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Authors
Shigeyasu, Kunitoshi
Toden, Shusuke
Ozawa, Tsuyoshi
et al.

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The PVT1 IncRNA is a novel epigenetic enhancer of MYC, and a promising risk-stratification biomarker in colorectal cancer

Kunitoshi Shigeyasu 1,2, Shusuke Toden 1, Tsuyoshi Ozawa 1,3, Takatoshi Matsuyama 1,4, Takeshi Nagasaka 3, Toshiaki Ishikawa 4, Debashis Sahoo 5, Pradipta Ghosh 6, Hiroyuki Uetake 4, Toshiyoshi Fujiwara 2 and Ajay Goel 1,7*

Abstract

Accumulating evidence suggests that dysregulation of transcriptional enhancers plays a significant role in cancer pathogenesis. Herein, we performed a genome-wide discovery of enhancer elements in colorectal cancer (CRC). We identified PVT1 locus as a previously unrecognized transcriptional regulator in CRC with a significantly high enhancer activity, which ultimately was responsible for regulating the expression of MYC oncogene. High expression of the PVT1 long-non-coding RNA (lncRNA) transcribed from the PVT1 locus was associated with poor survival among patients with stage II and III CRCs (p < 0.05). Aberrant methylation of the PVT1 locus inversely correlated with the reduced expression of the corresponding the PVT1 lncRNA, as well as MYC gene expression. Bioinformatic analyses of CRC-transcriptomes revealed that the PVT1 locus may also broadly impact the expression and function of other key genes within two key CRC-associated signaling pathways – the TGFβ/SMAD and Wnt/β-Catenin pathways. We conclude that the PVT1 is a novel oncogenic enhancer of MYC and its activity is controlled through epigenetic regulation mediated through aberrant methylation in CRC. Our findings also suggest that the PVT1 lncRNA expression is a promising prognostic biomarker and a potential therapeutic target in CRC.

Keywords: PVT1, MYC, Enhancer, Epigenetic, Prognostic marker, Colorectal cancer

Main text

Accumulating evidence indicates that the pathogenesis of colorectal cancer (CRC) is influenced by epigenetic modifications. Among these, in the past decade, alterations in enhancer elements have garnered a significant attention [1], and are emerging as important players in cancer pathogenesis and being exploited as potential therapeutic targets. Recently, the FANTOM5 (Functional Annotation of the Mammalian Genome 5) project curated a genome-wide enhancer element atlas from normal tissues and tumor cells, using the cap analysis gene expression (CAGE) and next-generation sequencing (NGS) approaches [2–5]. Herein, we systematically examined the FANTOM5 database and identified the PVT1 locus as an epigenetic enhancer in CRC and provided a novel evidence for its role in specific targeting of the MYC oncogene. Furthermore, we found that PVT1 lncRNA was transcribed by the PVT1 locus via an enhancer-like activity. Multiple in silico datasets were utilized to evaluate the clinical significance of the PVT1 lncRNA. Pooling of seven such datasets improved the overall statistical power of the analysis and minimized potential cohort bias. Our analyses indicated that PVT1 is associated with cancer stemness and modulates key CRC-associated signaling pathways – the TGFβ/SMAD

* Correspondence: ajgoel@coh.org
1 Center for Gastrointestinal Research, Center for Translational Genomics and Oncology, Baylor Scott & White Research Institute and Charles A Sammons Cancer Center, Baylor University Medical Center, Dallas, TX, USA
2 Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute of City of Hope Comprehensive Cancer Center, 1218 S. Fifth Avenue, Suite 2226, Duarte, CA, USA

Full list of author information is available at the end of the article.
and Wnt/β-Catenin pathways. We additionally noted that high expression of the *PVT1* lncRNA associated with poorer survival in CRC patients. Taken together, our data provide first evidence that the *PVT1* locus plays a key role in CRC pathogenesis, and that it may serve as a prognostic biomarker and a potential therapeutic target in patients with CRC.

**A novel enhancer, the *PVT1* lncRNA, is frequently activated in colorectal cancers, and activates its enhancer potential via oncogenic *MYC***

We performed a systematic analysis of 43,011 enhancer elements that were recently reported in the FANTOM5 enhancer database (Supplementary Table 1). We observed that the strongest enhancer activity in CRCs was confined to the classic, CRC-associated chromosome 8q24 region – often referred to as the ‘gene desert’, including the genes such as the *PCAT1, CCAT1, CCAT2, PVT1* and *MYC* (Fig. 1a). In particular, *CCAT1-L* lncRNA, which is transcribed from the *CCAT1* locus, has been shown to stabilize loop structure between *CCAT1* and *MYC* via an “enhancer-like function” [6, 7]. Therefore, we were intrigued by the identification of a novel locus, *PVT1*, which has previously not been reported as an enhancer region in CRC. In the genome-wide FANTOM5 enhancer database, the *PVT1* locus exhibited cancer-specific enhancer activity, especially in CRCs. We analyzed this region carefully using the University of California Santa Cruz (UCSC) genome browser. We discovered that the *PVT1* locus indeed exhibited a strong H3K27ac enhancer signal, in a panel of cell lines (GM12878, H1-hESC, HSMM, HUVEC, K562, NHEK and NHLF), as well as in HCT116 CRC cells (Fig. 1b). Next, we analyzed Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChiA-PET) results from the UCSC genome browser and demonstrated that the *PVT1* region interacts with the *MYC* oncogene (Fig. 1b).

Our results from the FANTOM5 database and the UCSC genome browser analysis lead to the hypothesis that the *PVT1* region might have oncogenic enhancer activity that targets the *MYC* oncogene in CRC cells. Using a chromosome conformation capture (3C) assay in HCT116 cell lysates, we demonstrated that indeed the *PVT1* locus formed a loop structure in a *cis* conformation with *MYC* (Fig. 1c).

We next measured the ability of the *PVT1* lncRNA for its ability to drive the expression of *MYC* in CRC cells. Recent reports suggest that enhancers may produce lncRNAs that can stabilize a *cis* conformation between enhancers and promoters [7]. This mechanism of enhancer-related lncRNA activity primarily occurs inside the nucleus. We found that majority of the *PVT1* lncRNA was indeed present within the nuclear compartment (Fig. 1d). In addition, we were very encouraged to observe that a positive correlation between this noncoding RNA and the *MYC* gene in the CRC specimens (cohort 1, *p* = 0.43, *P* < 0.0001, Fig. 1e).

To further investigate the role for the *PVT1*‘s enhancer activity, we next performed knockdown experiments for the *PVT1* lncRNA. Although the *PVT1* lncRNA knockdown using siRNA has already been previously attempted, such efforts did not result in concomitant suppression of the *MYC* mRNA [8], because establishing an effective nuclear *PVT1* lncRNA knockdown using siRNAs is challenging. To overcome this issue, we established a specific antisense oligonucleotide (ASO) that targets the *PVT1* lncRNA and permits its knockdown in the nucleus. Our approach led to successful knockdown of the *PVT1* lncRNA in the cancer cell lines (*P* < 0.01 in Caco-2, *P* < 0.001 in HCT116), with a simultaneous transcriptional suppression of the *MYC* mRNA (*P* < 0.05 in Caco-2, *P* < 0.01 in HCT116, Fig. 1f) and MYC protein levels (Fig. 1g), suggesting an enhancer-like function.

**The *PVT1* lncRNA is frequently overexpressed in stage II and III CRCs**

Next, we analyzed the *PVT1* lncRNA expression in CRC specimens. We evaluated the expression levels of the *PVT1* lncRNA in stage II and III CRCs. In multivariate analysis, high-*PVT1* expression emerged as an independent prognostic factor in stage II and III CRC patients (Cohort-1: *P* = 0.0246; Cohort-2: *P* = 0.0196, Supplementary Table 2 and 3). Data derived from these clinical cohorts further highlight that the *PVT1* lncRNA expression levels may serve as an important prognostic biomarker for stage II and III CRC patients, and can facilitate stratification of appropriate patient subsets that are optimal candidates for benefitting from adjuvant chemotherapy and attenuate recurrence [9].

***PVT1* lncRNA expression increases in CRC metastases, and its expression is widely associated with genes within the TGFβ/SMAD and Wnt/β-catenin pathways**

We next evaluated the expression pattern of the *PVT1* lncRNA, and the related functional pathways to clarify its clinical significance in data gathered from 7 pooled datasets (see *methods*). The expression levels of the *PVT1* lncRNA were significantly higher in both lung and liver metastases compared with the primary lesions (Fig. 2b). Intriguingly, the *PVT1* lncRNA was overexpressed at the bottom of the colorectal crypt compared with the top, both in mice (*P* = 0.022) and humans (*P* = 0.011); suggesting that *PVT1* lncRNA may either favor or serve as a marker of stemness which exists at the bottom of the crypt (Fig. 2c-e). Together, these findings using unbiased bioinformatic approaches suggest that the *PVT1* lncRNA may promote distant metastasis, perhaps via its ability to promote stemness in the colon cells.
Finally, we asked what other genes may be impacted by the PVT1 lncRNA, or vice versa. To this end, we analyzed The Cancer Genome Atlas (TCGA) CRC datasets ($n = 698$; Fig. 2f) using Boolean equivalent correlated clusters (BECC) analysis [10]. We used the PVT1 lncRNA as a seed gene and identified a set of 67 genes (Fig. 2g) that displayed a tight, statistically significant Boolean Equivalent relationship to the PVT1 lncRNA across all 698 CRCs in the dataset, as determined by BooleanNet statistics; and indeed MYC appeared as one of the key genes even in these analysis (Fig. 2f-g). The Reactome pathway analyses revealed that most of the 67 genes served within two major signaling pathways, i.e., TGFβ/SMAD2/3/4 and...
Wnt/β-Catenin pathways (Fig. 2h). These findings further support our hypothesis that the PVT1 IncRNA widely impacts major signaling pathways in CRC by regulating the expression of key genes, such as the MYC.

The PVT1 locus is epigenetically regulated and its methylation status inversely correlates with its transcriptional levels in CRC

Aberrant methylation is one of the key regulators of enhancer activity in various genes. Interestingly, we
observed a significant loss in CpG sequence methylation in the vicinity of the PVT1 locus, including its enhancer cluster (Fig. 3a). Specifically, a CpG site (cg23898497) in the middle of its enhancer region (chr8:128822251–128,823,013) was significantly hypomethylated in CRC vs. normal mucosa, in all disease stages in the cohort-1 (P < 0.001, area under the curve: AUC = 0.99, Fig. 3b) and cohort-3 patients (P < 0.001, AUC = 0.81, Fig. 3c). These results suggest that the PVT1 enhancer activity and the expression of this lncRNA might be controlled through an epigenetic regulation of this region. In support of our other findings, the methylation status of the PVT1
region negatively correlated with its lncRNA expression ($\rho = -0.4894$, $P < 0.0001$), as well as with the MYC gene expression ($\rho = -0.3879$, $P = 0.0005$, Fig. 3d).

Conclusions
Previously, the PVT1 lncRNA was reported as a stabilizer of the MYC protein [8]. In addition, our study indicates that the PVT1 locus may directly controls the MYC mRNA expression as an enhancer. Based on these data, targeting of the PVT1 lncRNA may be a potential therapeutic approach in CRC patients, which could eventually lead to suppression of the oncogenic MYC, at both the RNA and protein levels.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12943-020-01277-4.

Additional file 1: Supplementary Table 1
Additional file 2: Supplementary Table 2–7
Additional file 3: Methods

Abbreviations
CRC: Colorectal cancer; FANTOM5: Functional Annotation of the Mammalian Genome 5; CAGE: Cap analysis gene expression; NGS: Next-generation sequencing; UCSC: University of California, Santa Cruz; ChIA-PET: Chromatin Interaction Analysis by Paired-End Tag Sequencing; 3C: Chromosome conformation capture; RISC: RNA-induced silencing complex; ASO: Antisense oligonucleotide; TCGA: The Cancer Genome Atlas; BECC: Boolean equivalent correlated clusters

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Authors’ contributions
Conceived and designed experiments: KS, TO, TM; Performed experiments: TN, TI, HU, AY, TF; Wrote the manuscript: KS, TO, TM, KS, PG, AG. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data derived from public database are available from these sites. FANTOMS_Human_Enhancer_Tracks: http://slidebase.binf.ku.dk/human_enhancers/presets
TCGA_Research_Network: http://cancergenome.nih.gov/
cBioPortal: http://www.cbioportal.org/index.do
UCSC_Genome_Browser: http://genome.ucsc.edu/
All other data are contained within this article.

Ethics approval and consent to participate
All study-related procedures were performed as per the Declarations of Helsinki, wherein a written informed consent was obtained from each patient, and the institutional review boards of all participating institutions involved approved the study.

Consent for publication
Not applicable. The manuscript does not contain any individual personal data.

Competing interests
The authors declare that they have no competing interests.

Author details
1Center for Gastrointestinal Research, Center for Translational Genomics and Oncology, Baylor Scott & White Research Institute and Charles A Sammons Cancer Center, Baylor University Medical Center, Dallas, TX, USA.
2Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan.
3Department of Surgical Oncology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.
4Department of Specialized Surgery, Tokyo Medical and Dental University Graduate School of Medicine, Tokyo, Japan.
5Departments of Pediatrics and Computer Science and Engineering, University of California San Diego, La Jolla, CA, USA.
6Departments of Medicine and Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA, USA.
7Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute of City of Hope Comprehensive Cancer Center, 1218 S. Fifth Avenue, Suite 2226, Duarte, CA, USA.

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