon cancer, it was reported that the presence of CD26 (+) cells in primary tumors could predict distant metastasis on the follow-up. Notably, isolated CD26 (+) cells, not CD26 (−) cells, caused distant metastasis when injected into the mice [15]. This finding was similar to what we had observed in vivo wherein the PVT1-enriched M-exo promoted distant metastasis in PDX mice inoculated with primary (nonmetastatic) clinical colon cancer samples. On the basis of these reports and our observations, it could be concluded that PVT1-enriched exosomes could function as the promoter of EMT and cancer stemness in colon cancer. Nevertheless, the exact mechanistic role of PVT1 in the promotion of stemness warrants further investigation and is currently under exploration in our laboratory.

VEGFA plays a vital role in promoting colon metastasis by engaging with Sox2-associated signaling [16]. This finding was concordant with our data, wherein the addition of exosomes isolated from metastatic colon cancer promoted the generation of tumorspheres in both HCT116 and LoVo cells in association with increased Sox2 expression. Notably, EGFR and VEGF mutations are often noted to be associated with increased metastatic incidence and poor prognosis in colon cancer [17]. Our observation of PVT1 downregulation leading to the reduced expression of VEGFA, EGFR, Twist1, and MMP2 provided an insight into PVT1’s role in promoting metastasis. On the basis of our database analysis, PVT1, VEGFA, and EGFR expression levels were significantly elevated in the HT29 spheres compared with their parental cells [18]. Even though the present study did not establish a causal relationship among the expression of these three molecules, a common regulatory molecule, namely miR-152-3p, was identified as the inhibitor of both PVT1 and VEGFA. First, the potential binding sites of miR-152-3p to PVT1 and VEGFA were identified and revealed that the increased expression of miR-152-3p led to the significantly reduced expression of PVT1, VEGFA, and EGFR in both HCT116 and LoVo cells. A previous report indicated that PVT1 was linked to the activation of STAT3/VEGFA signaling and promoted angiogenesis in patients with gastric cancer [19]. According to the available data, miR-152-3p has been indicated to be a tumor suppressor. For example, miR-152-3p was observed to negatively regulate PIK3CA expression to inhibit the activation of AKT and RPS6 in breast cancer cells [12]. In addition, the downregulation of miR-152, which targets DNMT1 (an oncogene or cancer stemness marker), was observed in breast cancer cells [20]. Notably, a negative correlation between the expression levels of PVT1 and miR-152-3p was identified in the TCGA colon cancer database consisting of 450 patient samples [12]. Recent reports have demonstrated that PVT1 regulates VEGFA/VEGFR1/AKT Axis and promotes tumorigenesis of colorectal
cancer and deletion of PVT1 can reduce tumor volume. It is well known that overexpression of VEGFA regulates the AKT signaling cascade by activating vascular endothelial growth factor receptor 1 (VEGFR1) [21]. Colorectal cancer patients with high expression of PVT1-214 have a shorter survival period and a poor prognosis. In vivo and in vitro studies showed that the effects of PVT1-214 have revealed a complex phenotype affecting cell growth, stem-like properties, migration and invasion [22]. PVT1 could indirectly regulate the expression of four jointed box 1 (FJX1) by targeting miR-106b-5p. By regulating the miR-106b-5p/FJX1 axis, knockdown of PVT1 can impair cell proliferation, migration and invasion in colorectal Cancer [23]. As has been shown in a large number of studies, PVT1 overexpression is related to cancer growth and proliferation in vivo and in vitro. It is obvious from human tissue and mouse xenotransplantation studies that the overall survival rate and tumor size of many cancer types are related to PVT1 overexpression [24, 25].

Long non-coding RNA (IncRNA) is one of the important types of ncRNA. In addition, the role of PVT1 in cancer development is closely related to microRNA (miRNA). PVT1 can be spliced and processed into a variety of miRNAs, such as miR-1204 and miR-1207. It also can act as a "sponge" of miRNA and inhibit the activity of miRNA, thereby affecting cancer proliferation, invasion and angiogenesis [26]. Cancers of the digestive system have a high risk of morbidity and mortality. The expression of PVT1 is elevated in a variety of digestive system cancers and is associated with poor prognosis. Therefore, PVT1 is likely to become a new biomarker for screening tumors, prognostic biomarkers and future targeted therapies to improve the survival rate of patients [27–29]. Liquid biopsy-the determination has become a novel method in all cancer tests. Circulating non-coding RNA (ncRNA) (such as miRNA, IncRNA) is a biomarker that is easily obtained from the blood and can be used for early diagnosis and prediction of prognosis and treatment response. Long non-coding RNA (IncRNA) can be passively released through tissue or carry exosomes [30]. Our research on exosomal IncRNA PVT1/VEGFA axis confirms this value. PVT1 in the patient's serum exosomes could affect the metastasis and invasion of in vivo and in vitro model.

In conclusion, as shown in schema abstract Fig. 6, we have provided evidence for the potential role of PVT1/VEGFA-enriched serum exosomes in promoting distant metastasis in colon cancer. Both the in vitro and in vivo experiments revealed that increased PVT1 expression was associated with increased metastatic potential and stemness, as reflected by the concomitantly increased expression of VEGFA, EGFR, and Sox2. Moreover, the downregulation of PVT1 resulted in the significantly suppressed metastatic ability in colon cancer cells, and it was observed to be regulated by the tumor suppressor, miR-152-3p. Nevertheless, future studies should explore this perspective further and analyze the possibility of designing agents that can increase miR-152-3p expression for treating metastatic colon cancer.

**Abbreviations**

Cancer stem cells (CSCs); docetaxel (DTX); epithelial-to-mesenchymal transition (EMT); extracellular matrix (ECM); 5-flurouracil (5-FU); fetal bovine serum (FBS); immunofluorescence (IFC); sulforhodamine B (SRB); Trichloroacetic acid (TCA); tumor-initiating cells (TICs).
Declarations

Authors’ contribution

Conceived and designed the study: Shiue-Wei Lai. Performed the experiments: Ming-Yao Chen. Analyzed the data: Ming-Shou Hsieh. Bioinformatics: Ting-Yi Huang. Wrote the manuscript: Chi-Tai Yeh and Wei-Hwa Lee. Provided reagents, materials, experimental infrastructure and administrative oversight: Yih-Giun Cherng. All authors read and approved the final version of the manuscript.

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Ethics approval and consent to participate

Written informed consents for the collection of clinical samples were given to all the participants and IRB was approved by the medical ethics committee of Tri-Service General Hospital (TSGH-2019-00168) (Taipei, Taiwan). Serum samples were collected from healthy individuals, patients with primary colon cancer and metastatic based on the histological examinations.

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author in response to reasonable requests.

Conflict of interest

The authors declare that they have no potential financial competing interests that may in any way gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no non-financial competing interests are involved in the manuscript.

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Conflict of interest statement

The authors declare that there are no potential conflicts of interest.

References


**Figures**
Figure 1

Expression analyses of PVT1 expression in clinical samples of colon cancer. (A) The expression of PVT1 in Pan-Cancer analysis from the Cancer Genome Atlas (TCGA) database. (B) Kaplan-Meier survival curve constructed from GSE17537 database. Patients with a higher PVT1 expression showed a significantly lower disease specific survival rate. (C) PVT1 expression in different stages of colorectal cancer. The expression of PVT1 in advanced colorectal cancer is more significant and statistically significant, P<
0.05. (D) The difference between PVT1 in primary and metastasis tumor from the GSE49355 dataset. (E) The correlation analysis between PVT1 and vascular endothelial growth factor A (VEGFA), PVT1 and exosome biomarker MCT-1 from the Cancer Genome Atlas (TCGA) database. * p < 0.05; ** p < 0.01.

Figure 2

Comparative analysis of exosomes from sera of patients with primary and metastatic colon cancer. (A) Serum exosomes were isolated from patients with primary tumor (P-exo) and distant metastasis (M-exo).
Representative electromagnetic images of the exosomes are shown. Scale bar: 1 μm. Western blots demonstrated increased CD9 and MCT-1 (exosome markers) in the sera collected from patients with metastatic colon cancer (M, M-exo) as compared to ones collected from patients with primary tumor (P, P-exo). (B) Comparative qPCR analysis showed that level of PVT1 and VEGFA was significantly higher in the M-exo as compared to the P-exo. (C) Sphere-forming assay showed that the addition of M-exo led to an increased number of tumor spheres formed in both cell lines as compared to their control and P-exo groups. (D) Exosomes and colon cancer cell line co-culture experiment. HCT116 and LoVo cells co-cultured with M-exo demonstrated enhanced migratory (upper panels) and (lower panels) invasive abilities. (E) Western blot analysis demonstrated an elevation in metastatic markers including Twist1, Vimentin, MMP2 and stemness marker Sox2, in the HCT116 and LoVo cells co-cultured with M-exo as compared to the counterparts with P-exo. * p < 0.05; ** p < 0.01; *** p < 0.001. Scale bar: 100 μm.
Figure 3

M-exo injection promotes tumor growth and distant metastasis in PDX mouse model. (A) PDX mice (n=3) were divided into control, M-exo and P-exo groups. Mice injected with M-exo showed a higher tumor volum and weight (week 4) as compared to the control and P-exo groups. Distant lung (B) and lymph node (C) metastasis analysis. Lesions in the lungs from all three groups were examined and counted. M-exo group showed the highest number of lung lesions followed by P-exo and control group. (D)
Comparative analysis of tumor sphere forming ability. Tumor samples harvested from M-exo injected mice were able to form a greater number of spheres; the P-exo and control groups were not significantly different. * p < 0.05; ** p < 0.01; *** p < 0.001.

Figure 4

PVT1-silencing suppressed colon tumorigenic and metastatic potential. (A) Colony formation assay revealed that si-PVT1-transfected HCT116 and LoVo cells formed a significantly lower number of colonies compared with the control parental cells. (B) Comparative tumor sphere-forming assay. HCT116 and LoVo cells transfected with si-PVT1 were significantly less potent in forming tumor spheres compared with their parental cells. (C) Comparison of expression between parental colon cancer cells and PVT1-silenced cells. Left panels: Quantitative PCR analysis demonstrated markedly reduced metastatic markers, VEGFA, Twist1, MMP2, and oncogenic marker EGFR in si-PVT1 colon cells. Right panels: Western blots of parental versus PVT1-silenced HCT116 and LoVo cells. Prominent reduction in VEGFA, Twist1, MMP2, and EGFR was seen after PVT1-silencing in both cell lines. Effect of PVT1 expression on cell migration (D) and invasion (E) of HCT116 and LoVo cells detected using Transwell assays. * p < 0.05; ** p < 0.01; *** p < 0.001. Scale bar: 100 μm.
Figure 5

Targets validation for miR-152-3p and its role in suppression metastasis. (A) Target binding sequences of miR-152-3p in 3' UTR of PVT1 and VEGFA. These binding sites were predicted using both miRmap and MiRanda software. (B) qPCR analysis of PVT1, EGFR, and VEGFA levels in response to sequential miR-152-3p mimic and inhibitor transfections. A significant decrease in mRNA levels of PVT1, EGFR, and VEGFA after miR16-5p mimic transfection and subsequent restoration with the addition of the inhibitor of
miR-152-3p. Both HCT116 and LoVo cells had a similar trend. (C) Tumorsphere formation assay. The tumorsphere-forming ability was greatly inhibited by the transfection of miR-152-3p in both HCT116 and LoVo cells; partial restoration of tumorsphere-forming ability was noted when the inhibitor of miR-152-3p was added. (D) Invasion assay revealed that an increase in miR-152-3p led to the significantly reduced invasive ability in both HCT116 and LoVo cells, which was, however, restored by decreasing miR-152-3p with the addition of its inhibitor. (E) Western blot analysis. The addition of miR-152-3p mimics suppressed the expression of EGFR, Vimentin, and VEGFA in both HCT116 and LoVo cells, and the inhibitor restored their expression. (F) A negative correlation was noted between miR-152-3p and PVT1 levels in colon cancer clinical samples from TCGA databases [12] (N = 450). * p < 0.05; ** p < 0.01; *** p < 0.001. Scale bar: 100 μm.
**Figure 6**

Schematic abstract showing that the role of exosomal PVT1 in promoting metastasis in colon cancer via its association with EGFR and VEGFA expression.

**Supplementary Files**

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- PictorialAbstractCMET.docx
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