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Research article

Establishment of quantitative nested-PCR of Abelson interactor 1 transcript variant-11

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ABSTRACT

Abelson interactor 1 (ABI1), which presents 18 Transcript Variants (TSV), plays an important role in CRC metastasis. Different ABI1-TSVs play synergistic or antagonistic roles in the same pathophysiological events. ABI1 Transcript Variant-11 (ABI1-TSV-11) functionally promotes lymph node metastasis of left-sided colorectal cancer (LsCC) and is an independent molecular marker to evaluate the prognosis of patients with LsCC. However, there is still lack of a quick and accurate method to detect the expression of ABI-TSV-11, distinguishing ABI1-TSV-11 from other 17 TSVs. To establish a rapid method specific for ABI1-TSV-11detection, we developed a quantitative nested-PCR method composed of pre-amplification regular PCR using ABI1 universal primer pair and the followed Real Time (RT)-qPCR using ABI1-TSV-11 specific primer pair spanning exon-exon junction. ABI1-TSV-11-overexpressed SW480 and LoVo cell lines were used to verify the quantitative nested-PCR assay, and the sequencing data was used to evaluate the accuracy of ABI1-TSV-11 quantitative nested-PCR assay. The detection limit was 5.24×10^4 copies/ml. ABI1-TSV-11 quantitative nested-PCR provides a new technical means for the detection of ABI1-TSV-11.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer death worldwide [1]. Metastasis is a dynamic biological process that includes separation from the primary site, degradation of extracellular matrix, invasion into blood and lymphatic vessels, migration and movement in vessels, and distant colonization and neovascularization [2, 3]. CRC metastasis is the leading cause of death, and Abelson interactor 1 (ABI1) is an important adaptor protein and its abnormal expression plays an important role in regulating the behaviors of tumor cells, such as proliferation, adhesion, migration and invasion, and thereby affecting the metastasis and progression of CRC [4, 5, 6]. Recent studies have shown that ABI1 is highly expressed in CRC tissues [7]. In addition to abnormal expression, different transcriptional variants (TSVs) of ABI1 may perform distinct biological functions [8]and play an important role in the progress of CRC metastasis [6, 9]. There are eighteen TSVs generated from ABI1 primary transcript by alternative splicing (https://www.ncbi.nlm.nih.gov/gene/10006). In our previous studies, we demonstrated that ABI1-TSV-11 is involved in the metastasis and poor prognosis of left-sided colorectal cancer (LsCC), and its elevated expression is related to lymph node metastasis and shorter overall survival (OS) [6]. In addition, based on the analysis results of clinical follow-up information from The Cancer Genome Atlas (TCGA) [10] and RNA-seq data from TSVdb (http://tsvdb.com) [11], ABI1-TSV-11 could be used as an independent biomarker to evaluate the prognosis of LsCC. For systematic and high-throughput detection and multivariable analyses, expressed sequence tag (EST) sequences [12], exon microarrays [13], and next-generation sequencing (NGS) are valid qualitative and quantitative methods for evaluating TSV expression, cancer mechanisms, and prognoses of patients. However, there is lack of quick and accurate method to detect the expression of ABI-TSV-11 mRNA [14, 15, 16]. Regular PCR using spanning exon-exon junction (EEJ) sequences as primers can effectively amplify TSVs specifically, quickly and accurately [15, 16]. However, this reaction may generate false-positive results in the presence of non-specific amplification. In this study, we developed a quantitative Nested-PCR method using ABI1 universal primer pairs in

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pre-amplification followed by second RT-PCR run using ABI1-TSV-11 specific exon-exon junction spanning primer pairs. GAPDH amplification is performed simultaneously to conduct relative quantitative analysis of ABI1-TSV-11 expression. Furthermore, the Sanger sequencing of the product from second RT-PCR run were performed to verify the repeatability and accuracy of ABI1-TSV-11 Nested-PCR assay. We developed a simple quantitative nested-PCR method with strategy using pre-amplification regular PCR step followed by qPCR assay with specific primer spanning the exon junction.

2. Materials and methods

2.1. Plasmid and cell lines

The coding sequence of ABI1-TSV-11 (NM_001178124.2) was subcloned into a MSCV-based pSM2 retroviral vectors to construct eukaryotic over-expression plasmids, which refers to our previous study [6].

Two CRC cell lines, the SW480 and LoVo were purchased from the Chinese Academy of Science (Shanghai, China) and cultured in DMEM (HyClone, Beijing, China) containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), and 100 units/mL penicillin plus 100 μg/mL streptomycin (Invitrogen, Beijing, China) in a humidified atmosphere of 5% CO2, 95% air at 37 °C. After transfection with the two retroviral vectors, control (empty vector, EV) and ABI1-TSV-11 overexpression (TSV-11-OE), stable cell lines of SW480 and LoVo were conferred resistance to puromycin. Puromycin (2 μg/ml) was added into the medium to identify the transformant cell clones.

2.2. Patient characteristics and sample selection

The tumor tissue and adjacent tissues from four CRC patients were collected from Peking University People's Hospital. For each CRC patient, a single core tumor biopsy and adjacent tissue greater than 5 cm were taken out. Patients with a confirmed diagnosis of colon carcinoma based on histopathology of resected material were included. The expression level of ABI1-TSV-11 in tumor tissues and adjacent normal tissues were then evaluated using quantitative nested-PCR assay. This research project was approved by the Research Ethics Committee of Peking University People's Hospital (Approval No: 2019PHB028-01) and informed consent forms were signed by all of the subjects prior to participation.

Total RNA was isolated with QIAGEN miRNeasy Mini kit (QIAGEN, Germany), according to the manufacturer's instructions.

2.3. Next generation sequencing and RNA-seq data analysis

To reveal the relative abundance of 18 transcript variants in different cell lines, including LoVo, SW480 and SW620 cell lines. RNA sequencing was performed using the Illumina HiSeq 2000 sequencing platform (MGI, Shenzhen, China). The first step in the workflow involves purifying the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads. Then the cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. The libraries were prepared and sequenced on the Illumina HiSeq 2000 sequencing platform for the following data analysis study.

The paired-end clean reads were aligned to the reference genome with HISAT2 (v2.0.4) [17]. Index of the reference genome was built using bowtie2 (v2.2.5) [18]. The mapped reads of each sample were assembled by StringTie (v1.3.1) in a reference-based approach [19]. StringTie uses a network flow algorithm as well as an optional *de novo* assembly step to assemble and quantitate full-length transcripts representing multiple splice variants for each gene locus. The TSVs expression profile of LoVo, SW480 and SW620 cell lines were normalized by Fragments Per Kilobase Million (FPKM) count. Cuffdiff (v2.1.1) was used to calculate FPKMs [20].

2.4. RNA isolation and complementary DNA preparation

For cell lines, total RNA was isolated with a QIAGEN miRNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's instructions.

Total RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript RT Reagent kit with gDNA Eraser (TAKARA, Japan) according to the manufacturer's instructions. To eliminate of DNA contamination, the gDNA Eraser reaction was performed in 10 μL, including 1000 ng of RNA, 2 μL 5×gDNA Eraser Buffer, 1 μL gDNA Eraser and RNase-free dH_2O at 42 °C for 2 min. Thereafter, each 20 μL reaction mix was performed, containing 10 μL of reaction product of last reaction, 4 μL 5× PrimeScript Buffer, 1 μL PrimeScript Enzyme, 1 μL oligo (dT) primer and 4 μL RNase-free dH_2O. This mixture was incubated at 37 °C for 15 min and then at 85 °C for 5 s. To obtain all mRNAs with high quality, oligo (dT) primers were used to perform reverse transcription.

2.5. Nested ploymerase chain reaction

For the pre-amplification, regular PCR was performed, cDNA obtained by reverse transcription was used as a template with the universal primer pair ABI1-TSVs-F and ABI1-TSVs-R. The reaction system of the first PCR run was carried out in 50 μL with 2 μL template cDNA, 25 μL PrimeSTAR Max Premix containing high-fidelity DNA polymerase, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) and 21 μL ddH_2O. The reaction conditions were 20 cycles of 10 s at 98 °C, 15 s at 60 °C and 30 s at 72 °C. Then, the PCR products were purified using a QIAquick PCR Purification kit (QIAGEN, Germany).

For the second RT-qPCR run, the purified PCR product from the first run was used as a template, with the specific primer pair ABI1-TSV-11-F and ABI1-TSV-11-R. The reaction system of the second RT-qPCR run was 20 μL with 10 μL TB Green Fast qPCR Mix, 1 μL PCR product from the first run, 1 μL forward primer ABI1-TSV-11-F, 1 μL reverse primer ABI1-TSV-11-R and 7 μL ddH_2O. The reaction conditions were 95 °C for 30 s, followed by 35 cycles of 5 s at 95 °C and 10 s at 56 °C and stopped with the melt curve stage according to the manufacturer's instructions.

The product from the second RT-qPCR run was sequenced by means of Sanger sequencing and then blasted with the BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Amplification products from the first and second PCR runs were observed under UV light after electrophoresis in a 1.5% agarose gel containing GeneGreen Nucleic Acid Dye (TIANGEN, China).

The level of ABI1-TSV11 in the cell lines was evaluated by the following formula: The relative value (RV) of ABI1-TSV-11 expression was determined by the $2^{-\Delta ct}$ method using GAPDH as a normalizer. The expression value (EV) of ABI1-TSV-11 was scaled to a reasonable range with the following equation [21]: $EV = \log_2 (RV \times 10000 + 1)$. The RT-qPCR assay was done in triplicate and the results shown represent their average.

2.6. Statistical analysis

SPSS version 22.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 8.0 (La Jolla, CA, USA) were used for statistical analysis. Data from all quantitative assays are expressed as the mean ± standard deviation and were analyzed statistically using two-sided Student's t test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Design of quantitative Nested-PCR primers

We aimed to set up a quantitative nested-PCR system to detect ABI1-TSV-11 variants for rapid and practical detection, including pre-amplification regular PCR and the followed RT-qPCR using TSV 11-specific primer spanning the exon junction. However, the exon

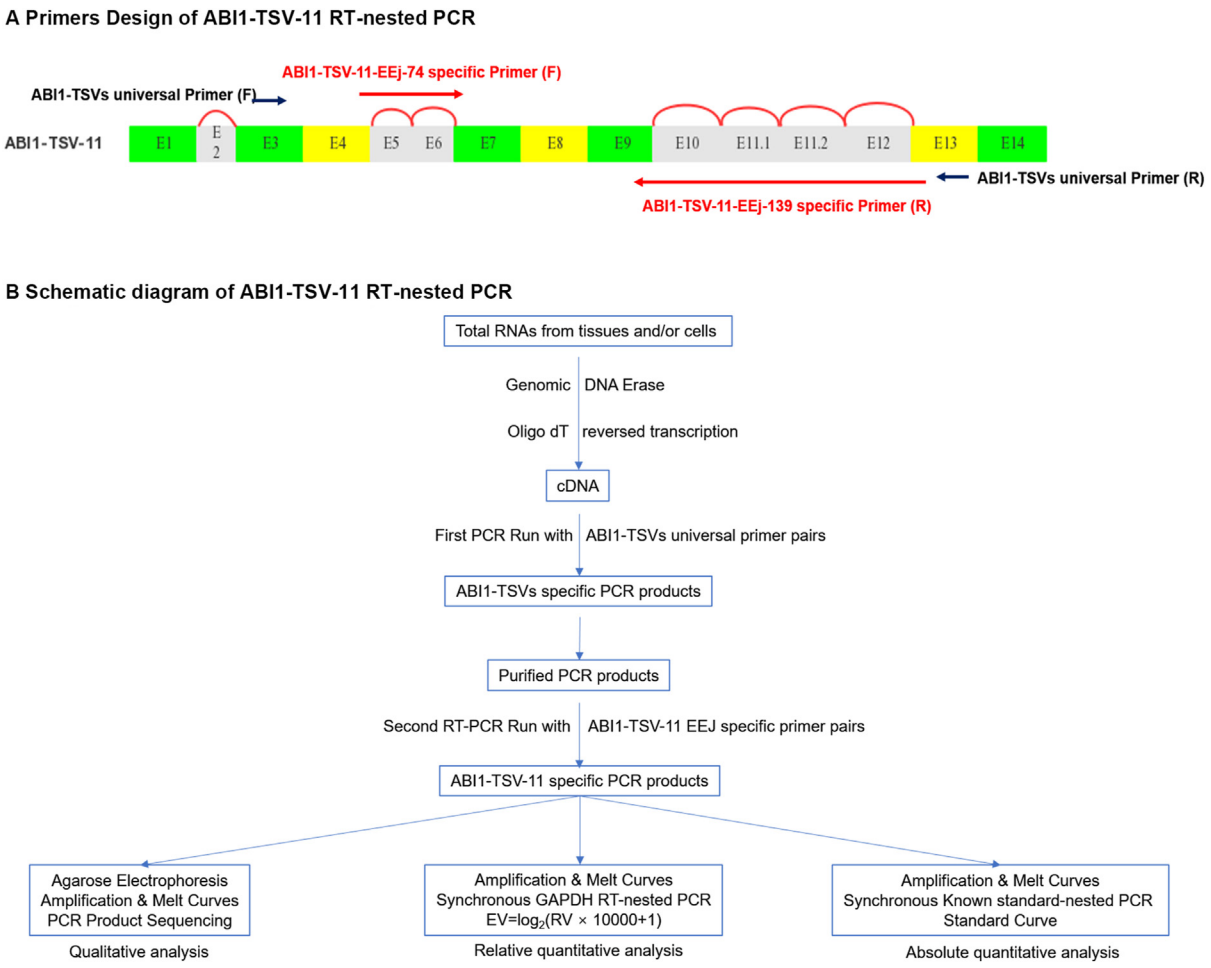


Figure 1. Primers design of ABI1-TSV-11 Quantitative Nested PCR and schematic diagram of ABI1-TSV-11 Quantitative Nested PCR. (A) Schematic showing locations of ABI1-TSVs universal primers and ABI1-TSV-11 specific primers. (B) Flowchart for qualitative analysis, relative and absolute quantitative analysis of ABI1-TSV-11.

combinations of the ABI1 gene are quite broad; there are 18 TSVs generated from ABI1 primary transcript, and all of the variants include retained exons 1, 3, 7, 8, 9, 13 and 14, which are common regions of ABI1 pre-mRNA (SupplementaryFigure 1). Therefore, a forward universal primer for ABI1 for pre-amplification was designed to bind exon 3, and a reverse universal primer for ABI1 for pre-amplification was designed to bind exon 13 (Figure 1A). A flow chart illustrating the recruitment strategy is shown in Figure 1B. The first regular PCR primer pair was designed to amplify the ABI1 product exclusively, harboring a total of 18 TSVs. The deduced size of each PCR product was 520–1057 base pairs (Table 1).

Then, the primer pair for second RT-qPCR run was designed to amplify the ABI1-TSV-11 variant specifically. ABI1-TSV-11 lacks several alternate in-frame coding exons, 2, 5–6, and 10–12. Exon 7-exon 4 junction (EEJ-74) and exon 13-exon 9 junction (EEJ-139) are the characteristic sequence structures that distinguish ABI1-TSV-11 from the other seventeen TSVs. Therefore, the forward specific primer for ABI1-TSV-11 was designed to span EEJ-74, and the reverse specific primer was designed to span EEJ-139. The deduced size of each PCR product was 367 base pairs (Figure 1A). Taken together, the universal primer pair covered all variants reported thus far, and the specific primer pair detected ABI1-TSV-11 specifically.

GAPDH was chosen as an endogenous housekeeping transcription control for nested PCR. Similarly, we designed outer and inner primer pairs for GAPDH for the pre-amplification regular PCR and the followed RT-qPCRs, respectively (Table 2).

3.2. Establishment of quantitative Nested-PCR method

To explore the specificity of nested PCR, we performed a nested-PCR assay using ABI1-TSV-11-overexpressed cell lines. The stable SW480 and LoVo cell lines were described in our previous study [6]. We performed the pre-amplification regular PCR run at 20 cycles, 25 cycles and 30 cycles. Light and clear bands were obtained with 20 cycles, the product of which was chosen for second RT-qPCR. Similarly, single and clear bands using the GAPDH outer primer pair were obtained with 20 cycles (Figure 2). To mitigate the influence of the background with more cycles, 20 cycles were selected for the following reaction. We performed the pre-amplification regular PCR run using the universal primer sets ABI1-TSV and the outer primer sets GAPDH, respectively. Products with an expected length of 520–1057 bp from the ABI1-TSV universal primer pair and 548 bp from the GAPDH outer primer pair were identified in the first regular PCR without a recognizable background (Figure 2).

Then, the product from the pre-amplification PCR with the expected length was amplified by a second RT-qPCR run using ABI1-TSV-11- and GAPDH inner primers. Then the product from second RT-qPCR were was determined on the 1.5 % agarose gel. A single band of the second RT-qPCR was detected with no background (Figure 3A), 367 bp from the

Table 1. The deduced size of each PCR product of eighteen ABI1 transcript variants from the first PCR run.

Transcript variants	Start site	End site	Amp. Length (bp)	TSV mRNA full length (bp)
1	333	1389	1057	3596
2	333	1308	976	3515
3	333	1221	890	3428
4	333	1293	961	3500
5	384	1353	970	3560
6	333	1305	971	3512
7	333	1218	886	3425
8	333	1203	871	3410
9	333	1131	799	3338
10	333	1044	711	3251
11	333	1029	697	3236
12	333	852	520	3059
13	333	1386	1054	3593
14	333	1206	874	3413
15	333	1125	793	3332
16	333	1302	970	3509
17	333	1299	967	3506
18	333	1212	880	3419

ABI1-TSV-11-specific primer pair, and 215 bp from the GAPDH inner primer pair. In addition, the second RT-qPCR products amplified by a specific primer pair were sequenced by Sanger sequencing. Blast analysis indicated that the ABI1-TSV-11 variant was sequenced (SupplementaryFigure 2). The amplification curve and melt curve indicated the primer sets for second RT-qPCR run were specific (Figure 3B and C).

3.3. Detection limit for the detection of ABI1-TSV-11

To detect the sensitivity and detection limit of nested-PCR assay, we performed nested-PCR assay using a plasmid vector containing ABI1-TSV-11 DNA as the template in the pre-amplification regular PCR run (Figure 4). The initial copy number of the first run product was calculated, considering that 1 mol of any substance contains 6×10^{23} molecules (Avogadro's number). Because the concentration of the first run product was approximately 14 ng/μL, 367 bp cDNA from ABI1-TSV-11-specific primers corresponded to approximately 5.24×10^{13} copies/ml.

Then, we performed a second RT-qPCR run using the specific primer and template from the first-round regular PCR product, which was diluted from 5.24×10^{13} copies/ml to 5.24×10^{4} copies/ml with a 10-fold series to conduct the standard curve. As shown in Figures 4A and B, the detection limit was 5.24×10^{4} copies/ml, equally 2.62×10^{3} copies/20 μl reaction. In addition, we observed a linear relation between the amount of ABI1-TSV-11 plasmid DNA and the Ct value necessary to provide the detection specificity (Figure 4C).

Table 2. Primers used for two-step PCR of ABI1-TSVs, ABI1-TSV-11 and GAPDH.

Name of primer	Sequence of primer (5′–3′)
ABI1-TSVs universal Primer-F	5′-GCCTCTCAGCTTCGGAGAATG-3′
ABI1-TSVs universal Primer-R	5′-TATACTGAACTACTGCAGCCTCCTCAT-3′
ABI1-TSV-11-EEj-74 specific Primer-F	5′-CATATCTCACAGCATGGAAGTAA-3′
ABI1-TSV-11-EEj-139 specific Primer-R	5′-GACTATCAGCAACTGGTCCAA-3′
GAPDH outer primer-F	5′-CCATGGGGAAGGTGAAGGTC-3′
GAPDH outer primer-R	5′-AGTGATGGCATGGACTGTGG-3′
GAPDH inner primer-F	5′-CCACATCGCTCAGACACCAT-3′
GAPDH inner primer-R	5′-TTCCCGTTCTCAGCCTTGAC-3′

3.4. Specificity for the detection of ABI1-TSV-11 in cell lines

To demonstrate our strategy for the specific detection of the ABI1-TSV-11 variant, we constructed two stable cell lines transfected with two retroviral vectors, control (empty vector, EV), and ABI1-TSV-11 overexpression vector (TSV-11-OE) containing ABI1-TSV-11 cDNA.

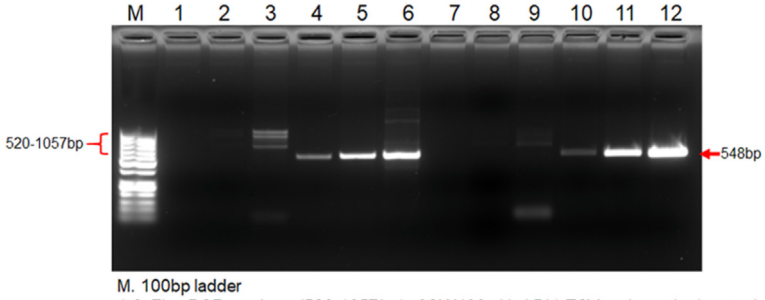
According to our previous study, the ABI1-TSV-11 variant in the SW480-ABI1-TSV-11 cell line was overexpressed compared with the SW480 and SW480-EV cell lines, as confirmed by Western blot analysis [6]. In this study, we developed the new strategy of detecting the mRNA level of the ABI1-TSV-11 variant by quantitative Nested-PCR. As shown in Figure 5, the expression of ABI1-TSV-11 in ABI1-TSV-11-overexpressed LoVo and SW480 cell lines was significantly increased compared with that in control cell lines by this assay (Figure 5A). In addition, we further detected the basic level of ABI1-TSV-11 in LoVo, SW480 and SW620 cell lines by this assay, the expression of the ABI1-TSV-11 variant in SW620 and SW480 cells was higher than that in LoVo cells, highly consistent with next-generation sequencing results (Figure 5B).

Furthermore, the level of ABI1-TSV-11 variant was detected in tumor and the matched adjacent colon tissues from four CRC patients (Supplementary Table 1), which showed higher level of ABI1-TSV-11 in the tumor tissues than that in the adjacent tissues as shown in Figure 5C ($P = 0.07$). The trend of high level ABI1-TSV-11 in tumor tissues is consistent with the result using TCGA dataset in our previous study [6].

4. Discussion

Based on TCGA RNA-sequencing data, our previous study showed that the up-regulated expression of ABI1-TSV-11 could promote lymph node metastasis of LsCC [6]. ABI1-TSV-11 could be used as an independent biomarker to evaluate the prognosis of LsCC.

Currently, exon-level microarrays and RNA sequencing are alternative technologies for high-throughput and precise detection of the



M. 100bp ladder
1-3. First PCR products (520-1057 bp) of SW480 with ABI1-TSVs universal primer pairs (20, 25 & 30 cycles)
4-6. First PCR products (548 bp) of SW480 with GAPDH outer primer pairs (20, 25 & 30 cycles)
7-9. First PCR products (520-1057 bp) of LoVo with ABI1-TSVs universal primer pairs (20, 25 & 30 cycles)
10-12. First PCR products (548 bp) of LoVo with GAPDH outer primer pairs (20, 25 & 30 cycles)

Figure 2. The first PCR products of SW480 and LoVo cell lines.

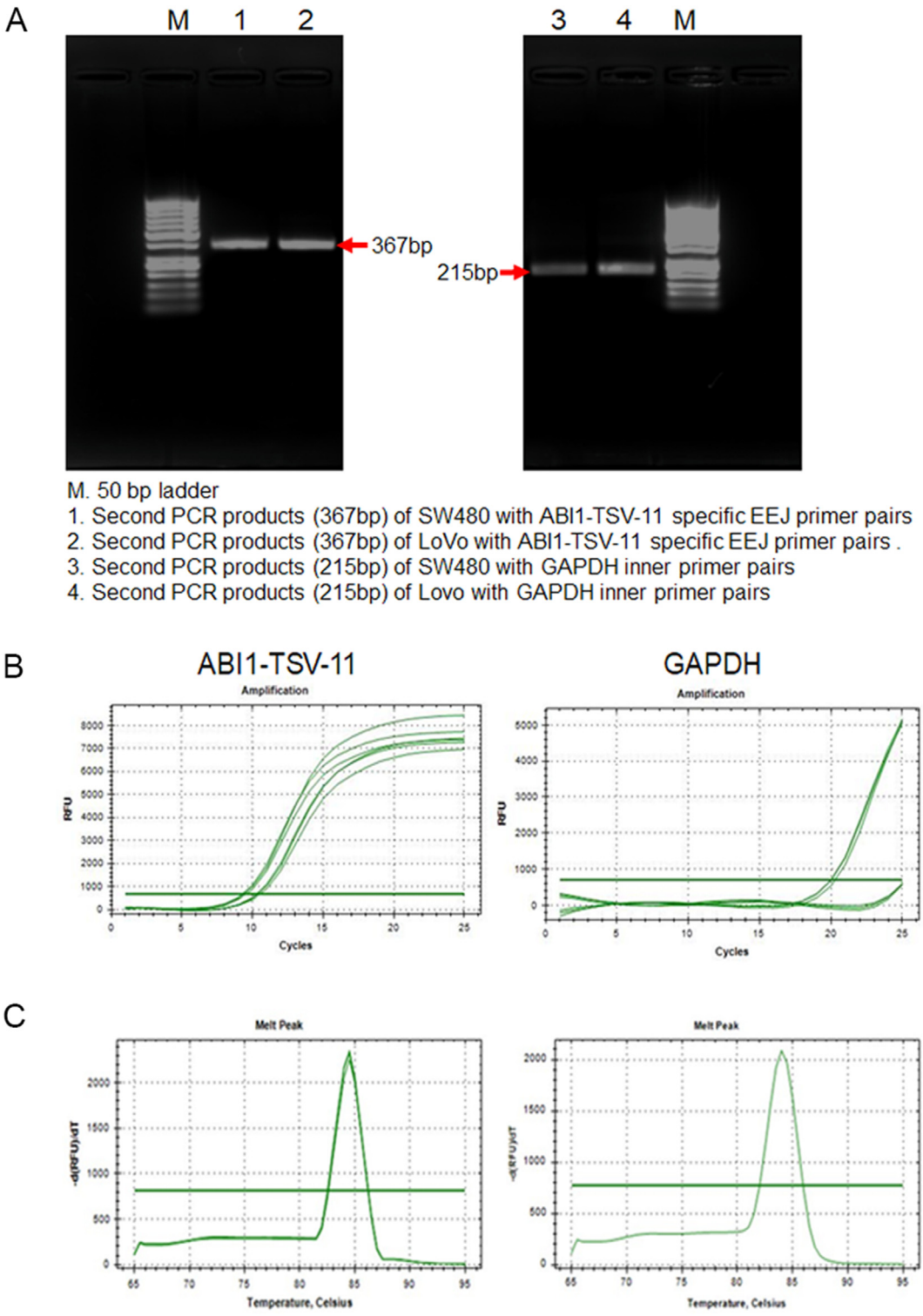


Figure 3. (A) The second PCR products of SW480 and LoVo cell lines. (B) The amplification curve of real-time PCR using ABI1-TSV-11 specific primers and GAPDH inner primer pairs, respectively. (C) The melt curve of real-time PCR using ABI1-TSV-11 specific primers and GAPDH inner primer pairs, respectively.

expression of cancer-related TSVs, but they cannot meet the clinical needs of rapid, convenient, and inexpensive methods [22]. RT-PCR is still the most rapid and sensitive method that can accurately quantify the expression of TSVs [23, 24, 25, 26, 27]. For ABI1-TSV-11, we established a rapid and sensitive quantitative nested-PCR detection technique based on quantitative nested-PCR strategy composed of pre-amplification and exon-exon junction (EEJ)-specific primers (SupplementaryFigure 1 and Figure 1) [15, 16].

In terms of the design strategy, the nested PCR method was employed, and the amplification products (containing all ABI1-TSVs) were obtained by using ABI1 universal primers through the first pre-amplification PCR run. Taking this as a template, ABI1-TSV-11 was specifically amplified

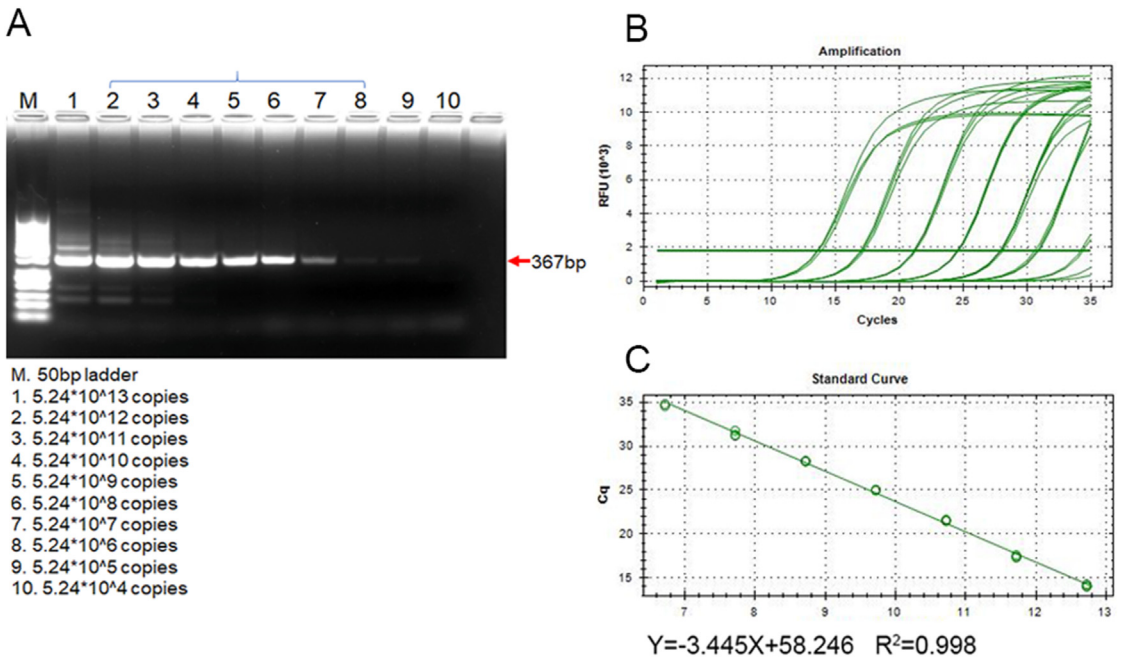


Figure 4. (A) The initial number of copies of the ABI1-TSV-11 fragment in second PCR is indicated above each lane. (B) The amplification plot in second PCR run with diluted template from 5.24×10^{12} copies/ml to 5.24×10^{6} copies/ml with a 10-fold series. (C) Efficiency plot of C_t versus log Quantity depicted in panel B (Slope = -3.445; R^2 = 0.998). Each point was reproduced in three independent experiments.

using primers spanning the Exon 7-exon 4 junction (EEJ-74) and exon 13-exon 9 junction (EEJ-139). The absolute quantitative detection were performed to evaluate the detection limit.

Regarding the methodology, to avoid nonspecific amplification, we first erased the trace genome of total RNA and then used oligo dT instead of random primers to synthesize cDNA. In addition, to eliminate of DNA

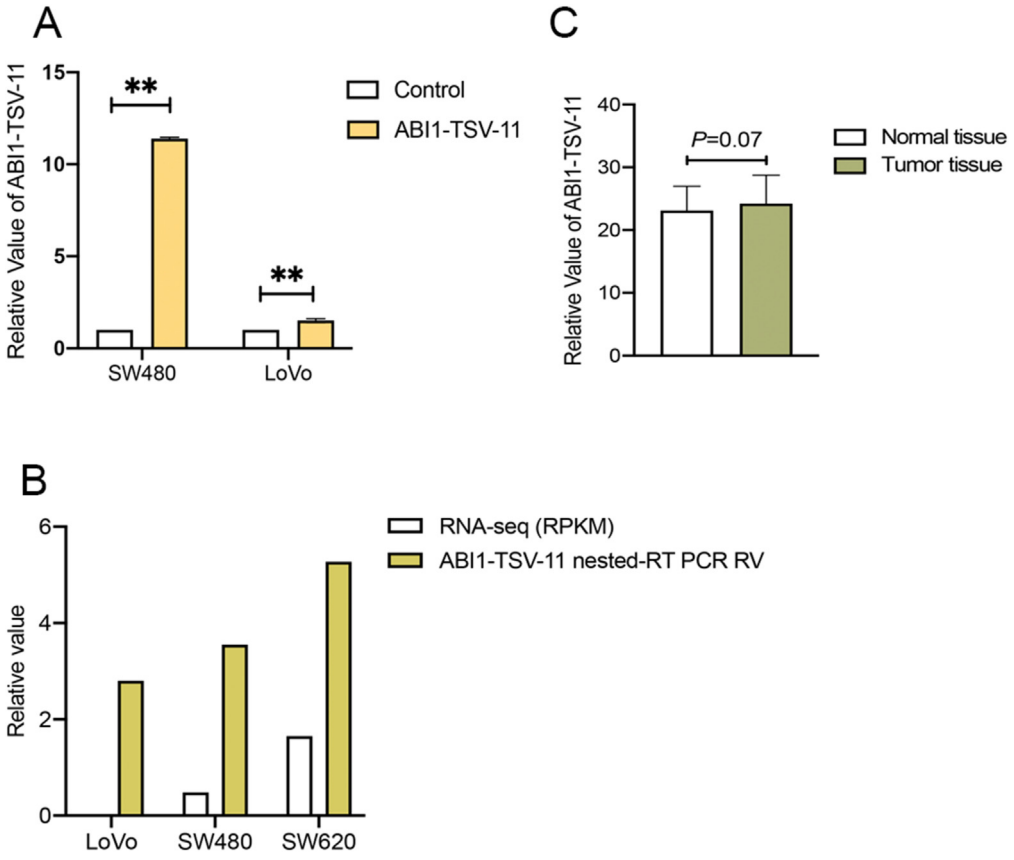


Figure 5. (A) Relative value of ABI1-TSV-11 in SW480-ABI1-TSV-11 and LoVo-ABI1-TSV-11 cell lines, as well as the controls. (B) The relative value of CRC cell lines (LoVo, SW480, SW620) with RNA sequencing and ABI1-TSV-11 quantitative-Nested-PCR. (C) The relative value of tumor and normal tissues from CRC patients.

contamination, the gDNA Eraser reaction was performed, as genomic DNA may cause a difficult challenge of nonspecific amplifications, and amplification of low expressed splice variant may be inhibited by competition with template from genomic DNA. Second, after the purification of PCR product from pre-amplification. The expression analysis of ABI1-TSV-11 was then determined by RT-qPCR. In this study, quantitative nested-PCR analytical system exhibits good analytical sensitivity (limit of detection; LOD = 2.62×10^3 copies/reaction) (Figure 4) and specificity (100%) (Figure 5A). Additionally, its reliability is determined and validated by cell lines, showing 100% analytical accuracy compared with RNA sequencing. We have also successfully applied this strategy for the detection in cell line and clinical tumor tissues.

Our study is distinct in taking a detection approach for splice variant specific detection by quantitative nested-PCR, given the strategy to avoid nonspecific amplifications for RT-PCR using pre-amplification and a primer spanning the exon junction. Our study is limited by (1) the lack of experiment to standardize the workflow in clinical practice; and (2) the limited sample size of patients' samples. Therefore, further studies using more clinical samples to evaluate this assay in clinical practice are required to confirm the findings here and to disentangle the ABI1-TSV-11 abundance in roles regulating tumor metastasis and progression.

5. Conclusions

Given the observation of important evidence for high level of ABI1-TSV-11 in the colon promoting the development of CRC, we developed a method which is specific, sensitive, repeatable and accurate to determine the expression of ABI1-TSV-11 in CRC cell lines and clinical samples, suggesting the potential for the clinical prognosis evaluation of LsCC.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Data availability statement

The authors are unable or have chosen not to specify which data has been used.

Declaration of interest's statement

The authors declare no competing interests.

Additional information

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