RESEARCH ARTICLE

Alternative Messenger RNA Splicing of Autophagic Gene Beclin 1 in Human B-cell Acute Lymphoblastic Leukemia Cells

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Abstract

Beclin 1 is a key factor for initiation and regulation of autophagy, which is a cellular catabolic process involved in tumorigenesis. To investigate the role of alternative splicing of Beclin1 in the regulation of autophagy in leukemia cells, Beclin1 mRNA from 6 different types of cell lines and peripheral blood mononuclear cells from 2 healthy volunteers was reversely transcribed, subcloned, and screened for alternative splicing. New transcript variants were analyzed by DNA sequencing. A transcript variant of Beclin 1 gene carrying a deletion of exon 11, which encoded a C-terminal truncation of Beclin 1 isoform, was found. The alternative isoform was assessed by bioinformatics, immunoblotting and subcellular localization. The results showed that this variable transcript is generated by alternative 3' splicing, and its translational product displayed a reduced activity in induction of autophagy by starvation, indicating that the spliced isoform might function as a dominant negative modulator of autophagy. Our findings suggest that the alternative splicing of Beclin 1 might play important roles in leukemogenesis regulated by autophagy.

Keywords: Beclin 1 - alternative splicing - autophagy - acute leukemia

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Introduction

Autophagy is a lysosome-mediated pathway responsible for degradation of damaged organelles and protein aggregates from the cytoplasm (Parkhitko et al., 2013). It keeps at basal level of autophagy under normal condition, but activated to higher level upon multiple physiological or pathological stimulus (Watson et al., 2011). Although much effort has been made in this field, the precise function and regulation of autophagy in cancer, such as leukemia, has not been fully elucidated. Recent studies suggest that autophagy plays an essential role in hematopoietic stem cells (HSCs) and might also be implicated in leukemia by using conditional knockout mouse models (Pua et al., 2007; Kundu et al., 2008; Sandoval et al., 2008; Zhang et al., 2009a; Mortensen et al., 2010a; Mortensen et al., 2010b; Mortensen et al., 2011). In others studies, activation of autophagy induced by pharmacological methods inhibits the growth of leukemia cells and degrades oncoproteins such as PML-RARA and BCR-ABL (Isakson et al., 2010; Elzinga et al., 2013; Yang et al., 2014), suggesting that autophagy might be an important pharmacological target in the prevention or treatment of leukemia.

Autophagy is initiated by the formation of a double membrane-bound vacuole known as the autophagosome, which is a multiple process that includes the biogenesis of the isolation membrane, followed by its elongation and closure (He et al., 2009; Parkhitko et al., 2013). The process is precisely regulated by ATG proteins, among which Beclin 1 is a major regulator in autophagy initiation and progression (Kang et al., 2011a). Originally identified as bcl-2 interacting proteins, Beclin 1 was the first autophagy related gene identified in mammalian system, and has been shown to be involved in several other biological functions in human diseases, including heart disease (Essick et al., 2013; Zeng et al., 2013), cancer (Jiang et al., 2012; Han et al., 2013; Karpathiou et al., 2013; Wu et al., 2013; Yu et al., 2013), pathogen infection (Liu et al., 2005; Patel et al., 2008; Huang et al., 2009; Vazquez et al., 2010), and neurodegeneration (Xing et al., 2012; Fields et al., 2013; Lucin et al., 2013; Nascimento-Ferreira et al., 2013). Beclin 1-/- mice die early in embryogenesis and Beclin 1+/- mice suffer from a high incidence of spontaneous tumors, including B-lymphoblast cell lymphoma (Yue et al., 2003), an aggressive disease characterized by excessive proliferation of immature B cells in lymphoid tissues. Recent studies with patient samples indicate that low Beclin 1 expression was significantly associated with worse overall survival and progression-free survival of natural T-cell lymphoma and non-Hodgkin lymphoma (Huang et al., 2010; Nicotra et al., 2010). These results imply that Beclin 1 might have an essential role in development and differentiation of lymphoid cells. Beclin

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1 harbors a BH3 domain, which binds to Bcl-2. This binding inhibits the association of Beclin 1 with class III PI3K and enhances the prevention of autophagy membrane nucleation (Liang et al., 1998; Itakura et al., 2008). In response to stimuli or stress, Beclin 1 disassociates with Bcl-2 and binds to autophagic proteins including class III PI3K, and subsequently induces autophagic response. However, the precise mechanism underlying autophagy regulation mediated by Beclin 1 in physiological and pathological processes are not fully understood.

Alternative splicing is a post-transcriptional process which controls almost all human gene expression and function. Abnormality in the splicing process may affect the progression of various human diseases (Kornblihtt et al., 2013; Tang et al., 2013). Eukaryotic pre-mRNAs are transcribed from genomic DNA and spliced to form mature mRNAs. The pre-mRNA alternative splicing, a process in which intronic regions and exonic sequences are destined to be removed, greatly increases the diversity and complexity of gene expression (Tang et al., 2013). Alternative splicing that affects the protein coding gene of mRNA gives rise to proteins that differ in their sequence and therefore in their activities. Two recent studies have investigated the implication of RNA splicing in the regulation of autophagic activity, one of which reported that transcription factor XBP1 mRNA splicing activates autophagy in endothelial cells through upregulating Beclin 1 transcription (Margariti et al., 2013); another study found that there are no canonical transcripts of full-length ATG5 but only two alternatively spliced ATG5 transcripts in prostate cancer DU145 cells, which lack one or two exon and lead to deficiency of autophagy in the cells (Ouyang et al., 2013). Our present study investigated alternative splicing of Beclin 1 in acute lymphoblastic leukemia cells. We indentified a transcript variant of Beclin 1 gene, of which the ORF sequence lacks exon 11 and thus results in premature termination of Beclin 1 gene translation and C-terminal truncation of Beclin 1 protein. Thus, our finding provides a new insight into the regulation of autophagy by Beclin 1. In this study we characterized alternative transcripts using subcellular locolization, co-immunoprecipitation (co-IP), and bioinformatics.

Materials and Methods

293T and HeLa cells were cultured in DMEM (Gibco, USA) containing 10% fetal calf serum (FBS), 2mM L-glutamine, sodium pyruvate and supplemented with 1% Pen-Strep. Human acute lymphoblastic leukemia cell line (697), human acute promyelocytic leukemia cell line (NB4), human leukemic monocyte lymphoma cell line (U937), human erythroleukemia cell line (HEL and K562), and human monocytic cell line (THP-1) were cultured in RPM1640 medium with 10% FBS and 1% Pen-Strep. All of these cells were incubated at 37°C with CO_2. Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-PaqueTM (ϱ=1.077g/mL) (GE Healthcare, USA). All antibodies used for western blotting or co-IP, including Beclin 1, p62, LC3β, Bcl-2 and VPS34 were purchased from Cell Signaling Technology (CST, USA).

cDNA cloning and bioinformatics

cDNAs from 6 different cell types of 697, NB4, U937, HEL, THP-1 and K562 cell lines were used as templates for PCR. The PCR primers were located at 5'UTR and 3'UTR of Beclin 1 gene, respectively. The sequence for sense primer was 5'-CTCCCGAGGTGAAGAGCATC-3', and the reverse primer sequence was 5'-AAGGCAAACCTCCC CCTAAGG-3'. The amplification was carried out with Phusion green high-fidelity DNA polymerase (Thermo Fisher, USA) and PCR products were analyzed using 1.5% agarose gel and purified with Cycle-Pure Kit (OMEGA Bio-Tek, USA). The purified amplicon was cloned into pEASY-blunt cloning vector (TRANS, Beijing, China), followed by transformation into DH5α competent cells. Bacterial single clones containing different transcripts were confirmed by sequencing. Sequence analysis and alignments were performed using DNAMAN and DNASTAR software, BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), ClustalW2 at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Vector construction and transfection

Beclin 1 wilt type ORF of 1535 bp and Del-E11 ORF of 1068 derived from 697 cells were subcloned into pCDNA3.1-EGFP (Invitrogen, USA), pLVX-CMV-IRES (Clontech, USA) expression vectors for transfection respectively. Lentivirus was produced according to the manufacture's protocol. 293T and HeLa cells were transiently transfected with Lipofactamine 2000 according to the manufacture's protocol. 2.5 μg plasmid and 5 μl Lipofactamine 2000 reagent were used for a 6-well plate.. For stable expression in 697 cells, 1000 plaque forming units of virus expressing Beclin 1 and Del-E11 were added into a 10mm cell plate, the medium was removed 2 hours later and replaced by complete medium. Stable expressing cells were sorted based on the green fluorescent protein (GFP) with BD FACS Aria III (BD, USA). Expression of Beclin 1 and Del-E11 in 697 cells were confirmed by western blotting.

Western blotting

Cells were washed three times with ice-cold PBS, and cellular protein were extracted by lysing cells in lysis buffer (50mM Tris-HCl, pH8.0, 150mM NaCl, 100 μg/ml PSMF, 1%TritonX-100) for 30 min on ice. After centrifugation at 12, 000g, 20 min, the supernatant was extracted as protein and quantified by Bradford method. 30μg protein of different groups were separated by electrophoresis in 10% SDS-PAGE and transferred onto PVDF membrane. Nonspecific reactivity was blocked by incubation for 1h in BSA buffer (10mM Tris-HCl, pH7.5, 150mM NaCl, 2%Tween-20, 4% bovine serum albumin). The membrane was then incubated with primary antibody overnight and secondary antibody for 1h The protein signals were detected using the ECL system (Biological Industries, Kibbutz Beit Haemek, Israel).

Co-Immunoprecipitation (Co-IP)

Lentiviral vectors containing Beclin 1 and Del-E11 sequence were transfected into 697 cells, transfected-positive cells were sorted against EGFP signal using

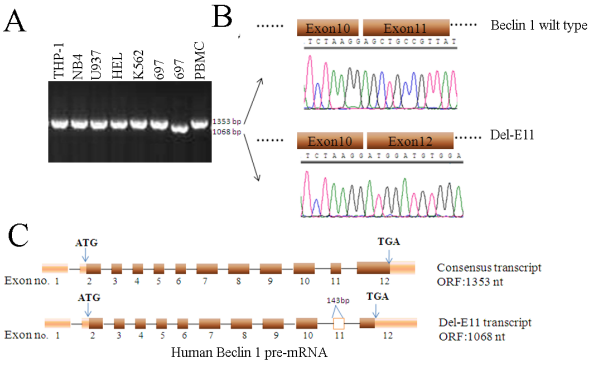

Figure 1. Identification of a Novel Human Beclin 1 Alternative Transcript. (A) PCR amplification products of plasmid clones containing alternatively splicing transcripts were analyzed in 1.2% (g/ml) agarose gel. (B) Sequence alignment analysis between wild-type Beclin 1 and Del-E11, showing the junction of exon 10 and 12. (C) Schematic diagram of deletion of exon 11 results a premature stop codon at the 5' terminal of exon 11 in Del-E11

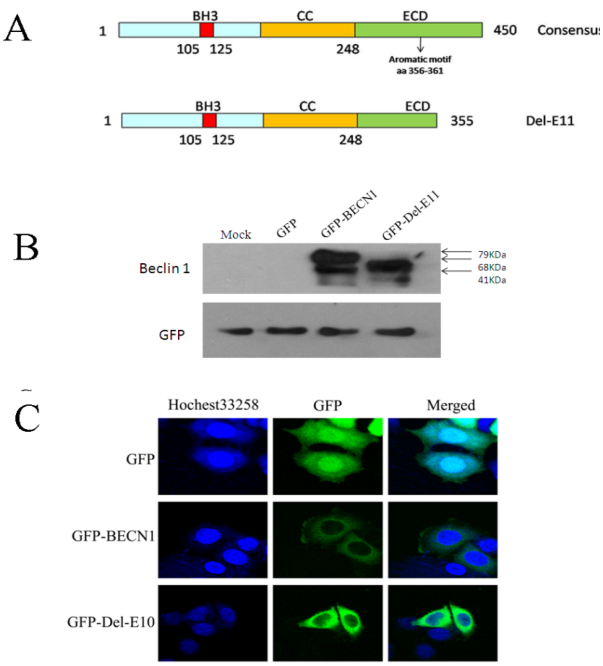

Figure 2. Characterization and Subcellular Localization of Beclin 1 and Del-E11 (A) Schematic diagram of Beclin 1 and Del-E11 protein structures. (B) After transfection with or without expression or control vector for 48 h, cells were lysed and subject to western blotting with Beclin 1 antibody. (C) Expression vectors were transfected into HeLa cell, and images were captured using confocal microscopy at 400 × magnification. Green fluorescent signals indicated GFP-tagged proteins, Blue fluorescence can be observed after nuclear staining with Hochest 33258. "Merged" represents the overlay of images

BD FACS Aria III (BD, USA). For starvation, medium was replaced by HBSS, and then cells were subjected to Co-IP Co-IP was performed using Pierce Co-Immunoprecipitation kit according to the manufacture's protocol (Thermo Scientific, USA). Briefly, cell lysates were prepared in a lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol, and protease inhibitor cocktail). The lysate was clarified by centrifugation at 13, 000 rpm for 10 min and subjected to immunoprecipitation by using antibody against Beclin 1. Precipitated immunocomplexs were washed 6 times using a wash buffer and boiled in sample buffer. Samples were subsequently separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Immunoblotting analysis was performed with specific antibodies and visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce Chemical, Rockford, IL).

Results

Identification of a shorter transcript of human Beclin 1 gene

RNA was isolated from all cells mentioned above, reverse transcribed, and the human Beclin 1 coding sequence (CDS) was cloned. Single clones were screened and plasmid clones containing copies of alternatively spliced transcripts were sequenced. A transcript variant of Beclin 1 gene carrying a deletion of exon 11, which termed as Del-E11, was only found in 697 cells (Figure 1A). Using the sequence of the transcript as a probe, we searched in database using Blast algorithm, and finally found that one transcript (XM_005257760.1) predicated from automated computational analysis loses exon 11. We have submitted this transcript of Beclin 1 to GeneBank, and received an accession number KC776730. Exon 11 in this transcript was alternatively spliced, and the splicing led to directly junction of exon 10 and 12 (Figure 1B), resulting in a translational frame shift that creates a premature stop codon in exon 12, which results in a C-terminal truncation of Beclin 1 protein (Figure 1C). Comparison with the consensus sequence, the putative product translated from Del-E11 is shorter for 95 amino acid residues (Figure 1D). An expressed sequence tag (EST) cloned from human lung cDNA library was found to be identical to Del-E11 sequence after database searching.

Characterization and Subcellular localization of Beclin 1 and Del-E11

Alternative splicing and exclusion of exon 11 causes a frame shift when translating from exon 10 to exon 12, resulting in a premature stop codon at 1066 bp at the 3' of exon 12. This results in a deletion of the region encoding the conserved C-terminal extension of Beclin 1 protein, which translated from Beclin 1 gene. The protein product translated from Del-E11 is 355 amino acids in length, in contrast to the 450 anima acids of full length protein (Beclin 1) with a long C-terminal extension (Figure 2A). The molecular weight of Del-E11 protein calculated by ProtParam software online (http://web.expasy.org/protparam/) is 41 KDa, which was confirmed by Western blotting result (Figure 2B).

Usually, subcellular localization of a protein is related to its biological function. To test whether deletion of C-terminal would affect the localization of Del-E11 transcript product, the coding sequences of Beclin 1 and Del-E11 transcripts were cloned into pEGFP-N1 expression plasmid, in which Beclin 1 and Del-E11 protein

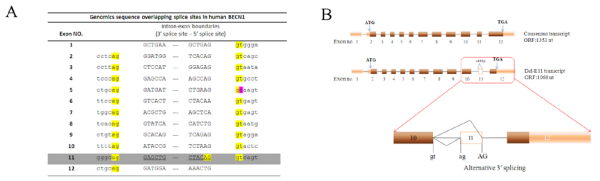

Figure 3. Analysis of Intron-exon Boundaries of Human BECN1. (A) Schematic diagram of junction of exon-intron in wild-type Beclin 1 genomic sequence. Coding region and un-coding region are shown in brown and pink boxes, respectively. Black lines represent intron. (B) Schematic diagram of the manner of alternative splicing of Beclin 1 pre-RNA

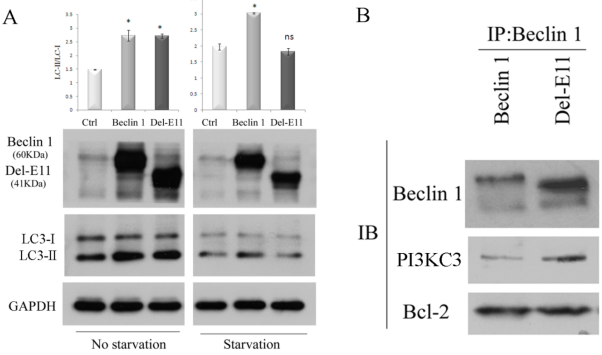

Figure 4. Del-E11 Displayed a Reduced Activity in the Induction of Autophagic Response. (A) Detection of LC3 by western blotting in 697 cells stably expressing Beclin 1 or Del-E11 under nutrient-deplete conditions. For making nutrient deplete condition, complete medium was removed and changed for HBSS, incubation for 4 hours, then cells were lysed and subject to analysis. (B) Cells expressing Beclin 1 or Del-E11 were incubated under nutrient deplete condition for 4 h, then total protein was extracted and subject to Co-IP analysis

were fused with green fluorescent protein (GFP). Vectors were transfected into NIH3T3 and Hela cells, respectively, and fusion proteins were confirmed by Western blotting. Both the Beclin 1 and Del-E11 fusion proteins were found to be distributed in the cytoplasm of the cells (Figure 2C), which indicated that there were no difference between Beclin 1 and Del-E11 protein in distribution in cells.

Analysis of intron-exon boundaries of Beclin 1 mRNA

Alternative splicing of mRNA precursors enables one gene to produce multiple isoforms with different functions. More than 95% of genes with multiple exons are involved in alternative splicing in human genome (Pan et al., 2008). Alternative splicing can occur due to many reasons, but both sequence based mechanisms and cis/trans elements that either positively or negatively regulate splicing are the predominant theories (Jeffries et al., 2013). Splice-site choice must therefore be tightly regulated in cells (Smith et al., 2000). Conservation at both 5' and 3' splice site junctions (AG GU/C) is required for efficient sequence based splicing mechanisms (Zhang et al., 2013a). The intron-exon junctions are essentially conserved in human Beclin 1 gene (Figure 3A), which indicated that when intron 10 was spliced, the alternative 5' splice-site was replaced, and the 3' boundary of the downstream exon 11 was changed, resulting in the excision of exon 11 in the mature mRNA (Figure 3B).

Del-E11 displayed a reduced activity in the induction of autophagic response

Beclin 1 plays an essential role for starvation-induced autophagic response via interacting with class III PI3K (also called VPS34). LC3, the product of MAP1LC3B gene, is generally considered as a specific autophagy marker. After the synthesis of LC3, this molecule is cleaved to form LC3-I, and upon induction of autophagy, LC3-I is conjugated to the lipid phosphatidylethanolamine to form LC3-II, which is tightly bound to the membrane of the autophagosome and to be a target protein of autophagy. Immunoblotting assessment of LC3 expression is usually used to evaluate autophagic activity in mammalian cells by evaluating the mount of LC3-II and the ratio of LC3-II/LC3-I (Mizushima et al., 2007; Torgersen et al., 2013). To examine whether Del-E11 is implicated in the regulation of autophagy, the ratio of LC3-II/LC3-I and the binding affinity of Del-E11 with class III PI3K during the process of starvation were tested by Western blotting and Co-IP assay, respectively. For this purpose, transgenic 697 cells which stably express Beclin 1 or Del-E11were generated respectively by transfecting lentiviral vectors. Autophagic responses were assessed by the transformation of LC3-I to LC3-II, a step critical for maturation of autophagosome and fusion of LC3-II with lysosomes to form autolysosomes. In nutrient-deplete conditions the amount of LC3-II was significantly higher in forced Beclin 1 expression cells than that in forced Del-E11 expression cells (Figure 4A), indicating that the alternative isoform protein has a reduced activity in the induction of autophagic response. This was confirmed by Co-IP results which showed that Del-E11 had lower binding affinity to class III PI3K, and failed to activate autophagic response to nutrient starvation (Figure 4B).

Discussion

Alternative splicing usually mediates changes in protein structure, function, and localization, which are implicated in diseases, cancers, and drug resistance (Reviewed in (Tang et al. 2013). Research on RNA splicing contributes to the understanding of cancer diagnosis, prognosis and treatment. Such as, the metastasis gene osteopontin is transcripted and yields 3 mRNA, osteopontin-a, osteopontin-b and osteopontin-c, the last one is specifically expressed in invasive, but not in noninvasive tumors, which implicate that osteopontin-c plays a role in tumor metastasis and can be taken as a potential diagnostic marker (Zhang et al., 2013b). Here, we show that alternative splicing of Beclin 1 pre-mRNA gives rise to a variable transcript in B-acute lymphoblastic leukemia cells, which carries a deletion of exon 11 and generates a C-terminal truncation of Beclin 1 isoform. Beclin 1 functions as a key factor in initiation of autophagy by interacting with class III PI3K. Beclin 1 contains three functionally structural domains: a BH3 domain (aa 114-123) at the N-terminus, a central coiled-coil domain (CCD, aa144-269) and an evolutionarily conserved domain (ECD, aa 244-450). The ECD is essential for induction of autophagy mediated by Beclin 1 (Reviewed in (Kang et al., 2011b). There is a critical motif comprising three aromatic amino acids (aa

356-361) in the ECD, whose mutation rendered Beclin 1 unable to fully rescue autophagy in Beclin 1-knockdown cells (Huang et al., 2012), indicating that the aromatic motif is essential for Beclin 1 in regulation of autophagy. Since this aromatic motif is deleted in the C-terminally truncated isoform, we predicted that the transcript variant would result in loss of autophagy-induction activity. This hypothesis was proven with assays by detection of LC3-II conversion and measurement of affinity to class III PI3K between wild-type Beclin1 and Del-E11.

In human, about 95% of multiexonic genes are alternatively spliced, which increases the production of various protein isoforms from the same genetic information, and resulting in different functional and regulatory role in cells. To further ensure fidelity of genetic information transition, many quality-control mechanisms have been evolved; one of which is nonsense-mediated mRNA decay (NMD). NMD is an evolutionarily conserved pathway whereby cells can selectively degrade aberrant mRNAs harboring premature stop codon (PTC) produced from alternative RNA splicing (Chang et al., 2007; Kervestin et al., 2012). In mammals, the recognition of PTC generally depends on the distance between premature termination codon and exon-exon junction close to 3' end. When this distance is more than 50 to 55 nucleotides, NMD is triggered and the mRNA is degraded, while this distance is less than 50 to 55 nucleotides, the alternatively spliced transcripts are not destroyed but produce truncated proteins (Zhang et al., 2009b). In this study, we have identified an alternatively spliced transcript of Beclin 1 gene Del-E11 in acute lymphoblastic cells and The PTC of Del-E11 is located in the terminal exon of Beclin 1 gene, 24-nt downstream of the last exon-exon junction. Therefore Del-E11 is not a classical target for NMD pathway. There are several examples of alternatively spliced transcripts carrying a PTC that are not cleaved but generating truncated proteins, and are implicated in diseases by functioning as dominant negative regulatory factor of wild isoform (Sztainberg et al., 2009; Batista et al., 2013; Lu et al., 2013; Salsman et al., 2013). The underlying mechanisms of different effects induced by a dominant negative regulator usually consist of aberrant dimerization, changed subcellular localization and altered affinity to binding ligand (Sabirzhanov et al., 2011; Batista et al. 2013). Since Del-E11 has more binding affinity than its wild-type isoform, it is highly probable that the product of Del-E11 transcript competitively bind to class III PI3K and interfere with the formation of Beclin 1-PI3K complex, consequently decreases autophagic response under nutrient-deplete condition. Although our observations demonstrate that Del-E11 plays a regulatory role in autophagy, its putative effect during leukemogenesis needs to be explored in our future study. Indeed, Del-E11 mRNA is also found in a fetal lung cDNA library and is associated with fetal life, which suggests that the alternatively spliced transcript might have additional yet unknown functions in development and differentiation. Usually, genes involved in differentiation and development are often associated with the occurrence of cancer. So the role of Del-E11 during leukemogenesis needs to be explored in our future study.

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