

Expression and clinical significance of the long non-coding RNA *PVT1* in human gastric cancer

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Background: Highly sensitive markers are urgently needed for the diagnosis and grading of gastric cancer and for managing drug resistance. The recent identification of long-non-coding RNAs (lncRNAs) has provided new approaches for resolving this challenge. The aim of this study was to screen and identify new biomarkers for human gastric cancer from lncRNAs.

Methods: First, we used lncRNA microarrays to conduct a preliminary screening for candidate lncRNAs of gastric cancer biomarkers in both human gastric cancer tissues and in two gastric cancer cell lines, SGC7901 cells and paclitaxel-resistant SGC7901 cells. The lncRNA plasmacytoma variant translocation 1 (*PVT1*) was found to exhibit higher expression in both gastric cancer tissues and the SGC7901 paclitaxel-resistant cell line. Quantitative polymerase chain reaction was used for large-scale analysis in a large number of human gastric cancer tissues to verify the involvement of *PVT1* in development of gastric cancer. The relationships between *PVT1* expression and clinical features were also analyzed.

Results: *PVT1* showed higher expression in human gastric cancer tissues than in adjacent non-cancerous tissues and in SGC7901 paclitaxel-resistant cells compared with SGC7901 cells. *PVT1* expression was correlated with lymph node invasion of gastric cancer.

Conclusion: *PVT1* is a new biomarker for human gastric cancer and may indicate lymph node invasion. Therefore, *PVT1* shows potential as a novel therapeutic target for the treatment of gastric cancer and enhancement of paclitaxel sensitivity.

Keywords: microarray analysis, quantitative polymerase chain reaction, lymph node invasion, tumor biomarkers, paclitaxel resistance

Introduction

Gastric cancer remains a major public health concern as it represents the fourth most common cancer and the second leading cause of cancer-related deaths worldwide.¹⁻³ Nevertheless, the incidence and mortality associated with gastric cancer have decreased considerably over the past 50 years in most areas of the world.¹ Although conventional diagnostic methods such as gastric endoscopy have contributed to this reduction by enabling early diagnosis, many patients are diagnosed with advanced gastric cancer and have a poor prognosis. Thus, more sensitive tumor markers are urgently needed to improve screening, diagnosis, prognostic evaluation, and tumor grading of gastric cancer. Several potential approaches have been proposed to find suitable marker candidates, and the recent discovery of long-non-coding RNAs (lncRNAs) provides a new opportunity to resolve this challenge. lncRNAs are non-protein-coding transcripts longer than 200 nucleotides⁴ and are widely distributed in the genome; indeed, more than 90% of the human genome is composed of non-coding RNAs.⁵ There is mounting evidence

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that many lncRNAs are dysregulated in various cancers and play important roles in tumorigenesis and invasion.⁶⁻⁸ Thus, lncRNAs may serve as useful tumor markers and therapeutic targets for controlling malignant tumors. In the current study, we investigated the expression of plasmacytoma variant translocation 1 (*PVT1*), a documented oncogenic lncRNA,⁹ in gastric cancer and examined its association with clinical features in patients with gastric cancer to reveal its potential as a biomarker and therapeutic target.

Materials and methods

Thirty-three tumor tissues and corresponding non-cancerous tissues were obtained at the time of surgery from June 2012 to October 2013 at the First Affiliated Hospital and Union Hospital of Fujian Medical University, Fuzhou, Fujian, People's Republic of China. Following excision, the gastric cancer and non-cancerous tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

The SGC7901 gastric cancer cell line was obtained from the China Center for Type Culture Collection (Wuhan, People's Republic of China). Paclitaxel-resistant SGC7901 cells were developed in our laboratory and cultured in $450\text{ }\mu\text{g/L}$ paclitaxel. Total mRNA was isolated using TRIzol[®] Reagent (Life Technologies Corporation, Carlsbad, CA, USA), according to the manufacturer's protocol.

The paclitaxel-resistant cell line was developed by culture of an SGC7901 gastric cancer cell line in vitro with an increasing gradient of concentrations of paclitaxel intermittently over 6 months. The initial concentration of paclitaxel was $25\text{ }\mu\text{g/L}$. Forty-eight hours later, medium containing $25\text{ }\mu\text{g/L}$ paclitaxel was replaced with routine medium until the SGC7901 cells were growing in exponential period, after which the paclitaxel concentration was increased to $50\text{ }\mu\text{g/L}$. Six months later, the final concentration of paclitaxel was $450\text{ }\mu\text{g/L}$, and the resistance in paclitaxel-resistant SGC7901 cells was detected with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and compared with normal SGC7901 cells. The MTT assay was performed after 48 hours of incubation with paclitaxel (five concentrations in a multiple proportion dilution from 160 to $10\text{ }\mu\text{g/L}$), the plates were centrifuged to pellet the cells, the supernatant was removed, and $20\text{ }\mu\text{L}$ of 5 mg/mL MTT was added in $180\text{ }\mu\text{L}$ of medium, followed by incubation for 4 hours at 37°C in a humid, 5% CO_2 atmosphere. Next, the plates were centrifuged again, the supernatant was removed, and the insoluble formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was read at 490 nm . The half maximal inhibitory concentration (IC_{50}) was calculated using the Spearman-Kärber cytopathic effect method.¹⁰

Before lncRNA microarray analysis, the MTT assay showed IC_{50} values for SGC7901 and paclitaxel-resistant SGC7901 of $22.16\pm 0.31\text{ }\mu\text{g/L}$ and $141.31\pm 0.9\text{ }\mu\text{g/L}$, respectively. The difference was statistically significant ($P<0.001$). The drug resistance index for paclitaxel-resistant SGC7901 cells was 6.38.

Microarray analysis

Tissue samples (two gastric cancer tissues and two corresponding non-cancerous tissues) and SGC7901 cells were used to synthesize double-stranded complementary DNA, which was labeled according to the protocol of the Amplification and Labeling Kit for Microarray (CapitalBio Corporation, Beijing, People's Republic of China). The labeled complementary DNA was hybridized to the Agilent Human lncRNA $4\times 180\text{ K}$ Expression Microarray (Agilent Technologies Inc., Santa Clara, CA, USA), which contains 37,000 human lncRNAs and 34,000 mRNA probes (CapitalBio Corporation). After hybridization and washing, the processed slides were scanned with a G2565CA microarray scanner (Agilent Technologies). Raw data were extracted as paired files using Feature Extraction software (Agilent Technologies). The raw data were normalized using GeneSpringGX11 software (Agilent Technologies) with default parameters. Differential expression of lncRNAs was screened in genes flagged as "detected" by the software. Differentially expressed genes were identified through the random variance model.

Quantitative polymerase chain reaction

Total mRNA from 31 gastric cancer tissues and 31 corresponding non-tumor tissues was isolated using TRIzol Reagent according to the manufacturer's protocol. Complementary DNAs from all samples were synthesized from $1.0\text{ }\mu\text{g}$ of total RNA using the PrimeScript[®] 1st strand cDNA synthesis kit (TaKaRa Bio, Shiga, Japan) following the manufacturer's protocol. *PVT1* expression levels were quantified using a LightCycler Brilliant SYBR Green quantitative reverse transcriptase polymerase chain reaction (PCR) kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol, with the following primers: forward, TTGGCACATACAGCCATCAT and reverse, GCAGTAAAGGGGAACACCA. The length of the quantitative PCR product was 102 base pairs. The *PVT1* expression level in each sample was normalized to the respective human β -actin expression level, with the following primers: forward, AGCGAGCATCCCCAAAGTT; and reverse, GGGCACGAAGGCTCATCATT (product length, 285 base pairs). The specificity of each PCR reaction was confirmed by melting curve analysis.

Statistical analysis

The statistical significance of differences between cancerous and adjacent non-cancerous tissues was determined using the Wilcoxon signed-rank test. The correlation between *PVT1* expression and patient clinicopathological variables was analyzed by the Fisher's exact probability test. A *P*-value <0.05 was regarded as being statistically significant. All statistical analyses were performed using Statistical Package for the Social Sciences version 11.0 software for Windows (SPSS Inc., Chicago, IL, USA).

Results

Microarray analysis

A total of 252 lncRNAs were upregulated in gastric cancer tissues identified from the microarrays. Twenty-eight of the upregulated lncRNAs had an obvious fold change in the gastric cancer tissues when compared with corresponding non-tumor

tissues (Figure 1 and Table 1). In total, 545 lncRNAs were upregulated in paclitaxel-resistant SGC7901 cells when compared with normal SGC7901 cells (data supplied). *PVT1* expression levels in the gastric cancer tissues were 5.128 and 5.755 times higher than those in the corresponding non-tumor tissues (Table 1). In addition, *PVT1* expression was 3.715 times higher in paclitaxel-resistant SGC7901 cells than in normal SGC7901 cells (data supplied). Based on these results and recent discoveries concerning the role of *PVT1* in tumorigenesis and resistance to chemotherapy,^{9,11} we studied the expression of *PVT1* further in patients with gastric cancer and its correlation with clinical features.

PVT1 expression

PVT1 expression levels in cancerous and adjacent non-cancerous tissues from patients with gastric cancer were examined by quantitative reverse transcriptase PCR and

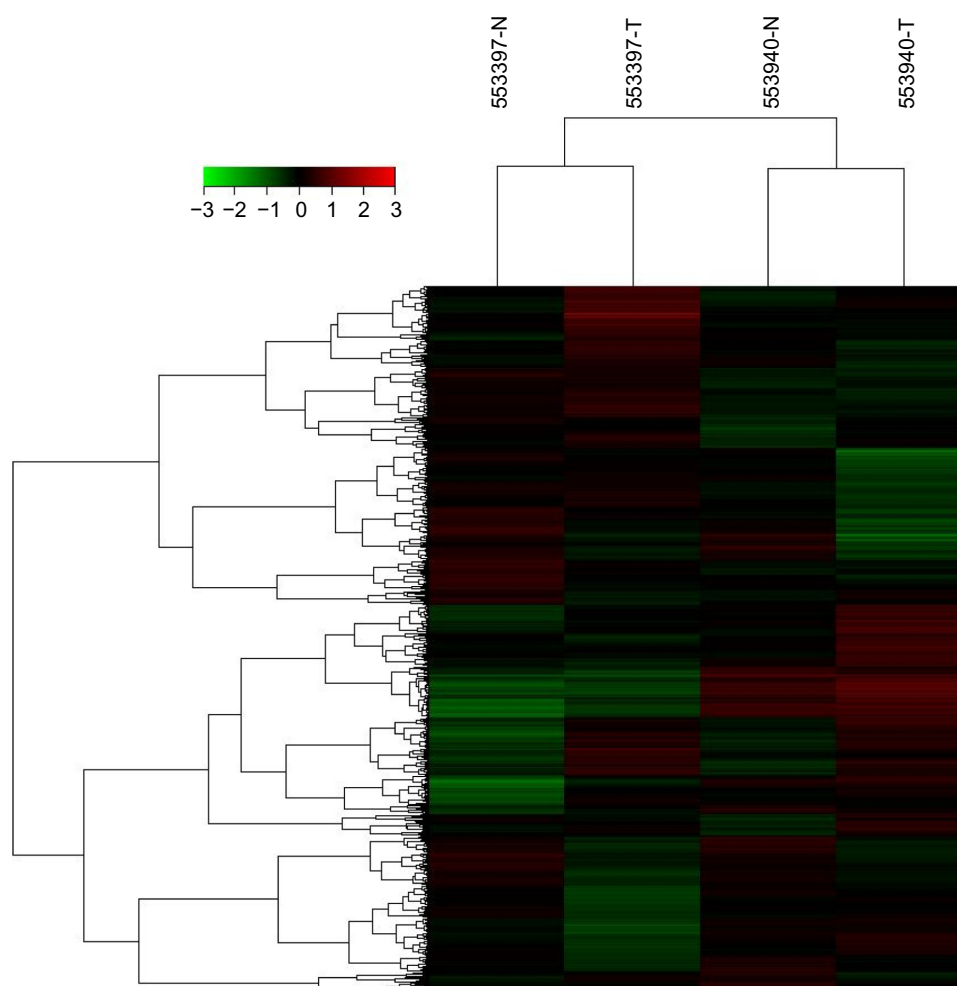


Figure 1 Cluster diagram of lncRNA expression data in gastric cancer and corresponding non-tumor tissues detected by the Agilent Human lncRNA 4×180 K Expression Microarray (Agilent Technologies Inc., Santa Clara, CA, USA).

Abbreviation: lncRNA, long-non-coding RNA.

Table 1 Twenty-eight candidate lncRNAs expressed with fold changes in two gastric cancer tissues, compared with corresponding non-tumor tissues (only the most obvious data are shown for the lncRNAs with different transcripts)

Fold change of T1 vs N1	Fold change of T2 vs N2	Gene biotype	Ensemble gene ID	HGNC symbol
27.20002	104.10264	Antisense	ENSG00000238133	MLK7-AS1
16.414074	4.819842	LncRNA	ENSG00000258294	RP11-314D7.1
12.021113	5.851729	LncRNA	ENSG00000236039	AC019117.2
10.219363	5.664894	LncRNA	ENSG00000241163	RP11-398A8.3
8.035696	3.0575392	LncRNA	ENSG00000222041	LINC00152
6.8010974	2.6033688	LncRNA	ENSG00000226598	AC017060.1
5.912395	4.000447	Antisense	ENSG00000224078	SNHG14
5.128479	5.754559	LncRNA	ENSG00000249859	PVT1
4.606247	4.3609843	LncRNA	ENSG00000167117	LINC00483
3.909583	2.458897	LncRNA	ENSG00000244306	CTD-2314B22.3
3.8943083	4.077277	LncRNA	ENSG00000224397	RP11-290F20.3
3.698091	5.106683	Antisense	ENSG00000243766	HOTTIP
3.5476947	4.3146453	Antisense	ENSG00000254560	RP11-1L12.3
3.3448012	4.3363175	Antisense	ENSG00000251179	RP11-893F2.9
3.2606792	2.2400966	Polymorphic_pseudogene	ENSG00000244682	FCGR2C
3.0598006	3.2264988	LncRNA	ENSG00000230061	AP001065.2
3.0492082	3.112142	LncRNA	ENSG00000233930	RP13-25N22.1
2.9770749	4.8328776	LncRNA	ENSG00000227888	FAM66A
2.8799016	2.383093	Processed transcript	ENSG00000233283	RP11-357H14.20
2.8781404	2.6564493	Processed transcript	ENSG00000250610	RP11-738E22.2
2.7795174	2.031886	LncRNA	ENSG00000230944	AC026202.5
2.7087636	25.26169	LncRNA	ENSG00000204241	RP11-713P17.3
2.5824153	2.942296	LncRNA	ENSG00000136315	RP11-84C10.2
2.453376	2.046279	LncRNA	ENSG00000214548	MEG3
2.4336877	3.0326896	LncRNA	ENSG00000248227	RP11-83C7.1
2.2753818	2.2845905	LncRNA	ENSG00000256969	RP11-320N7.2
2.1950364	4.0923414	LncRNA	ENSG00000245975	RP11-30K9.6
2.125026	2.8170245	LncRNA	ENSG00000235572	RP11-555H7.2

Abbreviations: HGNC, Human Genome Nomenclature Committee; lncRNAs, long-noncoding RNAs.

normalized by β -actin expression levels. The Wilcoxon signed-rank test showed that *PVT1* expression was significantly higher in cancerous tissues than in adjacent non-cancerous tissues ($P=0.0414$).

PVT1 expression and clinical features

In Figure 2 and Table 2, the human gastric cancer tissues are classified according to *PVT1* expression level into a high *PVT1* expression group (*PVT1* in cancer tissue/*PVT1* in non-cancerous tissue >1.0) and a low *PVT1* expression group (*PVT1* in cancer tissue/*PVT1* in non-cancerous tissue <1.0). The association of *PVT1* expression with the clinicopathological features of patients with intestinal-type gastric cancer is summarized in Table 2. A significant association was found between *PVT1* expression and lymph node metastasis ($P=0.022$). No correlation was found between *PVT1* expression and patient age, sex, tumor metastasis, or stage of gastric cancer.

Discussion

A set of lncRNAs showed differential expression between human gastric cancer and non-cancerous tissue. Thus, these

lncRNAs may play important roles in the carcinogenesis and invasiveness of human gastric cancer and are potential candidates for new biomarkers and therapeutic targets.⁶⁻⁹

PVT1, one such oncogenic lncRNA, is a large (>300 kb) locus located 57 kb downstream of *MYC* on human chromosome 8q24.¹²⁻¹⁵ Although no protein product or regulatory RNA has yet been identified among *PVT1* transcripts, the importance of this locus has caught the attention of many researchers, as it is a site of both tumorigenic translocations and retroviral insertions. Translocation breakpoints within either the *MYC* or *PVT1* locus are responsible for the characteristic lesions associated with Burkitt lymphoma and mouse plasmacytomas.¹³⁻¹⁸

Coamplification of human *MYC* and *PVT1* has been shown to correlate with rapid progression of breast cancer as well as poor clinical survival in postmenopausal women and in patients with HER2-positive breast cancer.¹⁹ Recent studies have discovered a cluster of miRNAs within the *PVT1* genomic DNA region, including miR1204, miR1205, miR1206, miR1207, and miR1208.^{20,21} The regulatory functions of these miRNAs within MYC-PVT1

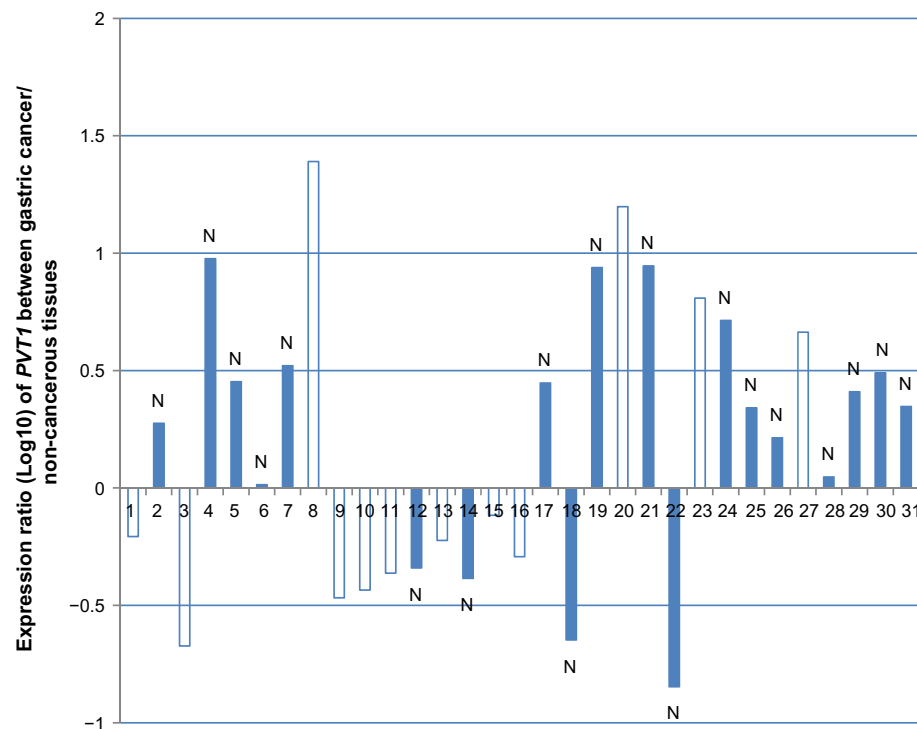


Figure 2 Ratio of relative *PVT1* expression in cancerous tissues versus adjacent noncancerous tissues from 31 patients with gastric cancer, examined by quantitative reverse transcriptase polymerase chain reaction. N indicates the number of patients with lymph node metastasis.

locus are complicated, only the function of miR1204 has been partially proved. MiR1204 plays an antiproliferative role, inducing cell death or cell cycle arrest, as a functional target of p53 at the *PVT1* locus. MiR1204 expression can also increase p53 levels, suggesting there is a positive feedback between miR-1204 and p53.²² So far, at least

one *PVT1* transcript microRNA product, miR1204, has been shown to be overexpressed and is thus defined as oncogenic.^{9,22} These additional non-coding RNA transcripts, exonic *PVT1* sequences, and other miRs are now thought to be either transcriptional byproducts of miR1204 regulation or additional functional targets, but no further evidence is provided. So, except for two aspects of the mechanism of *PVT1* in the prognosis of cancer (including a positive feedback regulation with p53 and coamplification with *MYC*), more studies need to be directed to the complex networks between *PVT1*, p53, *MYC*, and other genes.

In this study, we examined the expression of lncRNAs with 77×10^3 probes from the Agilent Human 4×180 K Microarray to identify specific lncRNA markers for human gastric cancers at the gene level. These probes correspond to 37,000 lncRNAs and 34,000 mRNAs. We also compared lncRNA expression between an SGC7901 human gastric cancer cell line and a paclitaxel-resistant SGC7901 cell line. The differential expression observed may reveal the mechanism of and biomarkers for paclitaxel resistance in human gastric cancer. Based on the microarray results and in light of recent reports,^{11,13–19} we ranked *PVT1* as the lncRNA with the highest potential as a biomarker and a therapeutic target for gastric cancer.

Table 2 Association between *PVT1* expression and clinicopathological factors in human gastric cancers

	High <i>PVT1</i> expression	Low <i>PVT1</i> expression	P-value ^a
Age	60.7895±7.0441	64.7500±9.0566	0.1827
Sex			0.1285
Male	11	10	
Female	8	2	
T			1.0000
T1, T2	4	3	
T3, T4	15	9	
N			0.0220
N0	4	8	
N1, N2, N3	15	4	
M			0.2645
M0	16	12	
M1	3	0	
Stage			0.0596
I, 2	5	8	
3, 4	14	4	

Note: ^aFisher's exact probability test.

In order to compensate for the deficiency of clinical cases in the microarray analysis and to develop a convenient and economical method for large-scale clinical studies, we used the quantitative PCR technique to confirm the relevance of *PVT1* as a biomarker for gastric cancer. Our results clearly show that *PVT1* expression is significantly enhanced in gastric cancer tissues when compared with non-cancerous tissues of the stomach. A high level of *PVT1* expression was also associated with lymph node metastasis in patients with gastric cancer. No correlation was found between *PVT1* expression and age, sex, tumor, metastasis, or stage of gastric cancer.

In conclusion, we demonstrated that *PVT1* is highly expressed in human gastric cancer. Enhanced expression of *PVT1* was associated with invasion to the lymph nodes and with resistance to paclitaxel. Therefore, these results indicate that *PVT1* is a potential candidate as a biomarker for gastric cancer. Screening for *PVT1* expression could potentially improve early diagnosis, therapy, and drug resistance, and thus improve quality of life for patients with gastric cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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