

# Identification of a novel transcript isoform of the *TLL12* gene in human cancers

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**Abstract.** Tubulin tyrosine ligase like 12 (*TLL12*), a member of the tubulin tyrosine ligase (TLL) family, has not been completely characterized to date. It is reported that histone methylation, tubulin modifications, mitotic duration and chromosome ploidy play crucial roles in a variety of cancers, and are related to tumorigenesis and cancer progression. A recent study showed that *TLL12* may be a pseudo-enzyme which has a SET-like domain and a TLL-like domain. In the present study, we first used 3'-rapid amplification of cDNA ends (3'-RACE) to amplify the transcripts of the *TLL12* gene from a human lung cancer cell line H1299, and unexpectedly discovered a new transcript isoform characterized with an additional 108-bp nucleotide sequence inserted at the location from 902 to 903 bases of the *TLL12* coding sequence (CDS), where it also locates between exons 5 and 6. Next, utilizing RT-PCR and Sanger sequencing, we further confirmed the existence of such a new transcript isoform of *TLL12* in more human cancer cells including lung cancer cells and other cancer cells. Moreover, several lung cancer cell lines were found to display a much higher proportion of the new isoform compared with *TLL12* wild-type transcript. These results suggest that the new *TLL12* isoform may be of importance for proper maintenance of lung cancer cells. Therefore, the new isoform of *TLL12*, with the inserted sequences probably acting as a disordered region, provides a novel perspective regarding *TLL12* functions in human cancers including lung cancer.

## Introduction

Tubulin tyrosine ligase like 12 (*TLL12*) is the least characterized and the most divergent member of the TLL family, which possesses catalytic functions in tubulin post-translational modifications (1-3). However, *TLL12* is said to be a pseudo-enzyme having a phylogenetically conserved association of two nonfunctional domains including a SET-like domain and TLL-like domain, for which several differences exist in the structure, compared with other members of the TLL family (4). These two domains are associated with histone methylation and tubulin modification, respectively. Recent studies have shown that *TLL12* could affect histone methylation, tubulin modification, mitotic duration and chromosome ploidy in human larynx cancer cells (5-8). In addition, there are numerous studies that have revealed that the TLL family is closely linked to human cancer, such as neuroblastomas (9-11). For example, the TLL family is often suppressed in human cancer (10) and is positively correlated with a poor prognosis in breast cancer (11). In prostate cancer, the level of *TLL12* expression was found to be increased in the proliferating layer of benign tissue and during cancer progression to metastasis (12,13). These findings suggest that *TLL12* may display the same pattern in other types of cancers, and it could play a crucial role in tumorigenesis and tumor progression.

In the present study, we found a new transcript isoform of the *TLL12* gene, namely the 36 amino acid (AA) plus isoform, with an additional 108-bp nucleotide sequence inserted between exons 5 and 6 of the wild-type. This new isoform may be the result of alternative splicing, and we identified it in several lung cancer cell lines as well as other cancer cell lines. Notably, some of the lung cancer cell lines presented a much higher proportion of the 36AA plus isoform compared with the wild-type *TLL12* transcript, implying the potential role of the new isoform in lung cancer cells. Moreover, the inserted 36AA was predicted to be part of a disordered region, which can alter the molecular structure of the whole protein, and could affect adaptive and deleterious-on gene expression and function.

Collectively, our discovery of a new transcript isoform of *TLL12* with an additional 108 bp, predicted to be a disordered

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region, provides an alternative prospective to study the functions of *TLL12* in tumor development and progression. In addition, our findings may also open a new window to explore potential targets related to post-translational modification of tubulin, which may eventually contribute to the development of more selective agents for cancer therapy.

## Materials and methods

**Cell culture.** Human lung cancer cell lines (H1299, 95-D, SPCA-1, A549, SK-MES-1, PC-9, H2170 and Hcc-827), human esophageal cancer cell lines (TE-11 and EC-109), human normal esophageal cell lines (HET-1A and HEEpiC), human acute monocytic leukemia cell line (THP-1), and human breast cancer cell line (MCF-7) were purchased from the Cell Bank of the China Academy of Science (Shanghai, China). THP-1, EC-109 and all the human lung cancer cell lines were grown in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gemini, Woodland, CA, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin. TE-11 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) culture medium (Life Technologies) supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. HET-1A and HEEpiC were grown in EpiCM-2 complete medium (ScienCell, Carlsbad, CA, USA) with 100 IU/ml penicillin and 100 µg/ml streptomycin. All of the cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**RNA isolation.** Total RNA was isolated from the cell cultures using TRIzol (Life Technologies) according to the manufacturer's instructions. The total RNA was quantified based on absorbance at 260 nm and the integrity of purified RNA was confirmed by agarose gel electrophoresis with a 28S/18S ratio not <1. The total RNA was stored at -80°C.

**3'-Rapid amplification of cDNA ends (3'-RACE).** As displayed in Fig. 1, the full length of *TLL12* cDNA was amplified by 3'-RACE (SMARTer RACE 3' kit; Takara, Dalian, China). First-strand cDNA synthesis from total RNA of H1299 was performed using a traditional reverse transcription procedure, but with a special oligo(dT) primer: [3'-RACE CDS primer, 5'-AAGCAGTGGTATCAACGCAGAGTAC (T) 30VN-3'; N=A, C, G or T; V=A, G, or C]. The first-strand cDNA synthesis reaction products were diluted with 10 µl Tricine-EDTA Buffer (Takara). The diluted first-strand cDNA was used as template and second-strand synthesis was amplified with a 3' gene-specific primer (3' GSP, 5'-GATTACGCCAAGCTTAG AGCACACAGACGGCGCGGGTG-3') and universal primer mix A (UPM; long, 5'-CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT-3' and short, 5'-CTAAT ACGACTCACTATAGGGC-3'). The 3'-RACE DNA samples were electrophoresed on an agarose gel. The position of the desired fragment was located under UV light and the products were extracted with the NucleoSpin Gel and PCR Clean-Up kit (Takara).

**In-fusion cloning of RACE products.** The 3'-RACE products were cloned into a pUC19 vector by the In-Fusion HD Cloning kit (Takara) according to the protocol. The recombinant vectors

were then transformed into Stellar Competent Cells (Takara) and the bacteria was spread on LB plates containing 100 µg/ml of ampicillin. All of the plates were incubated overnight at 37°C and individual isolated colonies were randomly selected from each experimental plate. Plasmid DNA was isolated using HiPure Plasmid EF Micro kit (Magen, Suzhou, China) according to the manufacturer's instructions.

**RT-PCR.** First-strand cDNA from total RNA was constructed by Transcript II All-in-One First-Strand cDNA Synthesis SuperMix kit (Life Technologies) following the manufacturer's instructions. The first-strand cDNA was then used as a template for PCR and the specific primers used were as follows: *TLL12* sense primer 1, 5'-GAAGATGCCGGTGTGGTATA-3' and *TLL12* antisense primer 1, 5'-CACGTCCGTGTAGACCT TGA-3'; *TLL12* sense primer 2, 5'-GAGACTTTGCCTACG GAGAGA-3' and *TLL12* antisense primer 2, 5'-GCTGAG TTTCCTGTAGTCCTTGA-3'. Meanwhile, we used pUC19 vectors inserted with *TLL12* wild-type or *TLL12* 36AA plus isoform as positive templates for PCR amplification of *TLL12* wild-type or 36AA plus isoform, respectively. PCR products were characterized by electrophoresing on agarose gel. The desired position was determined under UV light and PCR products were isolated by NucleoSpin Gel and PCR Clean-Up kit.

**Cloning and sequencing.** PCR products were cloned into a pMD18-T Simple Vector (Takara) and transformed into chemically competent cells. Individual colonies were grown overnight at 37°C and plasmid DNA was isolated using HiPure Plasmid EF Micro kit according to the protocol, and processed to Sanger sequencing (Life Technologies).

**Bioinformatics analysis.** Protein structures of the *TLL12* wild-type and new *TLL12* 36AA plus isoform were predicted by bioinformatics softwares. The mRNA CDS and AA sequence of wild-type *TLL12* were gained in NCBI (<http://www.ncbi.nlm.nih.gov/blast>). In addition, the new transcript of *TLL12* was translated into a new AA sequence using DNASTar EditSeq. Then, the AA sequence of the new *TLL12* 36AA plus isoform was aligned with the wild-type by NCBI protein blast (<http://blast.ncbi.nlm.nih.gov/>). Physicochemical properties of the wild-type and new isoform were predicted by online tools ProtParam and ProtScale (<http://www.expasy.ch/tools/protscale.html>), respectively. Secondary structures of the wild-type and new isoform AA sequences were predicted by DNASTar Protean and online tool CFSSP (<http://www.biogem.org/tool/chou-fasman/>). To further investigate the function of the new isoform of the *TLL12* transcript, we also used the online prediction tool DisPort search (<http://www.disport.org/search.php>) to predict whether the additional 36 AA sequence is in a disordered region of *TLL12*.

## Results

**The full length of *TLL12* cDNA amplified by 3'-RACE from total RNA of H1299 cells.** To gain the full length cDNA of *TLL12*, we first generated the first-strand cDNA from total RNA of H1299 cells, and then the first-strand cDNA was used as the template to amplify the second-strand cDNA using

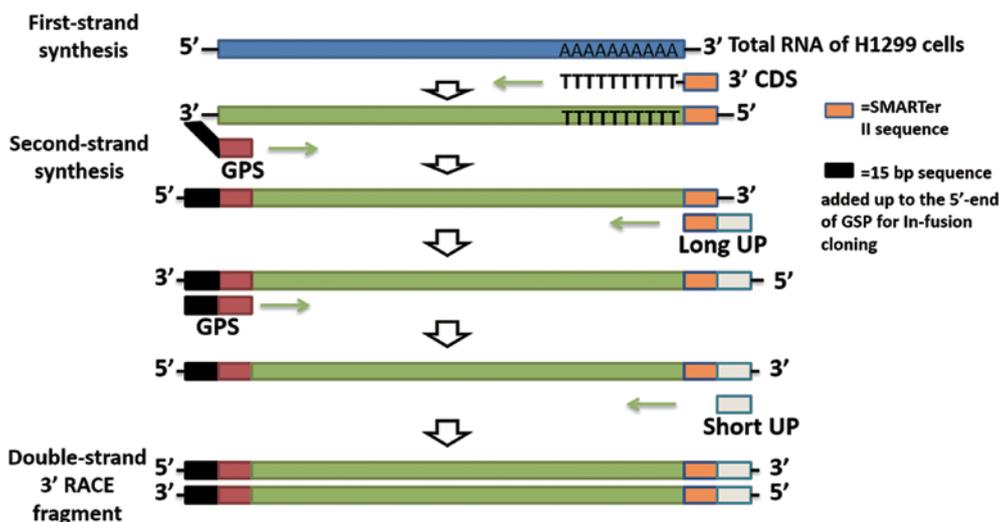


Figure 1. The procedure of the 3'-rapid amplification of cDNA ends. The 3'-RACE first-strand cDNA was synthesized using a traditional reverse transcription procedure, but with a special oligo(dT) primer (3' CDS). The first-strand cDNA was used as the template for the second-strand synthesis. The second-strand cDNA was generated with a distinct gene-specific primer (GSP) and universal primers mix A (UPM, containing long universal primer and short universal primer). The GSP and UPM ensure the full length of TTLL12 cDNA can be synthesized.

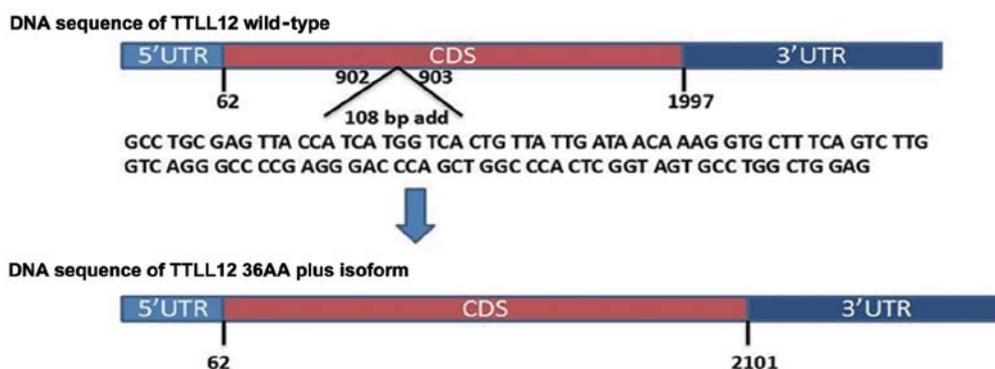


Figure 2. Diagrams for DNA sequences of the TTLL12 wild-type and the new TTLL12 36AA plus isoform. The new TTLL12 36AA plus isoform was found to contain an additional 108-bp nucleotide sequence which was located in the CDS from nucleotides 902 to 903, between exons 5 and 6, when aligned with the GenBank consensus *Homo sapiens* TTLL12 genomic sequence.

3'-RACE (Fig. 1). We aligned the 3'-RACE sequences with the consensus *Homo sapiens* TTLL12 genomic sequence (wild-type) from GenBank, and then found an additional 108-bp nucleotide sequence located at CDS from 902 to 903 bases, between exons 5 and 6 (Fig. 2). This new isoform was called the 36AA plus isoform of TTLL12.

**Detection of the new TTLL12 36AA plus isoform in both lung cancer cell lines and other cancer cell lines.** To determine whether the TTLL12 36AA plus isoform exists in human cancers, we isolated total RNAs from 8 human lung cancer cell lines (H1299, 95-D, SPCA-1, A549, SK-MES-1, PC-9, H2170 and Hcc-827), 2 human esophageal cancer cell lines (TE-11 and EC-109), 2 human normal esophageal cell lines (HET-1A, HEEpiC), human acute monocytic leukemia cell line THP-1 and human breast cancer cell line MCF-7. The reverse transcriptase-PCR products were then used as the template for PCR. In PCR, the plasmids harboring the 36AA plus isoform and wild-type of the TTLL12 gene sequence were used as the

positive templates for the 36AA plus isoform and wild-type, respectively. All the PCR products were electrophoresed on agarose gel. We then located the position of the desired fragment under UV light and extracted the PCR products from the gel. Extracted PCR products were subsequently cloned into a pMD18-T Simple Vector and transformed into chemically competent cells. All the plasmids from the positive clone were sequenced and aligned with the TTLL12 36AA plus isoform and TTLL12 wild-type. As a result, we found the product of the TTLL12 36AA plus isoform in all of the detected cell lines, and 3 lung cancer cell lines (H1299, H2170 and Hcc-827) showed the highest proportion of the 36AA plus isoform compared to the wild-type (Fig. 3). In addition, the sequencing results of the plasmids extracted from the positive clone were aligned, and demonstrated that the PCR products were from the TTLL12 36AA plus isoform (Fig. 4). Significantly, the TTLL12 36AA plus isoform was expressed more abundantly than the wild-type in the human cancer cell lines, particularly in the human lung cancer cell lines, as compared with the human normal

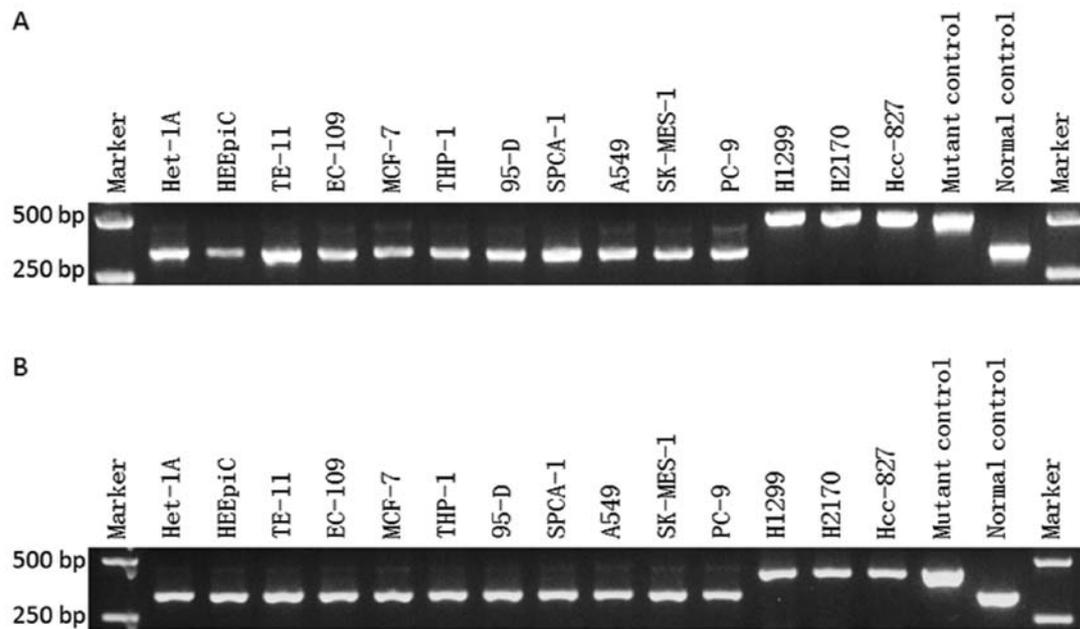


Figure 3. Detection of the new *TLL12* 36AA plus isoform in human cancer cell lines. RT-PCR was employed in a series of cell lines including human lung cancer cell lines (H1299, 95-D, SPCA-1, A549, SK-MES-1, PC-9, H2170 and Hcc-827), human esophageal cancer cell lines (TE-11 and EC-109), human normal esophageal cell lines (HET-1A and HEEpiC), human acute monocytic leukemia cell line THP-1, and human breast cancer cell line MCF-7. PCR was performed using (A) *TLL12* sense primer 1 and *TLL12* antisense primer 1, and (B) *TLL12* sense primer 2 and *TLL12* antisense primer 2. The PCR product for positive control of the 36AA plus isoform was 484 bp in size while the size of the wild-type product was 376 bp, when the pair of primer 1 was used. The product for positive control of the 36AA plus isoform was 434 bp in size while the wild-type was 326 bp in size as the pair of primer 2 was used.

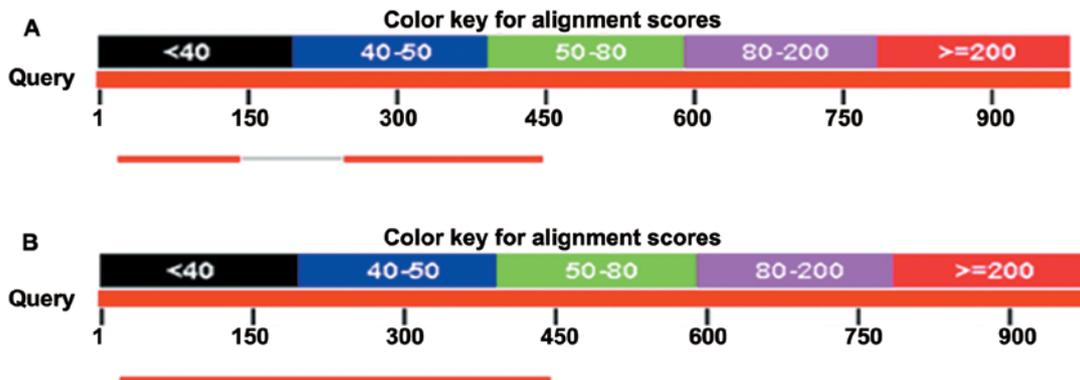


Figure 4. Alignment of DNA sequences of the *TLL12* PCR products. DNA sequences of the *TLL12* PCR products were aligned between (A) *TLL12* wild-type and (B) new *TLL12* 36AA plus isoform.

esophageal cell lines. The findings indicate that the new *TLL12* 36AA plus isoform may play an important role in the development of various tumors.

**Physicochemical properties of the *TLL12* wild-type and new 36AA plus isoform.** The AA sequence of the *TLL12* 36AA plus isoform was obtained using DNASTar EditSeq and translating the DNA sequences. Then, the AA sequence of the new isoform was aligned with the *TLL12* wild-type, and we found an additional 36AA inserted at the location of 281AA of the *TLL12* wild-type (Fig. 5A). Next, we predicted the physicochemical properties by ProtParam and ProtScale. The properties of *TLL12* wild-type and 36AA plus isoform AA sequences were predicted (Table I) as follows: the total number of negatively charged residues (Asp + Glu) was

93 and 96; and grand average of hydropathicity (GRAVY) was -0.390 and -0.338. In addition, the total hydropathicities of the two types of *TLL12* proteins are displayed in diagram (Fig. 5B and C). These results suggest that the two types of *TLL12* proteins are hydrophathical molecules, and the 36AA plus isoform is more stable than the wild-type.

**Secondary structures of *TLL12* wild-type and new 36AA plus isoform.** Secondary structures of the *TLL12* wild-type and *TLL12* 36AA plus isoform were predicted by DNASTar Protean and online tool CFSSP. As a result, the total residues of  $\alpha$ -Helix,  $\beta$ -sheet and  $\beta$ -turn in the *TLL12* wild-type were 476, 428 and 72, and the corresponding percentages were 73.9, 66.5 and 11.2%, respectively (Fig. 6A). While total residues of  $\alpha$ -Helix,  $\beta$ -sheet and  $\beta$ -turn in the *TLL12* 36AA plus isoform

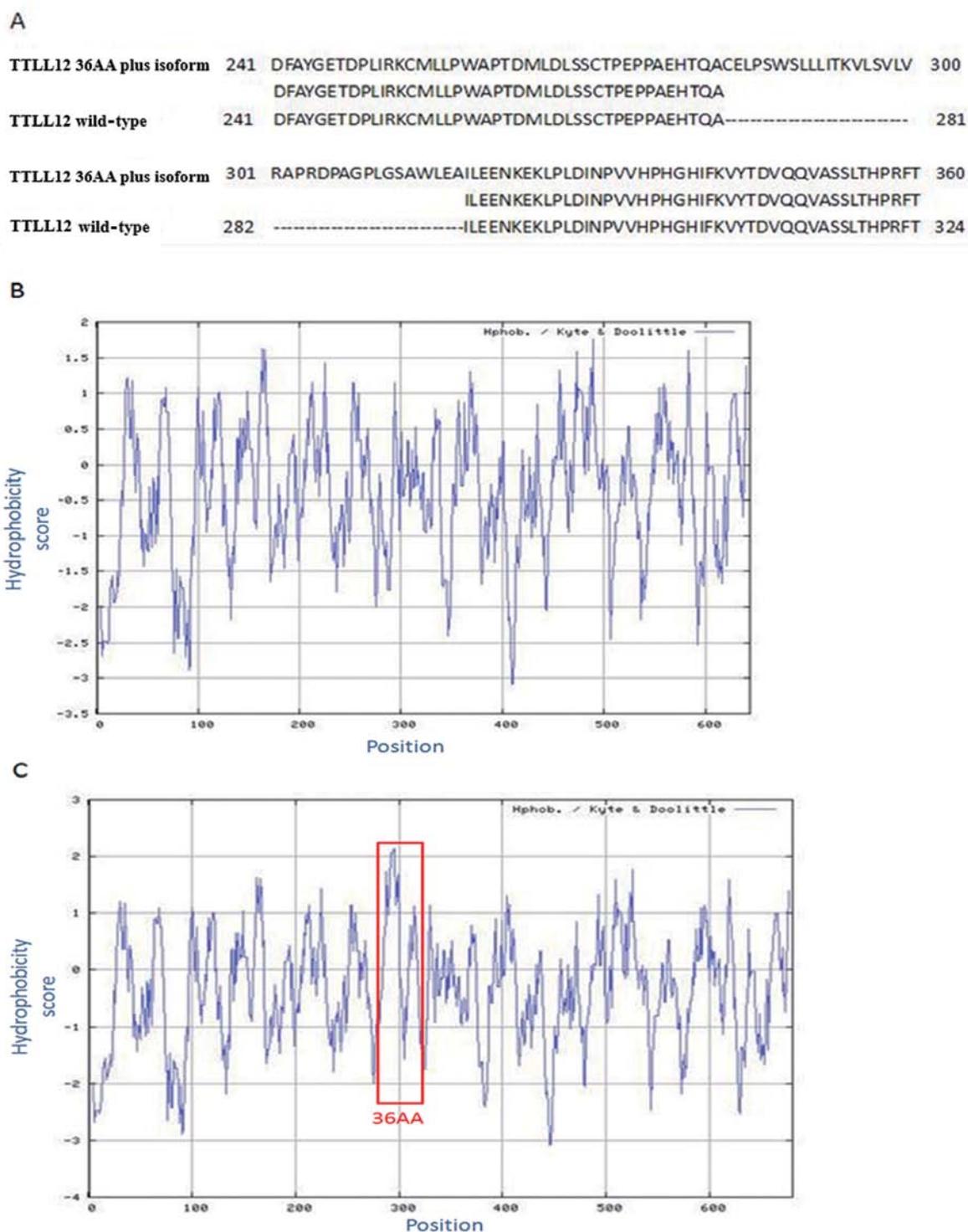


Figure 5. Analysis of the amino acid sequences of the TLL12 wild-type and new TLL12 36AA plus isoform. The amino acid sequence of the new TLL12 36AA plus isoform was aligned with the TLL12 wild-type by NCBI protein blast, and an additional 36 amino acid sequence was found (A). Hydropathicity of the wild-type (B) and the new 36AA plus isoform (C, red box indicates the inserted 36AA peptide) of TLL12 was predicted by ProtParam and ProtScale, respectively.

were 504, 455 and 75, and the corresponding percentages were 74.1, 66.9 and 11.0%, respectively (Fig. 6B). The results showed that the proportions of different types of secondary structures in these two TLL12 proteins were slightly different (Table II).

*The additional 36AA is involved in a disordered region of TLL12. To ascertain whether the additional 36AA in the*

TLL12 36AA plus isoform is in a disordered region of TLL12, we firstly sought the disordered regions of the TLL12 36AA plus isoform by the opening accessible online resources DisProt search, and found that the peptide of 260AA to 299AA in the TLL12 36AA plus isoform was highly identical to the disordered region of Bcl-2-like protein 11 (B2L11) (Fig. 7A), suggesting this peptide could act as a disordered region in the



Table I. Physicochemical properties of the wild-type and new 36AA plus isoform of TTLL12.

Physicochemical properties	Wild-type	New 36AA plus isoform
Number of amino acids	644	680
Molecule formulas	C <sub>3359</sub> H <sub>5101</sub> N <sub>905</sub> O <sub>959</sub> S <sub>28</sub>	C <sub>3537</sub> H <sub>5388</sub> N <sub>950</sub> O <sub>1006</sub> S <sub>29</sub>
Molecular weight	74,403.6	78,245.1
PI	5.33	5.34
Instability index	50.17	49.77
Aliphatic index	79.18	82.01
Negatively charged residues (Asp + Glu)	93	96
Positively charged residues (Arg + Lys)	65	68
GRAVY	-0.390	-0.338

TTLL, tubulin tyrosine ligase; PI, isoelectric point; GRAVY, grand average of hydrophobicity.

Table II. Secondary structures of the TTLL12 wild-type and new 36AA plus isoform.

TTLL12 type	$\alpha$ -Helix		$\beta$ -sheet		$\beta$ -turn	
	Total residues	%	Total residues	%	Total residues	%
Wild-type	476	73.9	428	66.6	72	11.2
New 36AA plus isoform	504	74.1	455	66.9	75	11.0

TTLL, tubulin tyrosine ligase.

that the additional 36AA was partly identical to the disordered region of brain natriuretic peptide-32 (BNP-32) (Fig. 7B), giving the possibility that the 36AA could endow TTLL12 with new functions by acting as part of the disordered region. However, the biological functions of the new TTLL12 isoform in cancers remain to be further investigated.

## Discussion

The tubulin tyrosine ligase (TTLL) family is a superfamily consisting of 14 members and they mainly catalyze ligation of amino acids to tubulins (1-3). TTLL12 is the least characterized and a most special member of the TTLL family, and it plays an important role in histone and tubulin modifications (14), mitotic duration and chromosome ploidy (5-8). It is the only member of the TTLL family without an assigned enzymatic function (15-17), and is said to be a pseudo-enzyme that has a phylogenetically conserved association of two domains including the SET-like domain and TTL-like domain in the N- and C- terminal of TTLL12, respectively. These two domains are related to histone methylation (18-22) and tubulin modifications (23-25) and then may contribute to tumorigenesis (4). The TTLL family is often suppressed in human cancers (10) and is closely connected with the poor prognosis of breast cancer (11), suggesting that TTLL12 could also play roles in human cancers. It is reported that TTLL12 increases its expression in the proliferating layer of benign human prostate and more apparently during cancer progression to metastasis (12), suggesting that TTLL12 may be of importance in tumor progression.

In the present study, we found an aberrant TTLL12 transcript with an additional 108-bp nucleotide sequence inserted between exons 5 and 6. This new isoform, named the TTLL12 36AA plus isoform, exhibited much more abundance, as compared with the TTLL12 wild-type, in human cancer cells as to normal cells, particularly in human lung cancer cells, suggesting it could be considered a new mechanism of human cancer development.

Alternatively splicing, by removing non-coding sequences (introns) and joining a coding part (exons) (26), plays an important role in gene expression (27-29). By the alternative splicing of precursor mRNAs (pre-mRNAs), multiple mRNAs and proteins can be generated from a single gene, and then the coding capacity of genomes is expanded through such a major mechanism (27-31). Furthermore, it has been reported that aberrant alternative splicing underlies various pathological processes, in particular tumorigenesis and tumor progression (32-34). The new TTLL12 36AA plus isoform may be the result of aberrant alternative splicing. Analysis by bioinformatics software showed that there are some differences in peptide physicochemical properties and protein secondary structure between the TTLL12 36AA plus isoform and the TTLL12 wild-type, suggesting that the new 36AA plus isoform could add some distinguished functions to TTLL12.

The traditional pattern analysis of protein structural biology indicates that the well-defined three-dimensional structures are pivotal to biological functions of proteins. However, it has been reported that the disordered regions of proteins are challenging this traditional structure-function paradigm (35,36). Disordered regions are ubiquitous in cellular processes and

human pathological conditions, but lack a well-defined, stable three-dimensional fold (37,38). Proteins with disordered regions often go through disorder-to-order when binding to their partners and they can remain partially or fully flexible in their bound state, and form fuzzy complexes (37,39-41). In contrast, proteins with disordered regions are mainly involved in enzymatic activity (37,42), including cell cycle regulation (43), cell division and differentiation (42,44), which play critical roles in different types of cancers (45). In the present study, we report that the additional 36AA in the newly found TTL12 protein isoform is a part of the disordered region, with the identity of those disordered regions identified in other functional proteins. Whether the 36AA is the binding site for certain proteins, such as those participating in post-translational modification of tubulin or other important cell process, still needs to be investigated.

The TTL family has been shown to be partially co-localized with vimentin and tubulin, which causes post-translational modifications of tubulin (5-8). Tubulin is a well acknowledged and important target for tumor therapy (46), but the precise functions of TTL12 in tubulin modification are still poorly understood. Proteins related to post-translational modification are targets for new cancer therapeutic agents (47,48), thus the new 36AA plus isoform of TTL12 protein may have the potential to become a novel strategy for cancer treatment involving tubulin post-translational modifications. Therefore, the functions and molecular mechanisms of the new 36AA plus isoform in cancer development remain to be investigated.

In conclusion, our findings unveil a novel transcript isoform of TTL12 with an additional 108-bp nucleotide sequence in the CDS. This TTL12 36AA plus isoform could shed light on a novel mechanism of TTL12 in human carcinogenesis and tumor progression, which may lead to a breakthrough of new potential targets for human cancer therapeutics.

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