

# Long noncoding RNA plasmacytoma variant translocation 1 is overexpressed in cutaneous squamous cell carcinoma and exon 2 is critical for its oncogenicity

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## Abstract

**Background** Cutaneous squamous cell carcinoma (cSCC) is one of the most common and fastest increasing forms of cancer worldwide with metastatic potential. Long noncoding RNAs (lncRNAs) are a group of RNA molecules with essential regulatory functions in both physiological and pathological processes.

**Objectives** To investigate the function and mode of action of lncRNA plasmacytoma variant translocation 1 (PVT1) in cSCC.

**Methods** Quantitative reverse transcriptase polymerase chain reaction and single-molecule *in situ* hybridization were used to quantify the expression level of PVT1 in normal skin, premalignant skin lesions, actinic keratosis (AK) and primary and metastatic cSCCs. The function of PVT1 in cSCC was investigated both *in vivo* (tumour xenografts) and *in vitro* (competitive cell growth assay, 5-ethynyl-2'-deoxyuridine incorporation assay, colony formation assay and tumour spheroid formation assay) upon CRISPR-Cas9-mediated knockout of the entire *PVT1* locus, the knockout of exon 2 of *PVT1*, and locked nucleic acid (LNA) gapmer-mediated PVT1 knockdown. RNA sequencing analysis was conducted to identify genes and processes regulated by PVT1.

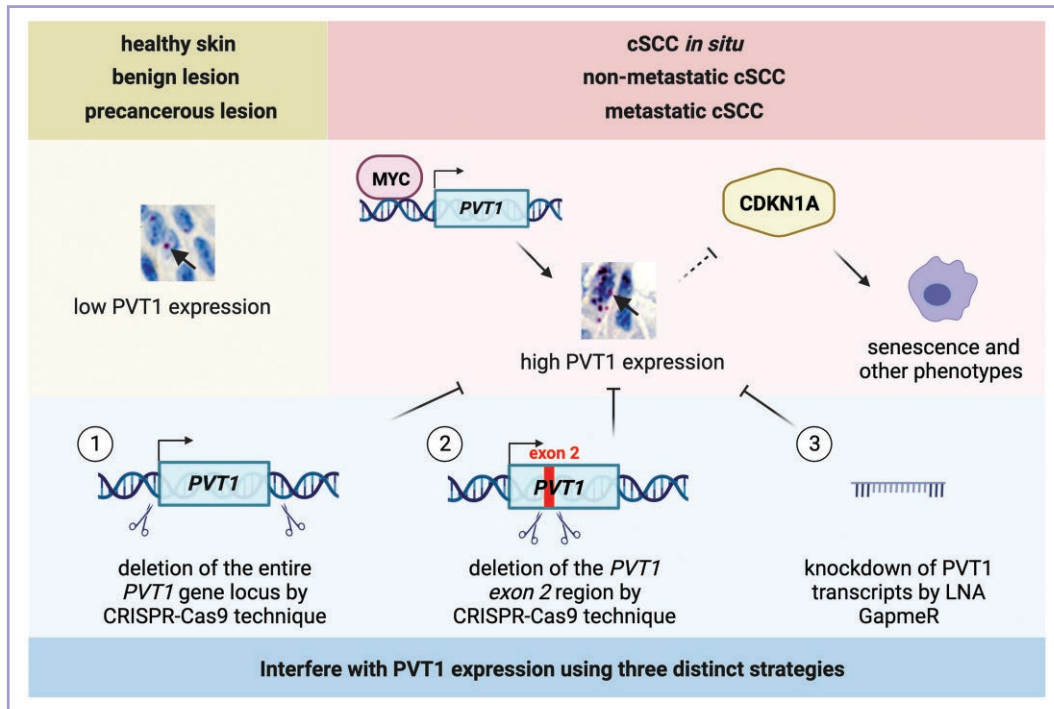
**Results** We identified PVT1 as a lncRNA upregulated in cSCC *in situ* and cSCC, associated with the malignant phenotype of cSCC. We showed that the expression of PVT1 in cSCC was regulated by *MYC*. Both CRISPR-Cas9 deletion of the entire *PVT1* locus and LNA gapmer-mediated knockdown of PVT1 transcript impaired the malignant behaviour of cSCC cells, suggesting that PVT1 is an oncogenic transcript in cSCC. Furthermore, knockout of *PVT1* exon 2 inhibited cSCC tumour growth both *in vivo* and *in vitro*, demonstrating that exon 2 is a critical element for the oncogenic role of PVT1. Mechanistically, we showed that PVT1 was localized in the cell nucleus and its deletion resulted in cellular senescence, increased cyclin-dependent kinase inhibitor 1 (p21/CDKN1A) expression and cell cycle arrest.

**Conclusions** Our study revealed a previously unrecognized role for exon 2 of PVT1 in its oncogenic role and that PVT1 suppresses cellular senescence in cSCC. PVT1 may be a potential biomarker and therapeutic target in cSCC.

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## Graphical Abstract

**What is already known about this topic?**

- Long noncoding RNA (lncRNA) plasmacytoma variant translocation 1 (PVT1) exhibits low expression in normal tissues but is highly upregulated in a variety of human cancers, and has been reported to exert oncogenic roles.
- Multiple modes of action of PVT1 have been reported in different cancers.
- The *PVT1* locus is complex and encodes >35 transcript variants, making the mechanistic understanding of PVT1 challenging.

**What does this study add?**

- Increased expression of PVT1 in cutaneous squamous cell carcinoma (cSCC) in comparison with normal skin and precancerous lesions indicates that PVT1 is associated with keratinocyte transformation and cSCC development.
- PVT1 expression is positively regulated by *MYC* in cSCC cells as well as in normal keratinocytes.
- PVT1 acts as an oncogenic lncRNA and a suppressor of cellular senescence in cSCC.
- Exon 2 is a critical element of PVT1 in exerting its oncogenic functions.
- Deletion of exon 2 impairs cSCC tumour growth *in vivo* and results in large-scale transcriptome changes in cSCC cells.

**What is the translational message?**

- With the advent of RNA-based medicine, PVT1 may be a promising molecular target for the management of inoperable or metastatic cSCC.
- Increased expression level of PVT1 might serve as a biomarker for cSCCs.

Cutaneous squamous cell carcinoma (cSCC) is one of the most common malignancies worldwide with a continuously increasing incidence.<sup>1</sup> Despite the high success rate of curative surgeries for localized cSCCs,<sup>2</sup> the high incidence and metastatic potential of cSCC makes it a significant health problem. To date, effective treatments for advanced cSCC

are lacking and the 5-year survival rate for metastatic disease is <30%; thus, further investigation into its pathomechanism is needed.<sup>3</sup>

The dominant risk factor for cSCC is chronic exposure to ultraviolet (UV) radiation and failed repair of UV-induced DNA damage resulting in the accumulation of somatic mutations.<sup>4</sup>

As one of the most highly mutated cancers, cSCC exhibits a mean somatic mutation rate of 50 mutations per megabase pair of DNA, corresponding to an approximate average of 1700 mutations per tumour exome.<sup>5–7</sup> Further understanding of cSCC pathogenesis at the molecular level will contribute to a better understanding of the disease and promote targeted therapy of cSCC.

Long noncoding RNAs (lncRNAs) are a group of transcripts with a length > 200 nucleotides and lack of a protein-coding functionality.<sup>8,9</sup> lncRNAs play important roles in regulating gene expression at the epigenetic, transcriptional and post-transcriptional levels,<sup>10</sup> and have been reported to participate in processes associated with tumorigenesis and development, such as cell stemness, epithelial–mesenchymal transition, immune escape and therapy resistance.<sup>11–14</sup> However, the specific functions and mechanisms of action of most lncRNAs are still poorly understood.

The *PVT1* locus was first identified in the early 1980s as variant (6; 15) translocations in murine plasmacytomas.<sup>15,16</sup> Human *PVT1*, orthologous to the mouse, is > 300 kb in length and located on chromosome 8q24.21, a well-known cancer-related region frequently aberrant in cSCC.<sup>17,18</sup> The oncogenic role of *PVT1* has been reported in multiple human cancers. However, the *PVT1* locus encodes > 35 isoforms of plasmacytoma variant translocation 1 (PVT1) due to alternative splicing (<https://genome.ucsc.edu>), posing a significant challenge to identifying the functional element in *PVT1* that maintains its oncogenic role. Multiple modes of action for PVT1 have been proposed, for example in the stabilization of target proteins [including transcription factors signal transducer and activator of transcription (STAT)1, STAT3, forkhead box protein M1 (FOXM1) and yes-associated protein 1 (YAP1)],<sup>19–22</sup> acting as a microRNA (miRNA) host gene and acting as a miRNA sponge.<sup>23</sup> Although substantial efforts have been made to explore the functions and modes of action of PVT1 in cancer, no single answer can be used to explain the role of PVT1 in different cancers, owing to the tissue-specific function and mechanism of lncRNA.

In this study, we analysed the expression of lncRNA PVT1 during cSCC development and investigated its function in cSCC through CRISPR-Cas9-mediated *PVT1* knockout and locked nucleic acid (LNA) gapmer-mediated lncRNA PVT1 knockdown experiments. Furthermore, we explored exon 2 as a putative functional element within the *PVT1* transcript and its molecular mechanism for the oncogenicity of PVT1 in cSCC.

## Materials and methods

### Clinical samples

Normal skin and cSCC samples used for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were collected at the Dermatology and Venereology Unit of Karolinska University Hospital (Stockholm, Sweden) and the University of Szeged (Szeged, Hungary). Actinic keratosis (AK) samples used for qRT-PCR were collected at the Department of Dermatology, Heinrich-Heine University (Düsseldorf, Germany). Archival formalin-fixed paraffin-embedded tissue blocks from the Department of Pathology, Turku University Hospital, and Auria Biobank, Turku University Hospital and

University of Turku (Turku, Finland) were collected and used to generate tissue microarrays consisting of samples from normal skin ( $n=59$ ), seborrhoeic keratosis (SK;  $n=12$ ), AK ( $n=60$ ), cSCC *in situ* (cSCCIS;  $n=57$ ), nonmetastatic cSCC ( $n=31$ ), metastatic cSCC ( $n=41$ ) and cSCC metastases ( $n=7$ ).<sup>24,25</sup> The clinical diagnosis was made by a dermatologist and confirmed by histopathology.

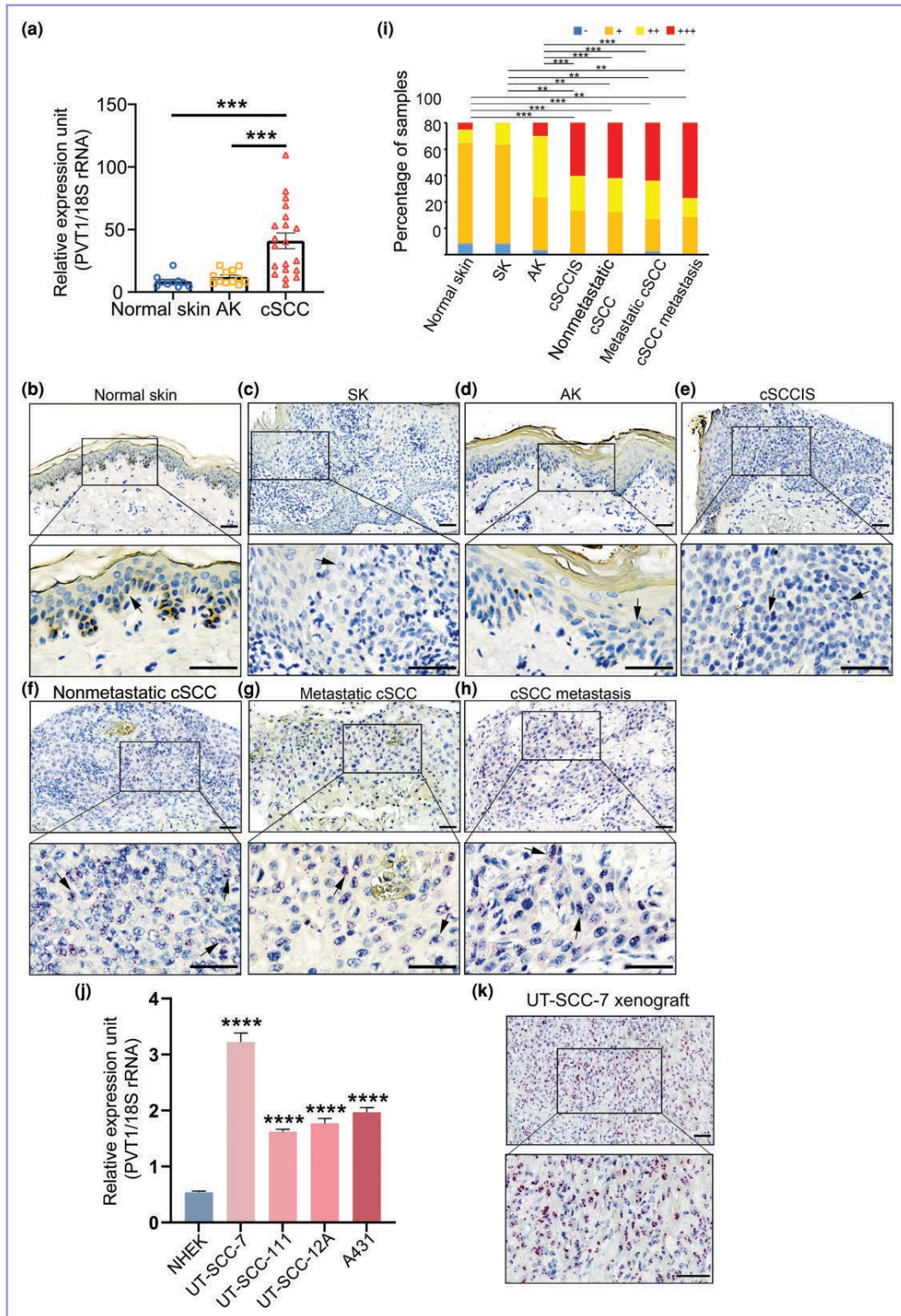
Detailed protocols for the RNA extraction, qRT-PCR, cell culture, RNAscope *in situ* hybridization, cell transfection, establishment of *PVT1*<sup>KO</sup> and *PVT1 E2*<sup>KO</sup> cSCC cell lines using CRISPR-Cas9, genomic DNA PCR and electrophoresis, competitive cell growth assay, 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, colony formation assay, tumour spheroid formation assay, tumour xenograft model, immunofluorescence staining analysis, Western blot assay and cycloheximide (CHX) chase assay, RNA sequencing (RNAseq), senescence associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) staining and statistical analysis are available in Appendix S1 (see Supporting Information).

## Results

### Increased expression of PVT1 is associated with the progression of cutaneous squamous cell carcinoma

Transcriptome analysis identified PVT1 as one of the top upregulated lncRNAs in cSCC (Figure S1a, Table S1; see Supporting Information).<sup>26</sup> The increased expression of PVT1 was validated in an independent cohort of cSCC samples by qRT-PCR (Figure 1a). Notably, PVT1 expression in cSCC was elevated not only compared with normal skin, but also compared with AK (Figure 1a) – a premalignant lesion caused by cumulative sun exposure that may proceed to cSCC.<sup>27</sup> To further analyse the expression of PVT1 in different stages of cSCC, sections of normal skin ( $n=59$ ), benign papillomatous proliferative SK ( $n=12$ ), AK ( $n=60$ ), cSCCIS ( $n=57$ ), invasive nonmetastatic cSCC ( $n=31$ ), metastatic cSCC ( $n=41$ ) and cSCC metastases ( $n=7$ ) were analysed using single-molecule *in situ* hybridization [RNAscope; Figure 1b–i (Figure S1b–d)]. PVT1 expression was quantitated by counting the visibly detectable PVT1 particles per cell in the microscopic images (for scoring, see Figure S1c). We saw that PVT1 was localized primarily in the nucleus and analysis revealed that strong (+++) PVT1 expression was significantly increased in cSCCIS (40% of cases), invasive nonmetastatic cSCC (42%), metastatic cSCC (44%) and in cSCC metastases (57%) compared with normal skin (5%), SK (0%) and AK (10%; Figure 1i). Furthermore, we employed an alternative scoring method based on the percentage of cells expressing PVT1. Once again, PVT1 expression was significantly increased in cSCC compared with healthy skin and benign and precancerous lesions, with the highest expression in cSCC metastases suggesting selection for high PVT1-expressing cells during tumour evolution (Figure S1d).

*In vitro*, PVT1 expression was higher in cSCC cell lines (UT-SCC-7, UT-SCC-111, UT-SCC-12A and A431) than in normal human epidermal keratinocytes (NHEK; Figure 1j). Furthermore, strong nuclear PVT1 expression was observed by single-molecule *in situ* hybridization in xenograft tissues



**Figure 1** Expression level of long noncoding RNA (lncRNA) plasmacytoma variant translocation 1 (PVT1) is elevated in cutaneous squamous cell carcinoma (cSCC). (a) Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of PVT1 expression in cSCC ( $n=20$ ), normal skin ( $n=10$ ) and precancerous skin lesions [actinic keratosis (AK);  $n=12$ ].  $***P<0.001$  (two-tailed Mann-Whitney  $U$  test). (b-h) Representative images of PVT1 detected by single-molecule *in situ* hybridization (RNAscope) on paraffin-embedded tissue sections of (b) normal skin ( $n=59$ ), (c) seborrheic keratosis (SK;  $n=12$ ), (d) AK ( $n=60$ ), (e) cutaneous squamous cell carcinoma (cSCC) *in situ* (cSCCIS;  $n=57$ ), (f) nonmetastatic cSCC ( $n=31$ ), (g) metastatic cSCC ( $n=41$ ) and (h) cSCC metastases ( $n=7$ ). Arrows indicate PVT1+ cells. Scale bar = 50  $\mu$ m. (i) Tissue samples were scored based on the number of PVT1 particles detected in the nucleus.  $**P<0.01$ ,  $***P<0.001$  (Fisher's exact test). (j) qRT-PCR analysis of PVT1 in cSCC cell lines (UT-SCC-7, UT-SCC-111, UT-SCC-12a and A431) and normal human epidermal keratinocytes (NHEK;  $n=3$ ).  $****P<0.0001$  (Student's  $t$ -test). (k) Images of PVT1 detected by single-molecule *in situ* hybridization (RNAscope) in xenograft tumours established with metastatic human cSCC cells (UT-SCC-7) in severe combined immunodeficiency mice. Scale bar = 50  $\mu$ m. For (a), (i) and (j), data are presented as mean ( $\pm$  SEM).

established by metastatic human cSCC cells (UT-SCC-7; Figure 1k).

Taken together, we observed an increased expression level of lncRNA PVT1 in *in situ* and invasive cSCC suggesting a role for PVT1 in squamous carcinogenesis in the skin.

### MYC regulates PVT1 expression in normal skin and cutaneous squamous cell carcinoma

MYC – one of the most commonly activated oncogenes in human cancers – is upregulated in cSCC. It has been reported that MYC activates PVT1 expression by binding to E-box elements located in the PVT1 promoter region.<sup>28</sup> In addition, our previous study identified MYC as one of the top upstream regulators of transcriptomic changes in cSCC.<sup>26</sup> Analysis of data from The Cancer Genome Atlas Head and Neck SCC cohort revealed a positive correlation between the expression of Myc and PVT1 transcripts (Figure S2a; see Supporting Information). In line with this, through detecting Myc expression in the same cSCC cohort that was used for PVT1 detection (Figure 1a), a positive correlation was found between the expression levels of Myc and PVT1 (Figure 2a); therefore, we hypothesized that PVT1 expression may be regulated by MYC in cSCC. Analysis of Myc chromatin immunoprecipitation sequencing data obtained from NHEK and multiple distinct cell lines revealed the direct binding of the Myc transcription factor to the PVT1 promoter region (Figure S2b, c), further suggesting that MYC can regulate PVT1 expression. We tested this hypothesis by performing endoribonuclease-prepared small interfering RNA (esiRNA)-mediated knockdown of Myc mRNA in NHEKs (used as a clean background devoid of most mutations potentially interfering with results), as well as cSCC cell lines. Depletion of Myc significantly suppressed PVT1 expression in NHEKs (Figure 2b, c; Figure S2d). Transfection of esiRNA targeting Myc (esiMYC) decreased the expression level of Myc 8, 24 and 48 h post-transfection (hpt) and also resulted in decreased PVT1 expression in UT-SCC-7 cSCC cells (Figure 2d, e; Figure S2e). Surprisingly, the Myc mRNA level (but not Myc protein; Figure S2e) was decreased only transiently (8 hpt) by esiMYC in A431 cells, after which it returned to a similar level as that of the control group (24 hpt); it then increased to an even higher level by 48 hpt (Figure 2f), possibly as a result of a rebound effect. Interestingly, the expression level of PVT1 followed a similar pattern: it was first slightly decreased by 8 hpt in A431, with an increase to a level comparable to that of the control group by 24 hpt; there was then a sharp increase at 48 hpt, paralleling the elevated Myc expression (Figure 2g). These results suggest that PVT1 transcription is regulated by MYC both in normal keratinocytes and in cSCC cells.

### Deletion of PVT1 results in decreased cutaneous squamous cell carcinoma cell growth, colony formation and tumour sphere formation

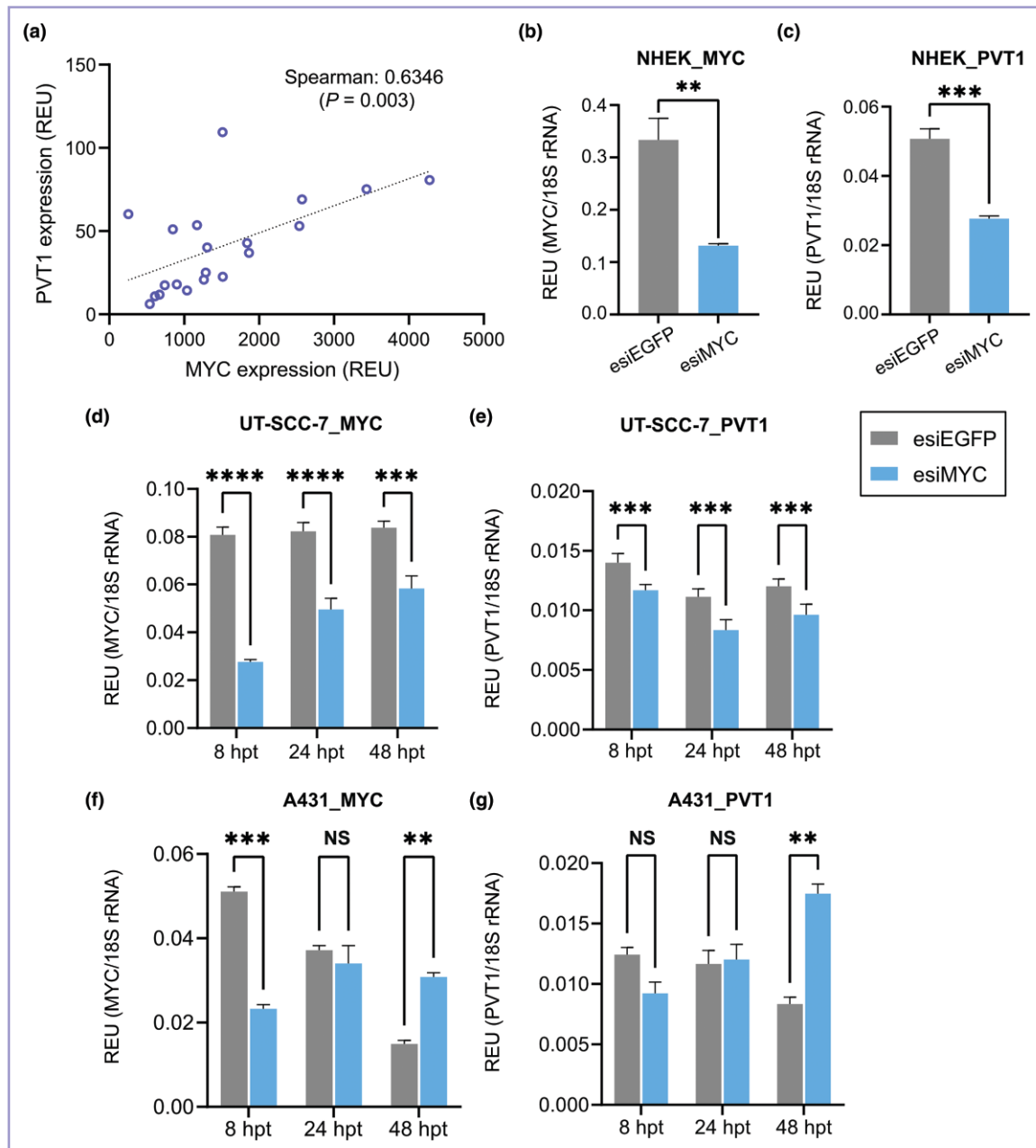
To investigate whether PVT1 plays a role in cSCC, we deleted the entire PVT1 locus in UT-SCC-7 cells by CRISPR-Cas9 gene editing with a pair of single-guide RNA

(sgRNA #1 and sgRNA #2) targeting sequences upstream and downstream, respectively, of the PVT1 locus [Figure S3a, b; Table S2 (see Supporting Information)]. To study the effect of PVT1 deletion on cSCC cell proliferation, we conducted a competitive cell growth assay in which we co-cultured fluorescently labelled wild type (WT; red) and PVT1<sup>KO</sup> (green) UT-SCC-7 cells and monitored them using video microscopy. The proliferative advantage of WT cells became significant after 48 h (Figure S3c, Video S1; see Supporting Information) and was even more apparent after 7 days of co-culturing, by which time they accounted for >85% of the cells in the culture (Figure S3d, e). The EdU incorporation assay consistently showed that the proliferation ability of PVT1<sup>KO</sup> UT-SCC-7 cells was significantly inhibited compared with WT cells (Figure S3f). Furthermore, PVT1<sup>KO</sup> UT-SCC-7 cells had an impaired capacity to form colonies and tumour spheroids *in vitro*, as determined by colony formation and tumour spheroid formation assays (Figure S3g, h). To minimize the risk that the results obtained were due to bias in single-cell cloning, spheroid formation assays were also performed with bulk (unselected) PVT1<sup>KO</sup> UT-SCC-7 cells, further confirming the impact of PVT1 deletion on cSCC cells (Figure S3i, j). Together, these data demonstrate that the PVT1 locus has an oncogenic role in cSCC.

### Deletion of PVT1 exon 2 mimics the tumour suppressive effect of entire PVT1 locus knockout in cutaneous squamous cell carcinoma cells

The intricate nature of the PVT1 locus, with its large size, overlapping functional elements and encoding of >35 isoforms, presents a significant challenge to studying PVT1. Considering that exon 2 (NCBI reference sequence: NR\_003367.3) was the most abundant exon of PVT1, based on our RNAseq analysis in cSCC,<sup>26</sup> and that it is contained by the majority of PVT1 isoforms, we hypothesized that exon 2 may play an important role in the oncogenicity of PVT1 transcript. To test this hypothesis, we generated PVT1 exon 2 knockout (PVT1 E2<sup>KO</sup>) UT-SCC-7 cells with the CRISPR-Cas9 system and a pair of sgRNAs (sgNA #3 and sgRNA #4) complementary to the sequences upstream and downstream of PVT1 exon 2 (Figure 3a, b; Table S2). Interestingly, while exon 2 could not be detected, the remaining PVT1 transcripts remained to be expressed (even increased) in PVT1 E2<sup>KO</sup> cells vs. WT cells (Figure S4a; see Supporting Information). At the functional level, knockout of exon 2 impaired the capacity of UT-SCC-7 cells to proliferate, as demonstrated by competitive cell growth and EdU incorporation assays [Figure 3c–e (Figure S4b, c; Video S2 (see Supporting Information))]. Furthermore, the deletion of exon 2 in PVT1 impaired the colony formation and tumour spheroid formation ability of UT-SCC-7 cells (Figure 3f, g; Figure S4d, e). To exclude potential cell line-specific effects, we also studied the effect of exon 2 deletion in A431 cSCC cells and obtained similar results in terms of impaired cell proliferation and tumour spheroid formation (Figure S4f, g).

Taken together, these results demonstrate that exon 2 is essential for the capacity of PVT1 transcript to sustain the growth and colony and tumour sphere formation ability of cSCCs *in vitro*.

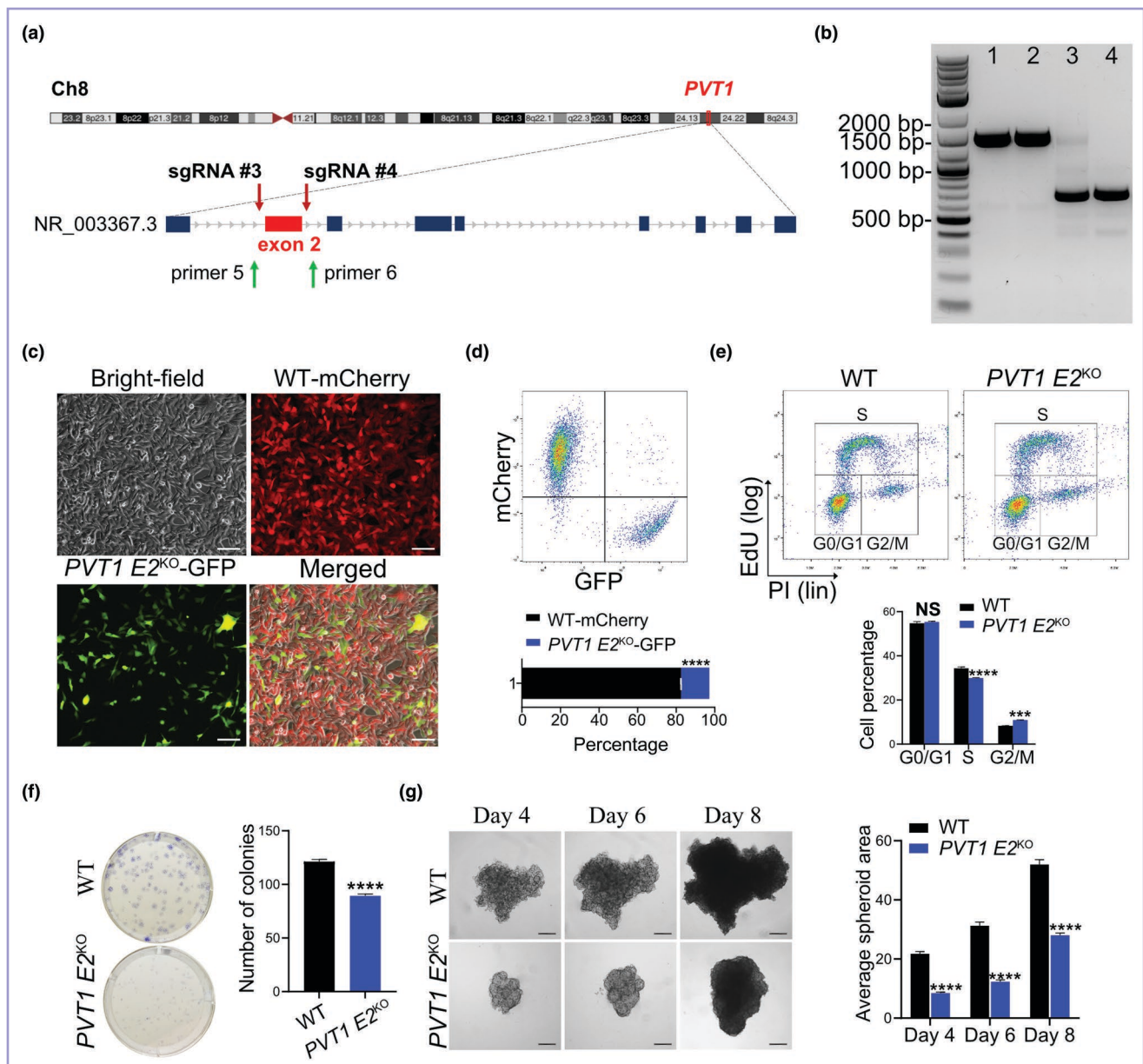


**Figure 2** The expression of plasmacytoma variant translocation 1 (PVT1) is regulated by MYC. (a) Correlation analysis between Myc and PVT1 expression in a set of 20 samples of cutaneous squamous cell carcinoma (cSCC) samples. (b, c) Expression levels of (b) Myc and (c) PVT1 in normal human epidermal keratinocytes (NHEKs) transfected with endoribonuclease small interfering RNA (esiRNA) targeting human Myc (esiMYC), with esiRNA targeting human enhanced green fluorescent protein (EGFP; esiEGFP) as a control ( $n=3$ ).  $**P < 0.01$ ,  $***P < 0.001$  (Student's *t*-test). (d, e) Expression levels of (d) Myc and (e) PVT1 in UT-SCC-7 cells transfected with esiMYC, with esiEGFP as a control, at 8, 24 and 48 h post-transfection (hpt) ( $n=3$ ).  $***P < 0.001$ ,  $****P < 0.0001$  (Student's *t*-test). (f, g) Expression of (f) Myc and (g) PVT1 in A431 cells transfected with esiMYC, with esiEGFP as a control, at 8, 24 and 48 hpt ( $n=3$ ). NS, not significant.  $**P < 0.01$ ,  $***P < 0.001$  (Student's *t*-test). For (b–g), data are presented as mean (SEM). REU, relative expression units.

### Disruption of PVT1 by deletion of exon 2 suppresses primary cutaneous squamous cell carcinoma tumour growth *in vivo*

Based on our *in vitro* results, we hypothesized that PVT1 and, in particular, exon 2 of PVT1 contribute to the malignant growth of cSCC. To test this hypothesis *in vivo*, a xenograft model of cSCC was established by subcutaneously injecting WT and PVT1 E2<sup>KO</sup> UT-SCC-7 cells into

immunocompromised mice (Figure 4a). In line with *in vitro* results, tumours formed by PVT1 E2<sup>KO</sup> UT-SCC-7 cells grew more slowly and were significantly smaller compared with those formed by WT cSCC cells (Figure 4b). After excising and weighing the tumours upon terminating the experiment, we found that deletion of exon 2 resulted in a decrease in weight of the tumour (Figure 4c). In line with the results of *in vitro* EdU incorporation assays, Ki-67 staining revealed that deleting exon 2 of PVT1 resulted in



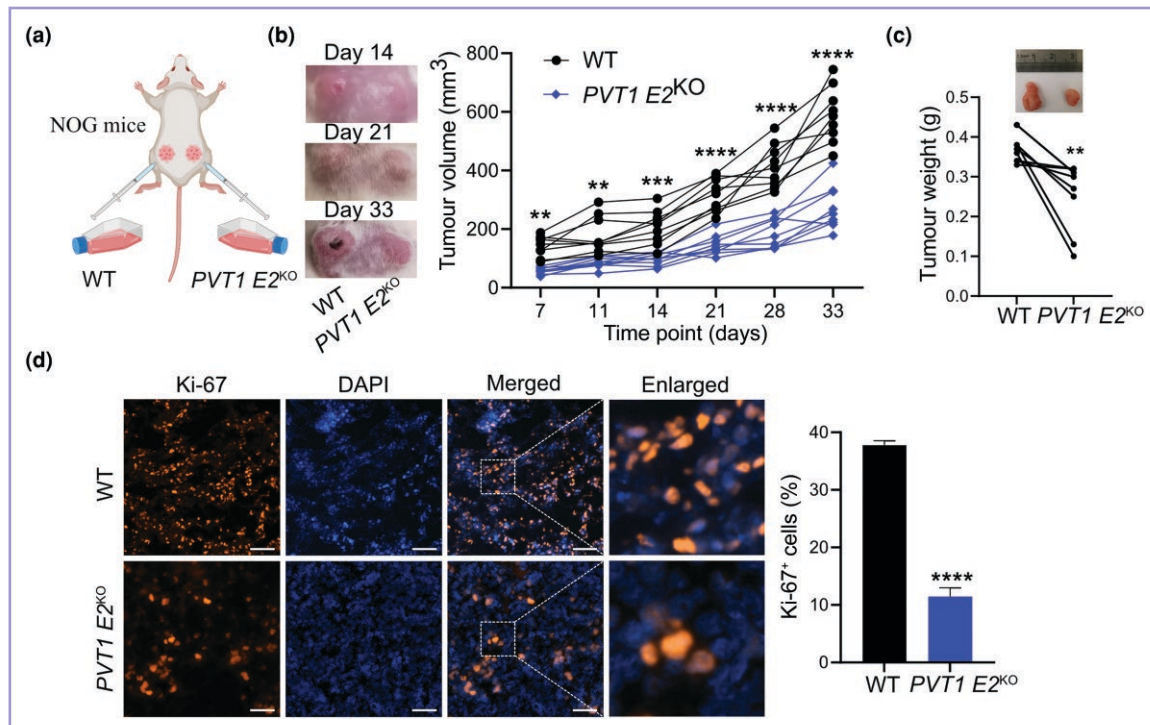
**Figure 3** Deletion of *PVT1* exon 2 mimics the tumour suppressive effect of *PVT1* ablation in cutaneous squamous cell carcinoma (cSCC) cells. (a) Graphical summary of the strategy used for CRISPR-Cas9-mediated knockout of the *PVT1* exon 2 locus. (b) Polymerase chain reaction [with primers shown in (a)] on genomic DNA followed by electrophoresis was used to validate the *PVT1* exon 2 knockout (*PVT1* E2<sup>KO</sup>) effect. Lanes 1 and 2: wild-type (WT) genomic DNA PCR using primers 5 and 6; lanes 3 and 4: *PVT1* E2<sup>KO</sup> genomic DNA PCR using primers 5 and 6. (c) Competitive cell growth assay performed on WT UT-SCC-7 cells expressing mCherry (red) and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells expressing green fluorescent protein (GFP; green). Images were taken 7 days after seeding cells ( $n=3$ ). Scale bar = 200 μm. (d) WT UT-SCC-7 cells expressing mCherry and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells expressing GFP co-cultured for 7 days were quantified by flow cytometry ( $n=3$ ). \*\*\*\* $P < 0.0001$  (Student's  $t$ -test). (e) 5-Ethynyl-2'-deoxyuridine (EdU) flow cytometry assay performed on WT and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells ( $n=3$ ). NS, not significant. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (Student's  $t$ -test). (f) Colony formation assay performed on WT and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells ( $n=3$ ). \*\*\*\* $P < 0.0001$  (Student's  $t$ -test). (g) Tumour spheroid formation assay performed on WT and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells ( $n=3$ ). \*\*\*\* $P < 0.0001$  (two-way ANOVA). Scale bar = 200 μm. For (d–g), data are presented as mean (SEM). bp, base pairs; PI, propidium iodide; sgRNA, single-guide RNA.

decreased cell proliferation *in vivo* (Figure 4d). These results demonstrate that *PVT1* – in particular exon 2 – has a significant role in cSCC tumour growth.

### PVT1 is an oncogenic transcript in cutaneous squamous cell carcinoma

To determine whether the *PVT1* transcript – rather than solely the underlying DNA element – is functional in cSCC,

we performed LNA gapper-mediated knockdown of the *PVT1* transcript in three human cSCC cell lines (Figure S5a; see Supporting Information). In line with the results obtained with *PVT1*<sup>KO</sup> and *PVT1* E2<sup>KO</sup> cells, LNA gapper-mediated *PVT1* knockdown suppressed proliferation of cSCC cells vs. control cells (Figure S5b). Furthermore, depletion of *PVT1* transcript impaired the colony formation and tumour spheroid formation ability of the cSCC cells (Figure S5c, d). Taken together, these results suggest that the *PVT1* transcript and



**Figure 4** Disruption of *PVT1* by deletion of exon 2 suppresses primary cutaneous squamous cell carcinoma (cSCC) tumour growth *in vivo*. (a) Xenograft models were established by subcutaneously injecting wild-type (WT; left) and *PVT1* E2<sup>KO</sup> (right) UT-SCC-7 cells into nonobese/severe combined immunodeficiency (NOD/SCID) and *Il2rg*-deficient NOD.Cg-*Prkdc*<sup>scid</sup> *Il2rg*<sup>tm1Sug</sup>/JicTac (NOG<sup>-</sup>) mice ( $n=9$ /group). (b) Primary tumour growth was monitored by measuring the greatest tumour length and width.  $**P<0.01$ ,  $***P<0.001$ ,  $****P<0.0001$  (two-way ANOVA). (c) Weight of tumours formed by WT or *PVT1* E2<sup>KO</sup> UT-SCC-7 cells were measured after the termination of the experiment at day 33.  $**P<0.01$  (Student's *t*-test). (d) Immunofluorescent staining of Ki-67 on sections of tumour formed by WT or *PVT1* E2<sup>KO</sup> UT-SCC-7 cells. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar=50  $\mu$ m. Cell proliferation was evaluated by determining the number of Ki-67<sup>+</sup> cells relative to 100 cells in 4 randomly selected fields. The analysis was performed on tissue sections from six pairs of xenografts.  $****P<0.0001$  (Student's *t*-test). For (b) and (d), data are presented as mean (SEM).

not only underlying DNA elements have an oncogenic role in cSCC.

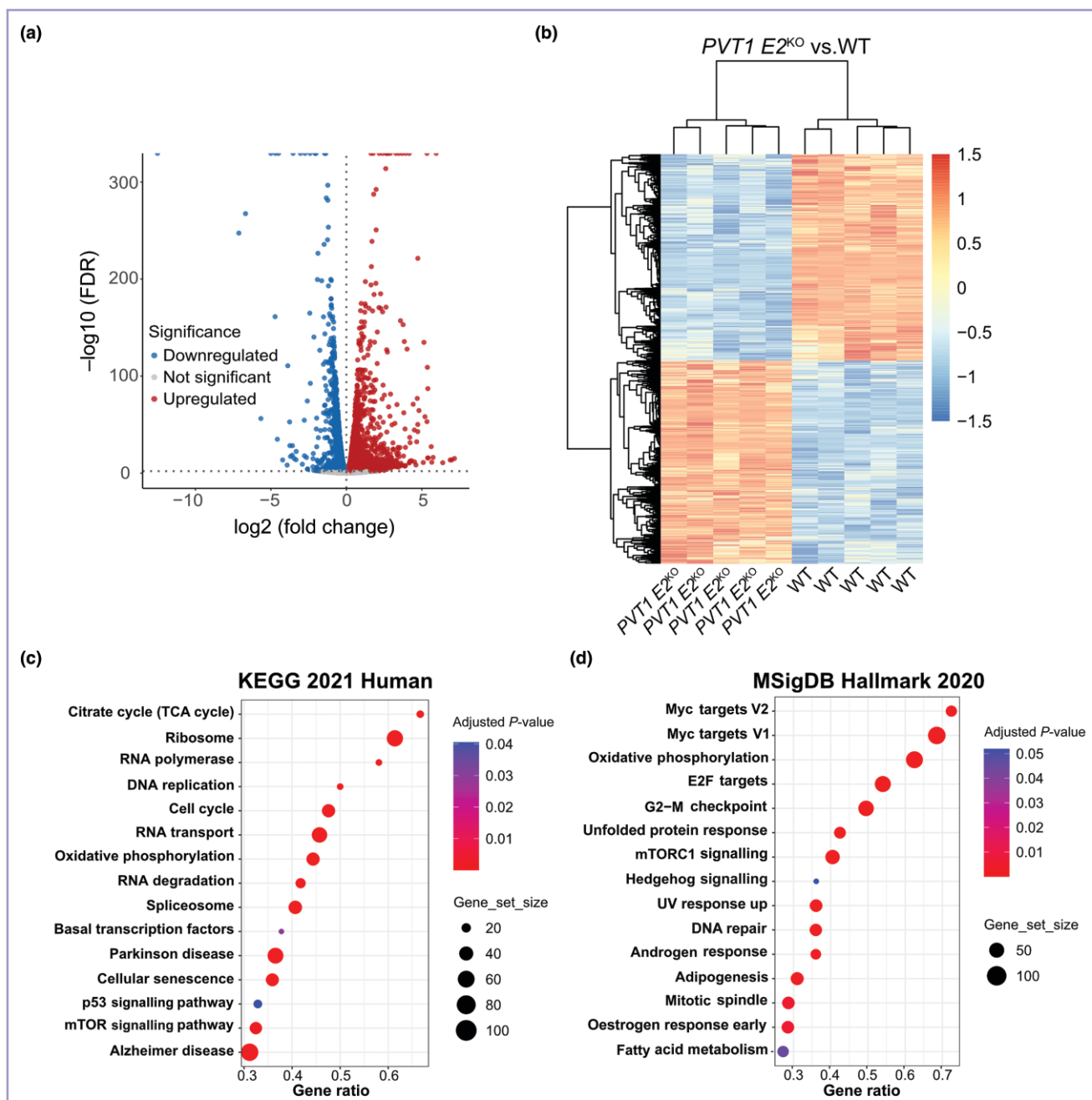
### PVT1 regulates cell cycle- and senescence-associated gene expression in cutaneous squamous cell carcinoma

It has been reported that *PVT1* expression is co-increased in most cancers with that of *Myc* oncogene;<sup>29</sup> therefore, the potential interaction between lncRNA *PVT1* and *Myc* has received widespread attention.<sup>29–31</sup> However, neither knockout of *PVT1* nor that of exon 2 resulted in significantly altered *Myc* protein levels in cSCC cells (Figure S6a; see Supporting Information). Moreover, we did not observe any significant difference in *Myc* protein half-life in CHX chase assays between *PVT1* E2<sup>KO</sup>, *PVT1*<sup>KO</sup> and WT cSCC cells (Figure S6b).

To obtain insights into the mode of action of *PVT1* and its effect on cellular pathways, we performed RNAseq analysis on WT and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells. Ablation of exon 2 of *PVT1* resulted in large transcriptomic changes with a total of 7789 differentially expressed mRNAs [false discovery rate < 0.01; Figure 5a, b (Table S3; see Supporting Information)]. The genes downregulated in *PVT1* E2<sup>KO</sup> cells were significantly enriched in pathways associated with cell cycle regulation, such as DNA replication, the cell cycle, the G<sub>2</sub>-M checkpoint and the mitotic spindle [Figure 5c, d;

Tables S4, S5 (see Supporting Information)], which is consistent with the results of our *in vitro* and *in vivo* experiments. Interestingly, processes related to cellular senescence and DNA repair were also enriched among *PVT1*-regulated mRNAs (Figure 5c, d; Tables S4, S5). Gene set enrichment analysis revealed that genes upregulated in senescent cells (gene set: FRIDMAN\_SENESCENCE\_UP) were enriched by genes upregulated in *PVT1* E2<sup>KO</sup> cells (Figure S7a, b; see Supporting Information). Interestingly, RNAseq detected fewer read counts of *Myc* in *PVT1* E2<sup>KO</sup> cells than in WT cells (Figure S6c). Notably, enrichment analysis conducted on the Molecular Signatures Database suggested that *Myc* targets (V1 and V2 lists) were markedly enriched in genes downregulated in *PVT1* E2<sup>KO</sup> cells (Figure 5d; Figure S6d), indicating that *PVT1* may be involved in the regulation of *Myc* activity, despite the fact that we did not observe differences in *Myc* at the protein level.

Looking further into the differentially expressed genes related to the cell cycle and cellular senescence, a number of important factors were found to be deregulated in *PVT1* E2<sup>KO</sup> cells (Figure 6a): cyclin-dependent kinase inhibitor 1 (CDKN1A) was significantly upregulated, while lamin B1 (LMNB1) was significantly downregulated. The dysregulated expression of CDKN1A and LMNB1 was verified by qRT-PCR in *PVT1*<sup>KO</sup> and *PVT1* E2<sup>KO</sup> cells (Figure 6b), suggesting a regulatory effect of *PVT1* on cellular senescence. To test this, we performed SA- $\beta$ -gal staining on

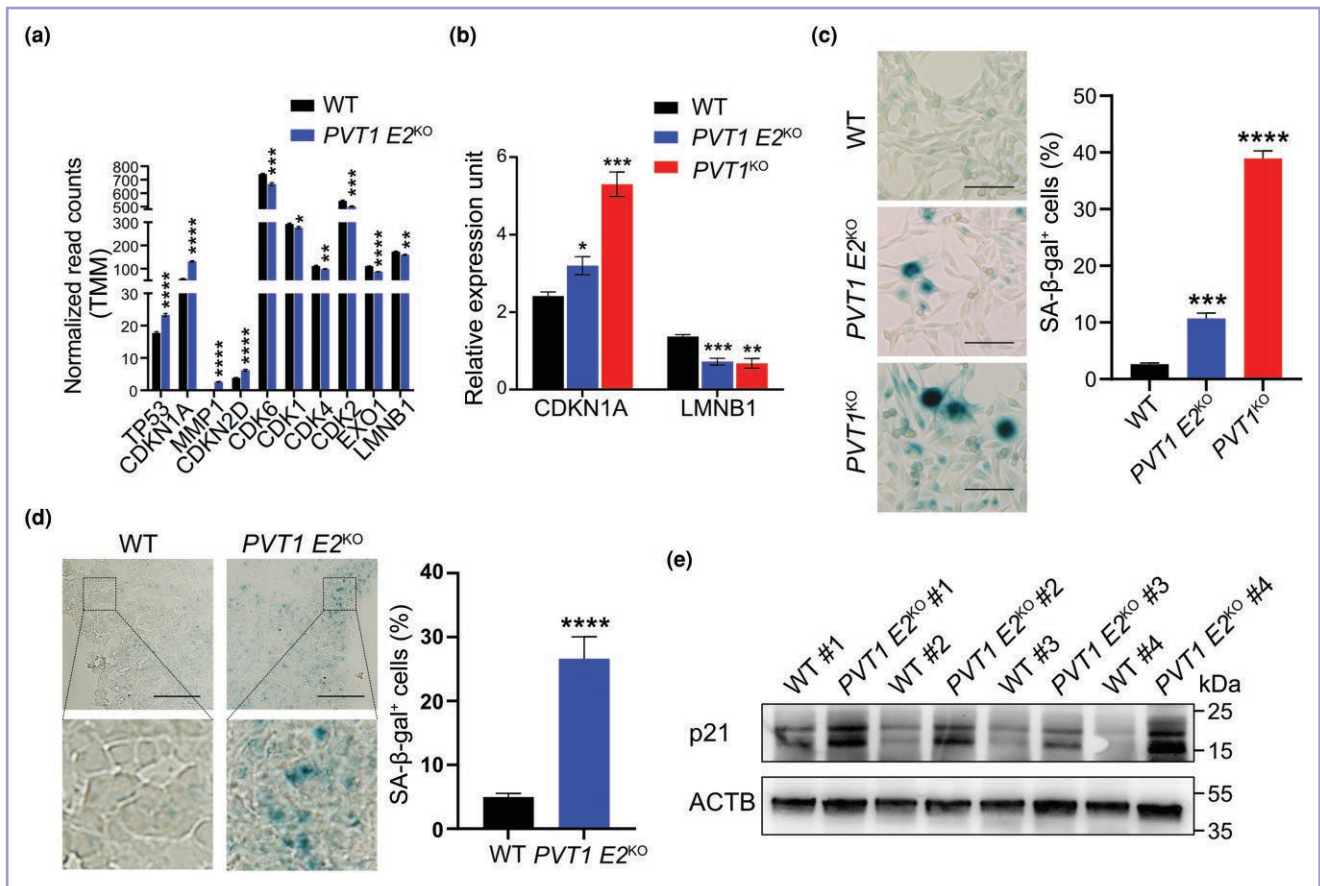


**Figure 5** Plasmacytoma variant translocation 1 (*PVT1*) regulates cell cycle- and senescence-associated gene expression in cutaneous squamous cell carcinoma (cSCC) cells. (a) Volcano plot showing transcripts differentially expressed in *PVT1* E2<sup>KO</sup> UT-SCC-7 cells vs. wild-type (WT) UT-SCC-7 cells. Vertical lines denote the fold change cutoff, while the horizontal line denotes the false discovery rate (FDR) cutoff (< 0.01). Red represents upregulated and blue represents downregulated coding transcripts. (b) Heatmap and hierarchical clustering of differentially expressed transcripts in *PVT1* E2<sup>KO</sup> UT-SCC-7 cells vs. WT UT-SCC-7 cells (FDR < 0.01). (c, d) Fifteen selected pathways significantly enriched among genes downregulated in *PVT1* E2<sup>KO</sup> UT-SCC-7 cells in (c) KEGG 2021 Human and (d) Molecular Signatures Database (MSigDB) Hallmark databases (FDR < 0.01). The colour of the nodes indicates the adjusted *P*-value and the size of the nodes reflects the number of overlapping genes in the gene sets. E2F, E2F family of transcription factors; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; TCA, the citric acid cycle; UV, ultraviolet.

WT, *PVT1*<sup>KO</sup> and *PVT1* E2<sup>KO</sup> UT-SCC-7 cells; the results demonstrated that both *PVT1*<sup>KO</sup> and *PVT1* E2<sup>KO</sup> resulted in increased cell senescence (Figure 6c). We next investigated the effect of *PVT1* E2<sup>KO</sup> on cellular senescence *in vivo*. SA- $\beta$ -gal staining identified more senescent cells in xenografts formed by *PVT1* E2<sup>KO</sup> UT-SCC-7 cells than those formed by WT cells (Figure 6d). Moreover,

xenografts formed by *PVT1* E2<sup>KO</sup> UT-SCC-7 cells showed an elevated level of p21 (Figure 6e).

Collectively, RNAseq analysis indicated that *PVT1* E2 deletion can abolish the oncogenic function of *PVT1* in cSCC through activating CDKN1A expression and regulation of cell cycle progression- and cellular senescence-related pathways.



**Figure 6** Plasmacytoma variant translocation 1 (PVT1) acts as a suppressor of cellular senescence through regulating cyclin-dependent kinase 1A (CDKN1A/p21) expression in cutaneous squamous cell carcinoma (cSCC). (a) Normalized read counts of 10 representative genes associated with cell cycle and cellular senescence. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (Student's *t*-test). (b) Expression levels of CDKN1A and lamin B1 (LMNB1) in wild-type (WT), PVT1 E2<sup>KO</sup> and PVT1<sup>KO</sup> UT-SCC-7 cells were detected by quantitative reverse transcriptase polymerase chain reaction ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (Student's *t*-test). (c) WT, PVT1 E2<sup>KO</sup> and PVT1<sup>KO</sup> UT-SCC-7 cells were stained for senescence associated  $\beta$ -galactosidase (SA- $\beta$ -gal;  $n = 3$ ). Scale bar = 100  $\mu$ m. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (Student's *t*-test). (d) WT and PVT1 E2<sup>KO</sup> xenografts were stained for SA- $\beta$ -gal. Scale bar = 100  $\mu$ m. Cellular senescence was assessed by quantifying the number of SA- $\beta$ -gal<sup>+</sup> cells relative to 100 cells in 4 randomly selected fields. The analysis was conducted on tissue sections obtained from four pairs of xenografts. \*\*\*\* $P < 0.0001$  (Student's *t*-test). (e) The expression level of p21 protein was detected in WT and PVT1 E2<sup>KO</sup> xenografts.  $\beta$ -Actin was used as loading control. For (a–d), data are presented as mean (SEM). TMM, trimmed mean of M values.

## Discussion

In this study, we found that the expression of lncRNA PVT1 is increased in cSCC and regulated by *MYC*. Our results showed that PVT1 functions as an oncogenic lncRNA in cSCC and plays a key role in maintaining the malignant behaviour of cSCC cells. Mechanistically, we identified exon 2 as a critically important part of the PVT1 transcript and we demonstrated that PVT1 acts as a suppressor of CDKN1A and cellular senescence.

AK – the most common precancerous cSCC lesion<sup>27</sup> – possesses strikingly similar genetic alterations to cSCC, according to molecular genetic studies based on whole-exome sequencing.<sup>32</sup> Most known cSCC driver gene mutations already exist in AK, such as *TP53*, *NOTCH1*, *NOTCH2* and *FAT*.<sup>27,32</sup> However, < 1% of AK lesions will progress to cSCC, which poses the question: What are the driving factors for the progression of AK to cSCC? In our study, we detected elevated expression level of PVT1 in cSCCIS and invasive cSCCs not only in comparison with normal skin and SK, but also with AK, suggesting that elevated PVT1

expression level contributes to keratinocyte transformation and plays a role in cSCC at both the preinvasive and invasive stages.

We found that PVT1 expression is regulated by *MYC* both in normal keratinocytes and in cSCC cells. *Myc* proto-oncogene is a transcription factor expressed in almost all proliferating cells and regulates the transcription of as much as 15% of the entire human genome.<sup>33</sup> *MYC* is deregulated in more than half of human cancers, including cSCC;<sup>34–36</sup> therefore, amplification or transcriptional activation of *MYC* may provide a mechanistic explanation for increased PVT1 expression. The direct binding of *Myc* to the PVT1 promoter suggests a potential direct regulatory effect of *Myc* on PVT1 expression, although indirect effects through other cellular pathways could also regulate PVT1.

In an effort to explore the mode of action of PVT1 in cSCC, we initially explored the hypothesis that PVT1 might serve as a host gene for miRNAs, as previously suggested.<sup>37</sup> Surprisingly, we did not detect miR-1204 in cSCC (data not shown); furthermore, this miRNA barely reaches the detection limit in any cell type investigated so far (data

not shown). It has been reported that PVT1 transcript stabilizes Myc protein by protecting it from ubiquitin proteasomal degradation;<sup>19,38</sup> however, we could not confirm these findings in cSCC, suggesting that this effect may be tissue- or context-dependent, or require more sensitive methods to detect it. Indeed, our observations revealed a distinct pattern in which PVT1 actively participates in the regulation of a diverse array of Myc targets. This highlights the potential involvement of PVT1 in modulating the intricate landscape of gene expression regulation driven by *MYC*. It is worth noting that, in contrast to two other studies that localized PVT1 into the cytoplasm in cSCC and in head and neck SCC,<sup>39,40</sup> our study demonstrated that the majority of PVT1 is localized in the cell nucleus, based on single-molecule *in situ* hybridization conducted on a large cohort of tissue samples and on mouse xenografts.

The *PVT1* locus encodes >35 PVT1 isoforms due to alternative splicing, posing a significant challenge for functional investigation. In this study, we established an important function for exon 2 in *PVT1*. Notably, knockout of exon 2 – which is a more precise way to disrupt the *PVT1* locus and keep the majority of the gene locus, including enhancers in the first intron, intact – mimicked the phenotypes caused by full-gene ablation. Based on this finding, we hypothesized that PVT1 exon 2 plays a pivotal role in PVT1 function, potentially by shaping its three-dimensional structure and/or influencing its interactions with its protein-, RNA- or DNA-binding partners. A limitation of our research is that the exact molecular mechanism by which PVT1 affected the cellular functions in cSCC was not identified, and further investigation is needed to clarify the exact mechanism, expanding the importance of exon 2 in the oncogenic functions of PVT1.

The precise mode of action of lncRNAs remains a subject of debate within the scientific community, with questions surrounding whether it is the lncRNA transcript itself, the process of lncRNA transcription or the underlying DNA that exerts functional effects. The results we obtained with LNA gapmer-mediated knockdown of PVT1 transcripts mimicked the effects of the entire *PVT1* ablation, as well as exon 2 deletion, in terms of cell proliferation and of colony and tumour spheroid formation capability, suggesting that PVT1 transcripts (especially exon 2-containing PVT1 transcripts) are functional in cSCC rather than a byproduct.

We found that ablation of *PVT1* or *PVT1* exon 2 resulted in increased cellular senescence. Emerging evidence suggests that cellular senescence is a physiological barrier that prevents tumour initiation and progression. *In vitro* and *in vivo* experiments have demonstrated that oncogene-induced senescence (OIS) acts as an anticancer mechanism in which *TP53* represents a key effector.<sup>41–43</sup> However, *TP53* mutation has been reported in 54–90% of patients with cSCC.<sup>44–46</sup> Mutated *TP53*, together with increased expression of PVT1 (acting as a suppressor of senescence), could provide an explanation as to why OIS is unable to prevent keratinocyte transformation in cSCC. Prosenescence therapy has been considered a novel and promising strategy for cancer therapy. Distinct strategies have been proposed for senescence induction, such as enhancing *TP53* activity.<sup>47</sup> Our data suggest that lncRNA PVT1 may be a potential target for cSCC treatment by inducing cellular senescence.

In conclusion, we report the increased expression of lncRNA PVT1 in cSCC and the association of increased

PVT1 expression with the progression from AK to cSCC. The results obtained with genetic deletion of the entire *PVT1* locus and antisense oligodeoxynucleotide-mediated knockdown demonstrate a prominent oncogenic role for *PVT1* and the PVT1 transcript. Our results suggesting that exon 2 is critical for the functionality of *PVT1* warrants further research to elucidate its exact role. Our results suggest that PVT1 may serve as a novel therapeutic target and a potential malignant transformation biomarker for patients with cSCC.

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## Conflicts of interest

The authors declare no conflicts of interest.

## Data availability

The RNA sequencing data that support the findings of this study have been deposited in the Gene Expression Omnibus under the accession codes GSE139505 and GSE235606. All other data supporting the findings of this study are available from the corresponding author.

## Ethics statement

Approval for use of archival human tumour material and clinical data was granted by the Ethical Board of Turku University Hospital (187/2006), Auria Biobank (AB15-9721), Turku University Hospital Clinical Research Center (T80/2018) and the Swedish Ethical Review Authority (Etikprövningsmyndigheten, 2008/1262-31/2). All patients gave their written and informed consent. Mouse experiments were approved by the National Government of the Region of Southern Finland (ESAVI15107/2020) and the Uppsala University Animal Protection Agency (DOUU-2020-024), and performed according to the institutional guidelines.

## Supporting Information

Additional [Supporting Information](#) may be found in the online version of this article at the publisher's website.

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