Mutation in the RNA binding protein TIS11D/ZFP36L2 is associated with the pathogenesis of acute leukemia

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Abstract. TIS11D is an AU-rich element binding protein that is involved in RNA metabolism and definitive hematopoiesis. Although disruption of genes related to hematopoiesis often leads to the development of leukemia and lymphoma, the involvement of TIS11D in hematological malignancies remains to be determined. In the present study, we identified a heterozygous frameshift mutation (I373fsX91) in the carboxy-terminus of the *TIS11D* gene in leukemic cells from a patient with acute myeloid leukemia. Moreover, biallelic inactivation of the *TIS11D* gene resulting from a hemizygous frameshift mutation (G107fsX80) was identified in the Burkitt's lymphoma cell line DG75. In HeLa cells, over-expression of wild-type TIS11D protein (TIS11D^{WT}) induced growth inhibition and an S phase checkpoint response, while the mutant protein (TIS11D^{I373fsX91}) showed a diminished effect. Interestingly, this response was accompanied by p21 down-regulation, which is frequently seen in the cellular response to ultraviolet irradiation. These data suggest that the dys-regulation of TIS11D function is associated with the patho-genesis of certain types of leukemia.

Introduction

Although several genetic abnormalities responsible for acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) have been identified, the concept of multistep leukemo-genesis suggests the presence of many unknown genetic alterations (1). It is therefore an important task to identify novel genetic abnormalities associated with AML and ALL for an accurate understanding of their pathogenesis and response to treatment. Transcription factors and tyrosine kinases are the most commonly-altered proteins in acute leukemia, while a recent study also suggests the involvement of RNA binding proteins in leukemia (2).

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The TIS family of proteins are classified as adenine-uridine-rich element binding proteins (ARE-BP) that inhibit the expression of target proteins by binding to the continuous AUUUA sequence located at the 3'-end of mRNA, and thereby degrade the mRNA or inhibit translation (3). These proteins are localized in a portion of the cytoplasm known as the processing body (P-body), along with Ago2, a main component of the RNA-induced silencing complex (RISC), and DCP1A, a protein involved in RNA decapping (4). Many of the ARE-containing genes are oncogenes and genes encoding proteins that undergo rapid turnover, such as cyto-kines (3). In humans, three TIS family proteins, TTP, TIS11B and TIS11D, have been identified (5). The genes encoding these proteins are suggested to serve as tumor suppressor genes in malignant tumors. However, there has only been indirect evidence to suggest an association between TIS family genes and malignant tumors, such as decreased expression of these genes and subsequently reduced degradation of oncogenic mRNA. The knock-out mice of *Tis11d*, also known as *Zfp36l2*, develop pancytopenia due to reduced numbers of hematopoietic stem and progenitor cells in the fetal liver, indicating that TIS11D is a crucial modulator of definitive hematopoiesis (6). The insertion of an immunoglobulin gene in the *TIS11D* locus has been reported in T-cell leukemia, but the significance of the finding remains unknown (7). The present study provides the first identification of a frameshift mutation in the *TIS11D* gene in leukemia patients. We also demonstrated the involve-ment of TIS11D in the cell cycle and the checkpoint pathway by inducing HeLa cells to express the wild-type and mutant TIS11D.

Patients and methods

Patients and cell preparation. A total of 257 patients with acute leukemia belonging to the following categories were subjected to a *TIS11D* gene mutation search: *de novo* AML (n=172), T-cell ALL (T-ALL) (n=21) and precursor B-cell leukemia (B-ALL) (n=64). Diagnosis of leukemia was based on cellular morphology, cell surface markers and chromosomal analysis according to the French-American-British (FAB) and WHO classifications. Mononuclear cells were isolated from peripheral blood or bone marrow samples at diagnosis. The samples were collected after informed consent was obtained from each patient in accordance with the guidelines based on the principle of the Revised Declaration of Helsinki. This study was approved by the ethics committee of the Kumamoto University School of Medicine.

Screening of TIS11D gene mutations. High-molecular-weight DNA was obtained from mononuclear cells treated with SDS-proteinase K according to the standard procedure, followed by phenol-chloroform extraction and ethanol precipitation. DNA (200 ng) was used to amplify all exons of the *TIS11D* gene. Polymerase chain reaction (PCR) was performed on a DNA thermal cycler (Whatman Biometra, Goettingen, Germany) using Taq polymerase (Invitrogen, Carlsbad, CA) and the following primer pairs: 5'-CGTTATT CGTCGTGGCTCAAG-3' and 5'-AGAAACTCAACCGCGA CACC-3' for exon 1; and 5'-GAAAAGAATCAGGCTGGG AGTTC-3' and 5'-CACAGCTCGGTCTTGTACTTCG-3' for exon 2 (GenBank accession NC_000002). PCR products were subjected to agarose gel electrophoresis, followed by direct sequencing. In addition, mutations in the *FLT3, NPM1, CEBPA, WT1* and *JAK2* genes were examined as previously described (8,9).

Cell lines used in the experiments. HeLa[tet pAcGFP], HeLa[tet pAcGFP TIS11D WT] and HeLa[tet pAcGFP TIS11D I373fsX91] cells (i.e., HeLa cells in which the expression of green fluorescent protein (GFP) fusion proteins can conditionally be induced using the tetracycline-off system) were cultured at 37˚C in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. DG75 cells were obtained from the Fujisaki Cell Center, Hayashibara Biochemical Labs Inc. (Okayama, Japan).

Construction of plasmid. PCR was performed to amplify the entire coding region of *TIS11D* using cDNA created from the K562 cell line and the following primers: 5'-GGGAAGCTT ACATGTCGACCACACTTCTGTCCGCCTTCTACGATG-3' and 5'-GGGGAATTCCTTCCTCCTCACTGGCGCCCTCT TGC-3'. PCR products were digested with *Hin*dIII and *Eco*RI, cloned into the pAcGFP1-C1 vector (Clontech, Mountain View, CA), sequenced, and then digested with *Nhe*I and *Bam*HI. The cleaved ends were smoothed with Phusion High-Fidelity DNA Polymerase (Finnzyme, Espoo, Finland). The pUHD10-3 vector was digested with *Eco*RI and *Bam*HI and the cleaved ends were smoothed. The obtained DNA fragment was subcloned into the digested pUHD10-3 vector to create a wild-type pUHD10-3 pAcGFP-TIS11D[WT] plasmid. A pUHD10-3 pAcGFP plasmid was obtained by digesting the pUHD10-3 pAcGFP-TIS11D[WT] plasmid with *Bsp*EI and *Bam*HI, smoothing the cleaved ends and causing the plasmid to self-ligate. A mutant pUHD10-3 pAcGFP-TIS11D[I373fsX91] plasmid was created via standard site-specific mutagenesis. More specifically, PCR amplification was performed using the pUHD10-3 pAcGFP-TIS11D[WT] plasmid and primers containing an 8-base insertional mutation (5'-cctcaAAGGGC CAtcacgccgctcgccatc-3' and 5'-gaTGGCCCTTgaggctgctgag ctccggac-3'). The template plasmid was then cleaved with *Dpn*I and DH5α was transformed to obtain the mutant plasmid. All clones were verified by sequencing.

Establishment of tetracycline-off cell lines. To induce HeLa cells to express GFP fusion proteins using the tetracycline-off system, pCAG20-1 and pUHD10-3 puromycin plasmids linearized by *Sca*I were transfected into 1x10^{7} HeLa cells by electroporation (10). The obtained puromycin-resistant stable cell line was then transfected with 10 μg of the pUHD10-3

pAcGFP, pUHD10-3 pAcGFP-TIS11D[WT] or pUHD10-3 pAcGFP-TIS11D[I373fsX91] vector linearized with *Sca*I, and 1 μg of the pPGKneo vector linearized with *Hin*dIII, simultaneously by electroporation. After a G418-resistant clone was obtained, tetracycline was removed and GFP expression was confirmed. The expression of fusion proteins in the GFP-positive clones was confirmed by Western blotting with an anti-GFP antibody (Clontech).

Cell proliferation analysis. Ten thousand cells were seeded onto a 6-well plate, cells were collected using trypsin every 24 h, and the viable cell count was assessed by trypan blue staining.

Fluorescence microscopy. HeLa cells were cultured in a glass-bottom dish in the absence of tetracycline for 24 h. Fluorescence images were obtained using an Olympus IX71 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and a BD CARV II confocal imager (BD Biosciences, Rockville, MD).

Western blot analysis. Proteins were extracted from cells using the M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) and subjected to SDS-PAGE. The separated proteins were transferred onto a PVDF membrane. The membrane was then incubated with Blocking one P (Nacalai Tesque, Kyoto, Japan) for 1 h for blocking and then with the following primary antibodies overnight: GFP (Clontech), CHK1, p-CHK1 (Ser317), p-CHK1 (Ser345), p-H2AX (Ser 139) (Cell Signaling Technology, Beverly, MA), CDC25A, Actin, p53, p21, Caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA) and PARP (Upstate, Lake Placid, NY). The membrane was then incubated with peroxidase-labeled secondary antibodies for 1 hour and detected using the ECL-plus system (GE Healthcare Life Sciences, Piscataway, NJ).

Cell cycle analysis. For synchronization, HeLa cells were cultured in the presence of tetracycline and 2 mM thymidine for 16 h. The cells were then washed three times with PBS and cultured in fresh medium for another 6 h. The cells were then trypsinized, washed twice with PBS, re-seeded onto a 6-well plate and cultured in the absence of tetracycline. After 6 h, 2 mM thymidine was added and cells were cultured for another 12 h. The cells were then washed three times with PBS and fresh medium was added. This time-point was defined as hour 0, and cells were collected every 2 h thereafter. For determination of DNA amounts, cells were fixed with ethanol, incubated with 100 μg/ml of RNase A and 50 μg/ml of propidium iodide for 15 min and analyzed using an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA). The cell cycle distribution was evaluated using FlowJo software (Tree Star, Inc., Ashland, OR).

SNP array analysis. The SNP array data for leukemia cell lines were downloaded from the NCBI gene expression omnibus http://www.ncbi.nlm.nih.gov/geo/ (accession no. GSM236783) and the ArrayExpress database http://www.ebi.ac.uk/ arrayexpress (accession no. E-MTAB-38). Raw data were genotyped using Genotyping Console Software version 4.0

Table I. Characteristics of *TIS11D* mutations.

Sample	Leukemia type	FAB	DNA mutation	Protein
DG75	BL		Hemi 320delG[a]	G107fsX80
Ramos	BL		Het 986C>T	A329V
Daudi	BL		Het 986C>T	A329V
Jurkat	T-ALL		Hemi 569C>T[a]	P190L
JM	T-ALL		Hemi 569C>T[a]	P190L
CEM	T-ALL		Het 986C>T	A329V
HPB-ALL	T-ALL		Het 986C>T	A329V
PEER	T-ALL		Het 986C>T	A329V
M46	AML	M4Eo	Het 186T>G	H62Q
M61	AML	M1	Het 186T>G	H62Q
M72	AML	M1	Het 186T>G	H62Q
M74	AML	M4Eo	Het 186T>G	H62Q
M42	AML	M4	Het 325G>A	G109S
M52	AML	M4Eo	Het 234C>A	F78L
M106	AML	M1	Het 657C>G	D219E
M30	AML	M2	Het 657C>G	D219E
			Het 1117_1118insAAGGGCCA	I373fsX91
M125	AML	M5a	Het 1156G>T	V386L
M155	AML	M5b	Het 1310T>C	L437P
T15	T-ALL		Het 186T>G	H62Q
T18	T-ALL		Het 186T>G	H62Q
B18	B-ALL		Het 186T>G	H62Q

Mutations are denoted relative to the *TIS11D* sequence, GenBank accession NM_006887.3. Underlined samples are cell lines, and the others are patient samples. [a]Hemizygosity was judged from sequence wave and SNP array. BL, Burkitt's lymphoma; Het, heterozygous; Hemi, hemizygous.

(Affymetrix, Santa Clara, CA), and normalization and copy number analysis were performed using dChip software (11).

Results

TIS11D gene mutations in leukemia. Many heterozygous missense mutations in TIS11D were detected in leukemias (Table I). One of the AML patients (M30) showed a heterozygous mutation, I373fsX91, caused by an insertion of 8 bases (Fig. 1A). This patient had deletion of the short arm of chromosome 6, but no mutations in NPM1, CEBPA, FLT3-ITD, FLT3-TKD, *WT1* exon 7, *WT1* exon 9 or JAK2V617F (data not shown). The Burkitt's lymphoma cell line DG75 was found to have a mutation (G107fsX80), which resulted from the deletion of one base (Fig. 1A), which was confirmed by SNP array analysis to be a hemizygous mutation (Fig. 1B). No nonsense mutations were detected. H62Q (seven cases) and A329V (five cases) mutations were most frequently detected. None of these mutations or polymorphisms, except for G109S, was reported in a previous report (12) or in existing databases. The Jurkat cell line and its derivative cell line JM had a mutation of P190L. SNP array analysis of the Jurkat cell line revealed only one copy of the *TIS11D* locus, suggesting that the P190L mutation was a hemizygous mutation (data not shown). All the other missense mutations were heterozygous. In all cases, whether these mutations were acquired or inherited remained to be determined because of the unavailability of samples obtained during disease remission or from normal tissue.

Wild-type TIS11D exhibits antiproliferative effects while the I373fsX91 mutant exhibits reduced antiproliferative effects. The TIS family has been shown to be involved in cell proliferation and apoptosis (5). To observe the effect of the TIS11D protein on cell growth, we induced HeLa cells to express TIS11D protein fused with GFP using the tetracycline-off system (Fig. 2A). Wild-type TIS11D exhibited a clear and strong antiproliferative effect on HeLa cells, lasting for at least 6 days (Fig. 2B). In contrast, the I373fsX91 mutant TIS11D exhibited only a limited antiproliferative effect, as compared to the wild-type protein. These findings suggest that the carboxy-terminal region of the protein, which is lost due to the I373fsX91 mutation, has an important role in inhibition of proliferation.
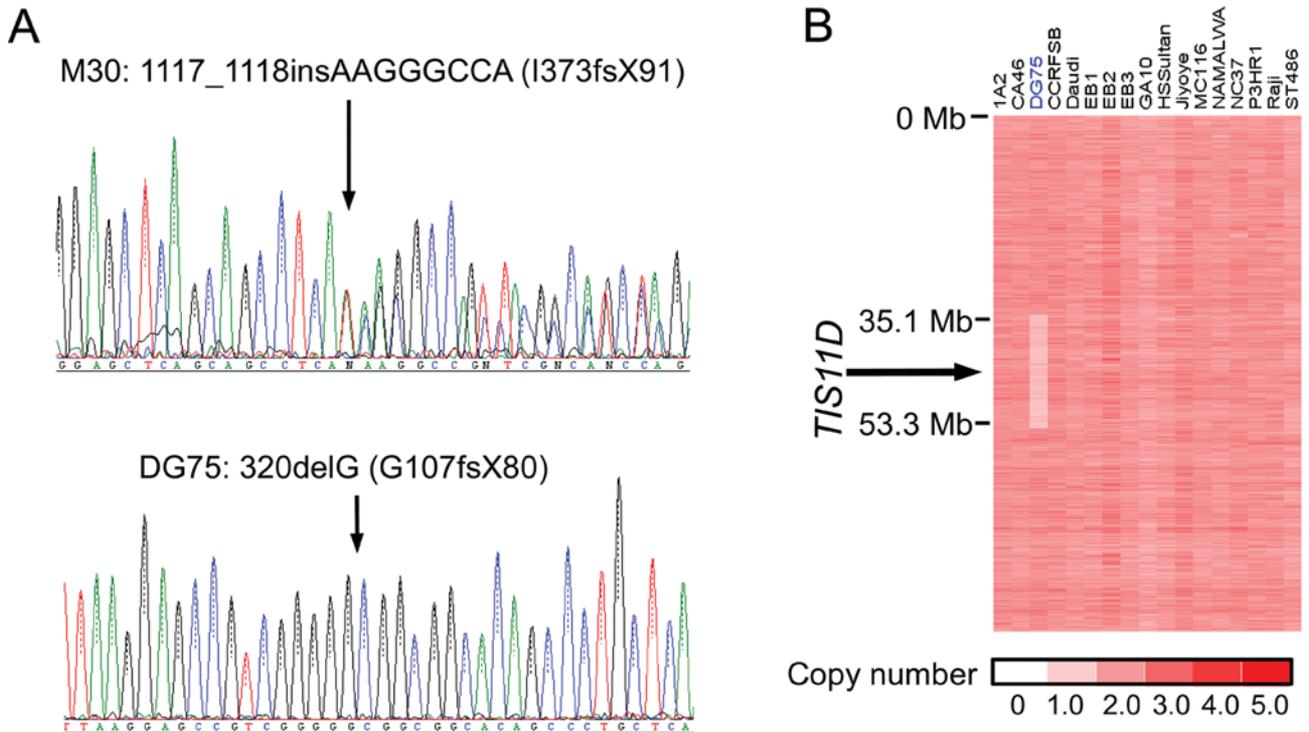
Figure 1. TIS11D is recurrently mutated in leukemias. (A) Direct sequence analysis of DNA from an AML patient (M30) and the DG75 cell line revealed frameshift mutations in *TIS11D*. (B) SNP array profile of chromosome 2 in the DG75 cell line showing an 18 Mb deletion including the *TIS11D* locus, indicating hemizygous mutation. Seventeen Burkitt's lymphoma cell lines were analyzed simultaneously.
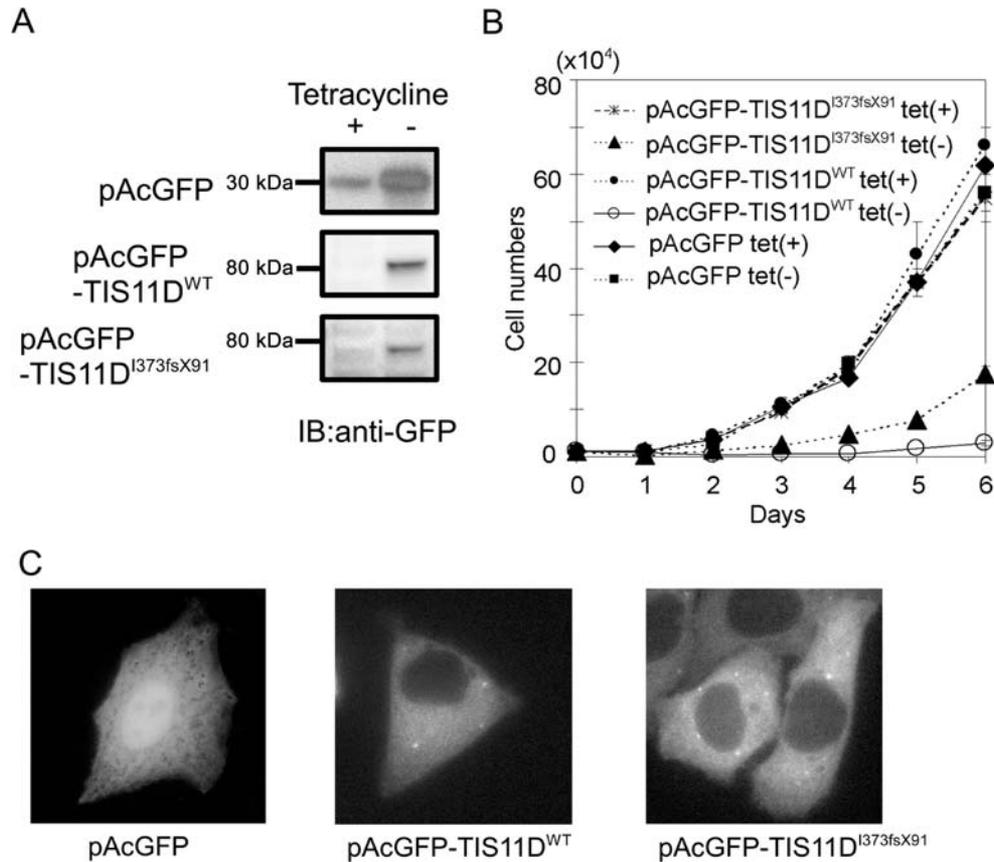

Figure 2. Growth suppression effects and localization of TIS11D. (A) Protein expression of TIS11D and its mutant fused with pAcGFP using the tetracycline-off system was confirmed by Western blotting. (B) Proliferation of HeLa cells expressing pAcGFP-TIS11D^{WT}, pAcGFP-TIS11D^{I373fsX91} and pAcGFP in the presence or absence of tetracycline. Data are expressed as the mean counts of live cells from two independent experiments. (C) Localization of pAcGFP fusion proteins was assessed by fluorescence microscopy. Cytoplasmic localization is conserved between wild-type and mutant TIS11D.
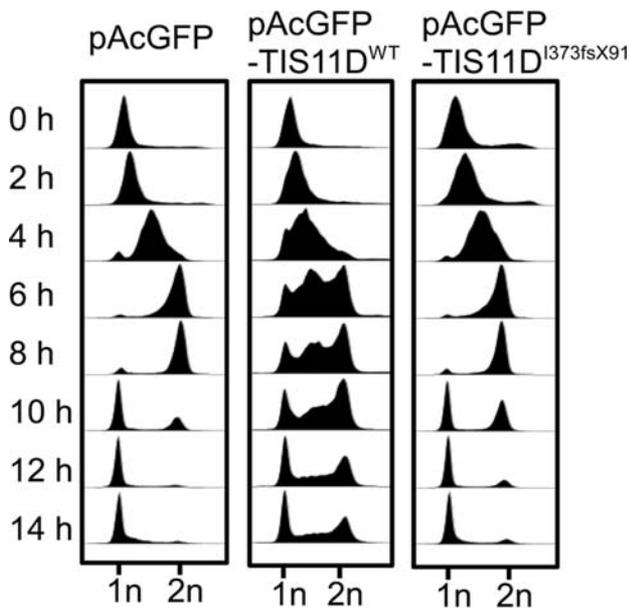
Figure 3. pAcGFP-TIS11D^WT slows down S phase progression. Synchronized HeLa cell lines carrying wild-type and mutant TIS11D were subjected to flow cytometry in the absence of tetracycline at indicated time-points.

I373fsX91 mutant protein shows a predominant cytoplasmic localization. A previous report has demonstrated the presence of a nuclear export signal (NES) motif at the carboxy-terminus of TIS11D (13). This motif is deleted in the I373fsX91 mutant. We performed fluorescence microscopy to examine the correlation between the intracellular localization and function of the mutant protein. Both the wild-type and I373fsX91 mutant TIS11D were localized predominantly in the cytoplasm (Fig. 2C). This finding suggests the presence of another signal, in addition to the NES motif at the carboxy-

terminus, which allows the localization of the protein in the cytoplasm.

Wild-type but not I373fsX91 mutant TIS11D delays progression of S phase. We then performed cell cycle analysis using double thymidine block synchronization to determine on which phase of the cell cycle the antiproliferative effect of TIS11D is dependent (Fig. 3). Cells expressing wild-type TIS11D protein showed a delay in the S phase while those expressing mutant TIS11D protein did not show significant delay in the S phase. This finding suggests that TIS11D inhibits the proliferation of HeLa cells by delaying progression of the S phase.

TIS11D activates apoptosis-related proteins. The forced expression of TIS11D has been shown to induce cell apoptosis (5). We performed Western blot analysis of apoptosis-related proteins to elucidate the mechanism of apoptosis induction by TIS11D (Fig. 4A), and found that the expression of wild-type TIS11D was accompanied by the activation of caspase-3 and PARP. Phosphorylation of H2AX is induced by cleavage of double-strand DNA and thus can be an indicator of apoptosis and DNA-damaging response (14). Cells forced to express wild-type TIS11D showed strong phosphorylation of H2AX while those expressing mutant TIS11D showed a marked decline in this phosphorylation.

TIS11D activates the S phase checkpoint pathway and thereby decreases p21 expression. The S phase checkpoint activated by DNA damage can be divided into an immediate response involving a CHK1/CHK2-CDC25A pathway and a delayed response involving a p53-dependent pathway (15). Based on the hypothesis that TIS11D delays progression through the S phase by activating the S phase checkpoint, we performed Western blot analysis of related factors (Fig. 4B).
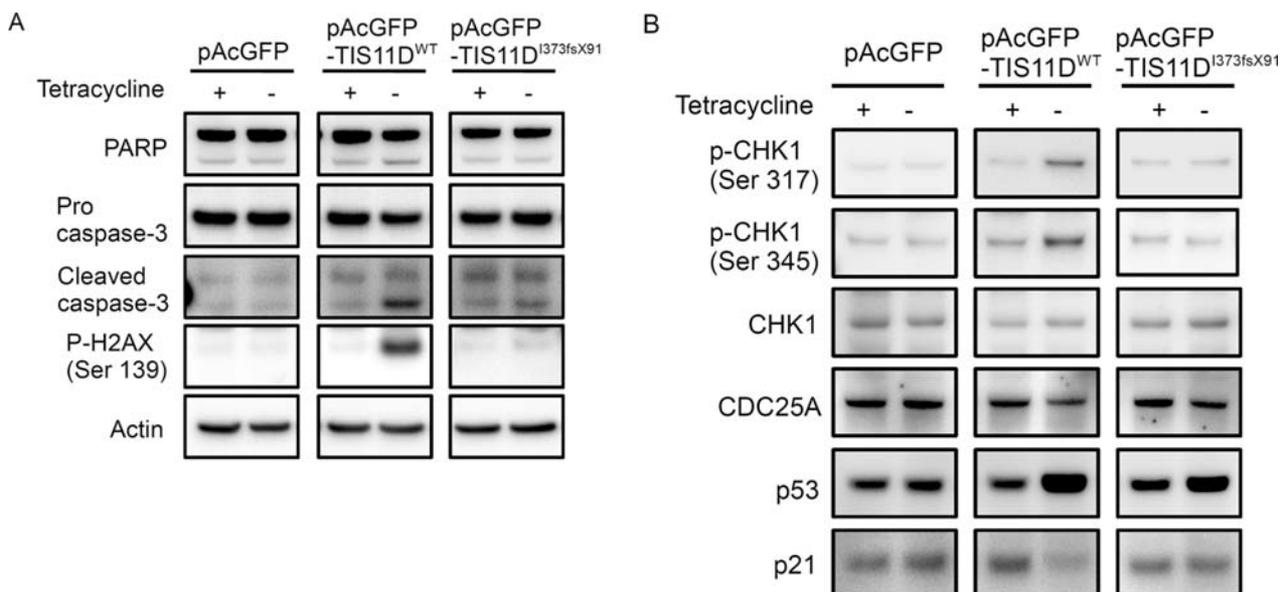

Figure 4. Reduced effects of mutant TIS11D on the apoptotic (A) and checkpoint pathways (B). Western blot analysis was performed using whole cell extract of asynchronized HeLa cell lines harboring wild-type and mutant TIS11D, with or without tetracycline, 24 h after induction.

Cells overexpressing wild-type TIS11D exhibited increased CHK1 phosphorylation and decreased CDC25A expression. These responses were not observed in pAcGFP control cells or cells expressing I373fsX91 mutant TIS11D. The expression of wild-type TIS11D was accompanied by a marked elevation of p53 protein levels and, interestingly, a decrease in the expression level of p21 protein, a downstream target of p53. Cells expressing mutant TIS11D exhibited a slight increase in p53 expression, but no decrease in p21 expression. This discrepancy in expression of p53 and p21 is observed during DNA-damaging reactions following ultraviolet (UV) irradiation (16). These findings indicate that wild-type TIS11D induces an S phase checkpoint reaction similar to that induced by UV irradiation in HeLa cells, while mutant TIS11D does not have this effect.

Discussion

In this study, we identified frameshift mutations in the *TIS11D* gene in leukemia patients. SNP array analysis revealed disruption of both alleles of *TIS11D* in the Burkitt's lymphoma cell line DG75. The double thymidine block system of HeLa cells demonstrated that wild-type TIS11D significantly inhibits cell proliferation by activating the S phase checkpoint pathway and thereby delaying progression through the S phase.

The present study identified two frameshift mutations (G107fsX80 and I373fsX91) in TIS11D. Because *Tis11d* knock-out mice die of pancytopenia (6), a hemizygous G107fsX80 mutation, which results in loss of functional TIS11D protein, may affect the proliferation and differentiation of hematopoietic cells. The I373fsX91 mutation is also considered to be of great significance, as it leads to deletion of preserved domains in the carboxy-terminus of the protein and exhibits a limited antiproliferative effect on HeLa cells. The loss of the carboxy-terminal domain may have contributed to the lower p53-inducing effect relative to wild-type, and the absence of clear evidence of CHK1 phosphorylation.

In samples obtained from AML patients, several missense variants were detected including H62Q and D219E. Both H62 and D219 are conserved in mammals and are considered functionally important. Therefore, future studies should compare the sequences at these amino acids between samples collected from patients with active disease and those collected during disease remission. The A329V mutation was detected in five cases collected from ALL samples. This amino acid is not conserved in mammals and its biological significance has not been established.

Myeloid tumors are known to show uniparental disomy (UPD) of chromosome 2p, to which *TIS11D* is mapped (17). UPD regions often contain homozygous gene mutations related to leukemogenesis. It is also commonly observed that a gene lacking one allele has a mutation in the other allele (18). Therefore, those patients found to have 2p deletion or UPD by SNP array analysis should be examined for *TIS11D* mutation. In view of the fact that *Tis11d* knock-out mice develop pancytopenia, hematopoietic progenitor cells from patients with myelodysplastic syndrome and aplastic anemia might be affected by TIS11D alterations. The association of decreased expression of the TIS family with various types of malignant tumors was previously observed (19). On the other hand, in t(8;21) AML, the *RUNX1-RUNX1T1* fusion gene induces overexpression of TIS11B, which mediates myeloid cell proliferation in response to granulocyte colony-stimulating factor, suggesting that expression of TIS11B contributes to t(8;21)-associated leukemogenesis (20). These observations also suggest the importance of measuring TIS11D expression levels in hematopoietic neoplasms.

In localization analysis, it is important to determine the locations of signals that induce the nuclear export of TIS11D. Phillips *et al* (13) demonstrated the presence of a NES signal at the carboxy-terminus of murine TIS11B, and found that TIS11B lacking the NES signal localizes to the nucleus. However, in the present study, I373fsX91 mutant TIS11D, which lacks the NES at the carboxy-terminus, was found predominantly in the cytoplasm. Whether the NES at the carboxy-terminus of TIS11D performs any function should be determined in a deletion mutant experiment involving TIS11D.

It is an interesting phenomenon that TIS11D activates the checkpoint pathway in the absence of external DNA damage. The observed strong induction of H2AX phosphorylation and activation of the CHK1/CDC25A pathway suggest the presence of ongoing DNA damage other than an apoptotic reaction. This may be explained by the mechanism through which the external manipulation of proteins involved in histone biosynthesis and chromatin remodeling factors induces cell cycle arrest at the S phase and DNA damage response (21). Dysregulation of histone chaperones is critical in hematological malignancies; for example, histone chaperone NPM1 is mutated in approximately half of AML patients with normal karyotype, and NPM1 knock-out mice develop myeloid, B-cell, and T-cell neoplasms (22). Similarly, TIS11D was found to be altered in both myeloid and lymphoid leukemias. These findings indicate that examining the relationship between TIS11D and leukemia from the viewpoint of histone bio-synthesis may provide clues towards elucidating the function of TIS11D.

It is of note that the expression of wild-type TIS11D induced a significant elevation of p53 protein level and a marked reduction of p21 protein level in HeLa cells. While activation of the p53-dependent checkpoint pathway is typically accompanied by increased expression of the target p21 protein, the checkpoint reaction following exposure to hydroxyurea or UV irradiation is accompanied by decreased expression of p21 protein, which is due to either decreased mRNA expression or CRL4^{CDT2}-dependent proteolysis (16). Decreased p21 expression following the DNA damage response is assumed to be related to an inaccurate DNA repair mechanism known as translesion DNA synthesis (16). TIS11D may also be involved in such DNA repair mechanisms, given that it induces a checkpoint reaction and a decrease in p21 expression. DG75 cells, in which both alleles of *TIS11D* are inactivated, are more prone to homologous recombination than are other B cell-derived cell lines (23). Whether TIS11D is involved in such abnormal DNA repair mechanisms remains to be addressed in future studies.

In conclusion, this is the first report of TIS11D mutations in leukemia cells. TIS11D inhibits S phase progression and is also involved in the checkpoint pathway in HeLa cells. Further studies are needed to analyze the function of TIS11D in hematopoietic cells and leukemia cells.

Acknowledgments

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