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GENETIC AND MOLECULAR ANALYSES OF P53 IN A JAPANESE FAMILY WITH LI-FRAUMENI SYNDROME

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Abstract
Li-Fraumeni syndrome (LFS) is an autosomal dominant cancer predisposition syndrome associated with a broad spectrum of early-onset tumors including bone and soft-tissue sarcoma, central nervous system tumors, leukemia, adrenocortical carcinoma, and breast cancer. This syndrome is associated with the *P53* gene in 80% of families that fulfill the clinical criteria. Exome sequencing analysis of the *P53* gene was performed on a Japanese family with LFS that includes a 2.5-year-old boy with rhabdomyosarcoma, his mother with osteosarcoma and lung cancer at 11 and 37 years old, respectively, and his maternal grandfather with osteosarcoma and gastric cancer at 12 and 42 years old, respectively. A heterozygous germline mutation in the *P53* gene, c.997delC or p.R333Vfs*12 P53, causing a frame-shift and premature termination was identified. p53 protein is a transcription factor that binds to DNA as a tetramer. Each P53 monomer comprises a transactivation domain, a proline-rich domain, a central DNA-binding domain, an oligomerization domain, and a C-terminal domain. The truncated protein, p.R333Vfs*12 P53, was transiently expressed in COS-7 cells and studied for subcellular localization and immunoblotting. The results show that the truncated p.R333Vfs*12 P53 protein is appropriately localized into the nucleus. However, immunoblotting analysis suggested that the truncated p.R333Vfs*12 P53 protein would be unstable and degraded into nonfunctional lower molecular proteins. The predicted defects in oligomerization caused by loss of the C-terminus after residue 333, which is located within the tetramerization domain, may account for LFS in this family. Further study is needed to clarify the molecular explanation for oncogenic predisposition in this family.

Key words : Li-Fraumeni syndrome, P53, Subcellular localization

Introduction

Li-Fraumeni syndrome (LFS) as well as its variant form, Li-Fraumeni-like, is an autosomal dominant cancer predisposition syndrome with clinical heterogeneity. LFS is associated with a broad spectrum of early-onset tumors including bone and soft-tissue sarcoma, central nervous system tumors, leukemia, adrenocortical carcinoma (ACC), and breast cancer[1,2)]. LFS is clinically defined by three clinical criteria : a proband with a sarcoma diagnosed before the age of 45 years ; a first-degree relative with any cancer with onset before the age of 45 years ; and, a first- or second-degree relative with any cancer diagnosed during these age intervals or a sarcoma diagnosed at any age[2-4)]. The clinical definition of Li-Fraumeni-like also includes three clinical criteria : a proband with any childhood cancer or a sarcoma, brain tumor or ACC with onset before the age of 45 years ; a first-de-

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gree or second-degree relative with a core LFS cancer (sarcoma, breast cancer, brain tumor, ACC or leukemia) with onset at any age ; and, a first- or second-degree relative with any cancer with onset before the age of 60 years[2-4].

Currently, this syndrome is known to be associated with the *P53* gene in 70% of families and in 30% of Li-Fraumeni-like families that fulfill the clinical criteria for each disease entity[2,5]. To date, more than 300 mutations of the *P53* gene have been described throughout the gene[1]. However, in many families with LFS, a specific gene mutation responsible for the disease has not been identified.

We report here a three-generational Japanese family with LFS for which conventional Sanger sequencing analysis had failed to identify a genetic variant in the *P53* gene. We applied whole-exome sequencing using next-generation sequence technology to a proband-father-mother trio after a 2-year-old-boy presented with a LFS tumor.

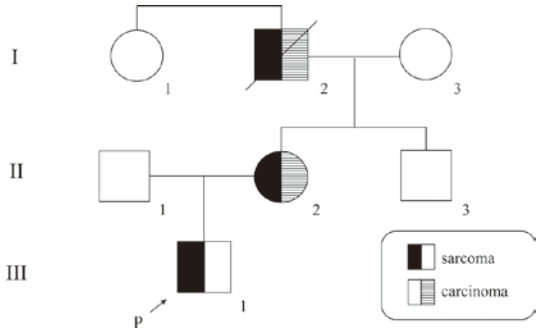


Fig. 1. A pedigree of the family.
III-1 represents the patient presented in this article. I-2 suffered from osteosarcoma at the age of 12 years and died of gastric cancer at the age of 42 years. II-2 suffered from osteosarcoma at the age of 11 years and lung adenocarcinoma at the age of 37 years.

Materials and Methods

Ethical approval for this study was obtained from the Ethics Committee of Akita University, Graduate School of Medicine in Akita, Japan. Blood were collected after obtaining written informed consent from a guardian of the patient and the parents.

Patient and clinical course

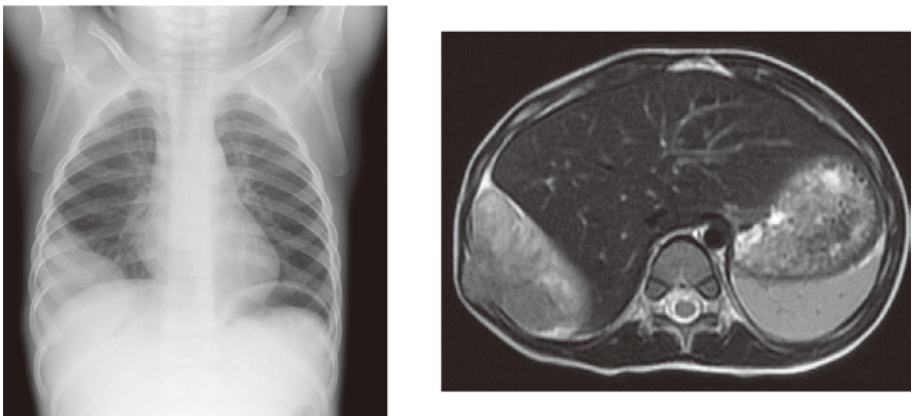
A 2.5-year-old male was admitted to our hospital with a palpable subcutaneous nodule (2 centimeters in diameter) on his right 8th intercostal space (Fig. 1 III-1). Thoracic X-ray showed a mass shadow on the lower lateral of the right thorax and MRI revealed a single oval tumor about 8 centimeters in diameter (Fig. 2A and B).

The family history of this patient was noteworthy (Fig. 1). The patient's maternal grandfather suffered from osteosarcoma at the age of 12 years and well-differentiated tubular adenocarcinoma of the stomach (which was immediately treated with a subtotal gastrectomy) at the age of 19 years (Fig. 1 I-2). He died of carcinomatous peritonitis originating from signet ring cell carcinoma of the remnant stomach at the age of 42 years. The patient's mother also suffered from osteosarcoma of the left upper arm at the age of 11 years (Fig. 1 II-2). The mother was treated with a combination of chemotherapy (with methotrexate, vincristine, and doxorubicin) and surgical excision, resulting in the remission of the disease. When she was 30 years old, she gave birth to the patient. Recently, at the age of 37 years, the mother has been diagnosed with lung adenocarcinoma.

For diagnosis of the patient, a biopsy of the subcutaneous nodule was performed. The tumor consisted of closely packed spindle-shaped cells with anaplastic change (Fig. 3A and B). The tumor was confirmed to be positive for myogenin and desmin using immunohistochemistry (data not shown). The cells were also partially positive for p53 immunostaining (Fig. 3C). The MIB-1 positive count was 70% (Fig. 3D). Based on these findings, the patient was diagnosed with anaplastic type spindle cell rhabdomyosarcoma. The patient was refractory to subsequent treatment. Despite chemotherapy with various anticancer drugs, radiation therapy, surgical treatment, and cancer immunotherapy, the patient died of exacerbation of lung metastases 43 months after the initial diagnosis.

Exome analysis and Sanger sequencing

Genomic DNA was extracted from the peripheral blood leukocytes of the patient, his father, and his mother. A



A B

Fig. 2. Images of tumor in the patient.
(A) Chest X-ray image showed mass shadow on the lower lateral of the right thorax. (B) T2-weighted MRI image showed a single oval tumor about 8 centimeters in diameter in the right thorax.

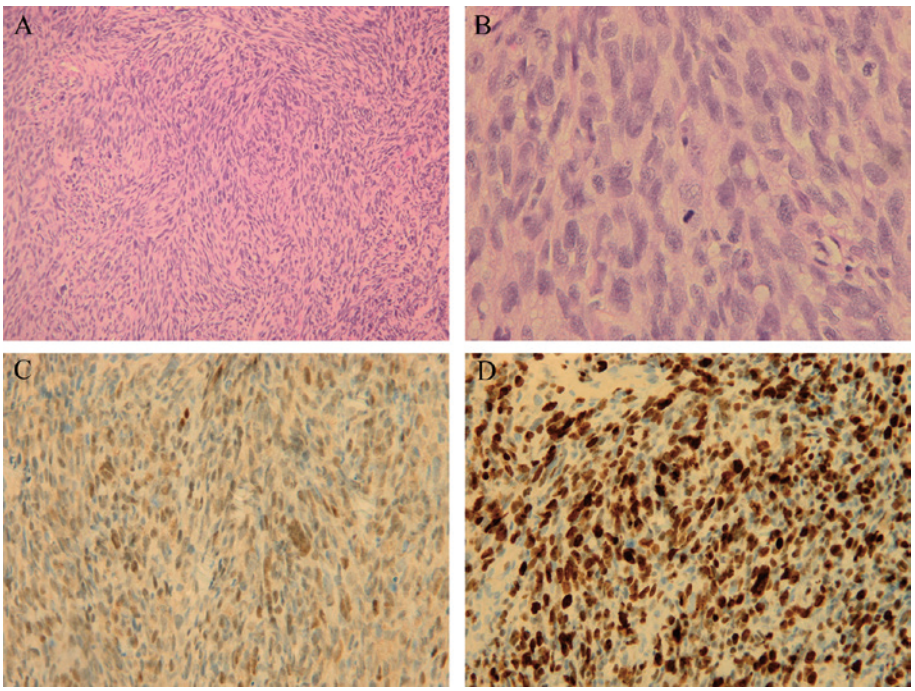


Fig. 3. Pathological findings of tumor in the patient.
(A) Hematoxylin Eosin stain (HE) shows closely gathered spindle-shaped tumor cells and a specific bundled structure. (B) HE shows that tumor tissue contains anaplastic components such as abnormal mitosis, nuclear polymorphism, and apoptosis. (C) The cells of tumor were partially positive for p53 immunostaining. (D) The MIB-1 was positive for about 70 % of the cells of tumor.

trio whole-exome analysis was performed in Keio University on the family using SureSelect XT Human All Exon V6 (Agilent Technolo-gies, Santa Clara, California) for whole exome enrichment and the MiSeq platform (Illumina, San Diego, California) for Next-Generation sequencing. We obtained 10 giga bases per sample providing a mean coverage of 118-fold across the targeted coding regions. The sequencing reads were aligned to the reference human genome sequence (hs37d5) with the Burrows-Wheeler Transform, while local realignment around the indels and base quality score recalibration were performed using the Genome Analysis Toolkit. The mutation identified was confirmed by Sanger sequencing. *P53* variants were analyzed and described using GenBank Accession Number NM_001126112.2 as a reference sequence.

Site-directed mutagenesis and vector construction

A full-length cDNA encoding wild-type P53 was custom-synthesized by FASMAC Co. Ltd (Atsugi, Japan). The *P53* gene variant was introduced into the wild-type cDNA using a previously described site-directed mutagenesis method[6]. The construct was re-sequenced using the BigDye Terminator method to ensure no non-specific mutations had been incorporated into the cDNA for p53 during the mutagenesis procedure. Wild-type and mutant P53 cDNAs were separately subcloned into the pCL-neo mammalian expression vector (Promega Corporation, Madison, WI, USA) for the P53 immunoblotting study. In addition, wild-type and mutant P53 cDNAs were separately subcloned into the pAcGFP-c1 vector (TaKaRa, Otsu, Japan) for subcellular localization of mutant P53 proteins using a pAcGFP - P53 N-terminal fusion protein.

Cell transfection and confocal microscopy

The wild-type and mutant constructs were transfected into COS-7 cells using Lipofectamine 2000 reagent according to the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA). COS-7 cells transfected with the pAcGFP-c1 vectors were examined under a Zeiss LSM 780 confocal microscope equipped with a UV laser. To visualize the nucleus, cells were stained with Hoechst 33,342 (Nacalai Tesque, Kyoto, Japan). For the immunoblotting, cells were transfected with wild-type P53 cDNA, mutant-type P53 cDNA, and wild-type and mutant-type p53 cDNA for co-expression studies.

Immunoblotting analyses of transiently expressing wild and mutant P53 in COS-7 cells

Immunoblotting analysis was performed to evaluate p53 protein expression levels. Briefly, protein lysates from the COS-7 cells were prepared using RIPA buffer (Nacalai Tesque, Kyoto, Japan). The protein extracts (20 μg) were resolved by SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was blocked with 1% bovine serum albumin (Nacalai Tesque, Kyoto, Japan) at room temperature for 1 h, and then incubated overnight at room temperature with primary polyclonal antibodies against human P53 (dilution 1 : 1,000 ; Bioss Antibodies, Woburn, MA, USA) and human GAPDH (dilution 1 : 1,000 ; Cell Signaling Technology, Danvers. MA, USA). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 5,000) at room temperature for 2 h, the protein bands were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, Waltham, MA, USA).

Results

Exome analysis and Sanger sequencing

Using exome analysis, a heterozygous one base deletion in exon 10 of the *P53* gene, designated as c.997delC, was identified in the proband and the mother (relative to the reference sequence) but not in the father. The mutation identified was confirmed by Sanger sequencing using DNA from the patient (Fig. 4). The c.997delC mutation is predicted to cause a frame shift at codon 333, which results in a arginine (CGT) to valine (GTG) substitution at codon 333, and a termination codon at 344. As a result, the mutated DNA is predicted to encode a premature truncated P53 protein, designated as p. R333Vfs*12 (Fig. 5).

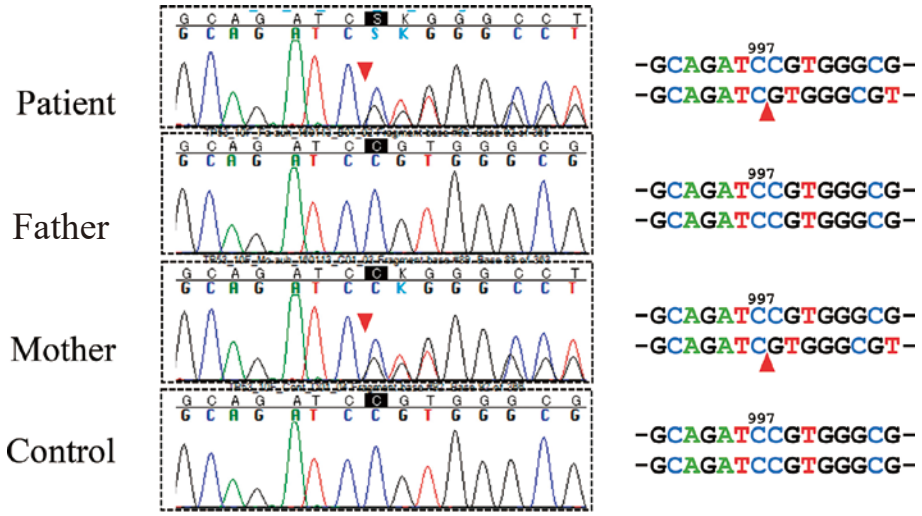


Fig. 4. Genomic exon 10 sequences of *P53* gene in a patient, his father, his mother, and a control.
A heterozygous one base deletion, designated as c.997delC, was identified in the patient and his mother.

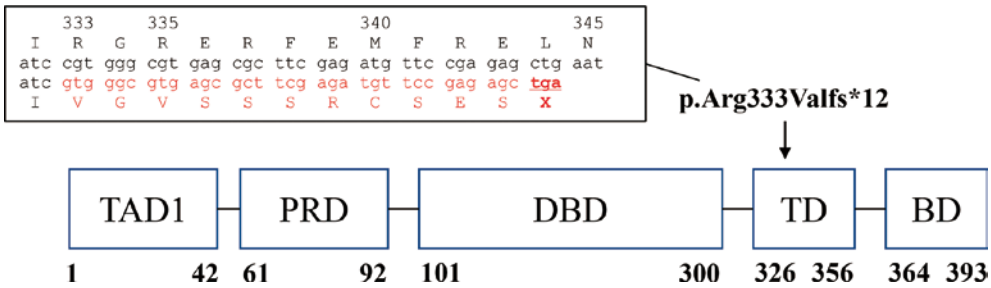


Fig. 5. A c.997delC mutation in structure and function of P53.
A c.997delC mutation was identified in exon 10 of *P53* gene, which is predicted to cause a frame shift at codon 333, resulting in making a termination codon at 344. TAD1 : the N-terminal transaction domain, PRD : the proline rich domain, DBD : the central DNA binding domain, TD : the tetramerization domain, BD : the C-terminal basic domain.

Cell transfection and confocal microscopy

In separate experiments, fluorescent proteins comprising AcGFP fused to the N-terminus of wild-type P53, and AcGFP fused to the N-terminus of mutant-type p53, were transiently expressed in COS-7 cells. The fluorescent signal from the wild-type P53 fusion was observed exclusively in a compartment of the nucleus (stained with Hoechst 33,342). The AcGFP-mutant P53 fusion protein was also exclusively localized in the nucleus of COS-7 cells (Fig. 6).

Immunoblotting analyses of transiently expressed wild-type and mutant P53 in COS-7 cells

The expression of wild-type and mutant-type P53 protein in COS-7 cells was also investigated by immunoblotting analyses. With un-transfected COS-7 cells, immunoblots showed weak staining for P53 (Fig. 7 ; lane 1). After expression of wild-type P53 protein, immunoblots showed strong staining for P53, revealing an approximately 50 kDa protein band and a second lower molecular weight band (Fig. 7 ; lane 2). By contrast, after expression of mutant-type P53 protein, immunoblots

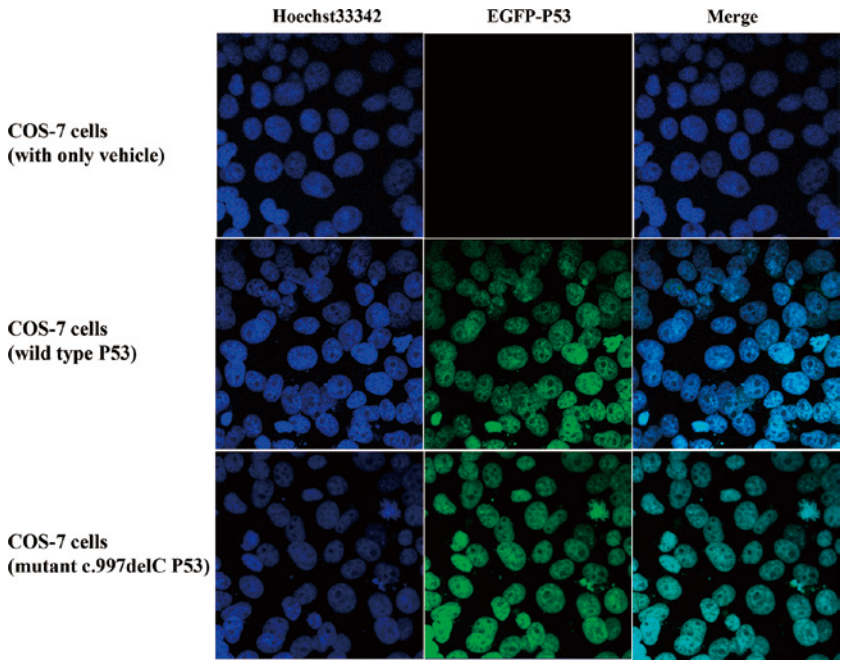


Fig. 6. Transient expression and subcellular localization studies of wild-type and mutant-type P53 in COS-7 cells. pAcGFP was fused to the N-terminus of wild-type P53 or p.R333Vfs*12 mutant-type P53 protein. The nucleuses of the cells were stained with Hoechst33342. The pAcGFP signals both of wild-type and p.R333Vfs*12 mutant-type P53 protein were observed exclusively in a compartment of the nucleus.

showed weak staining for P53, revealing a weak band of around 50 kDa protein (which could represent endogenous P53 protein in COS-7 cells) and several additional weak lower molecular weight bands, consistent with degradation of the mutant P53 proteins (Fig. 7. ; lane 3). After co-expression of wild-type and mutant-type P53, immunoblots showed a composite pattern of bands reminiscent of both the wild-type and mutant-type P53 studies (Fig. 7 ; lane 4).

Discussion

A germline mutation in the *P53* gene, c.997delC, was identified in a three-generational Japanese family of LFS through exome sequencing (and then verified with Sanger sequencing), contrary to our expectations. A somatic c.997delC *P53* gene mutation is registered as genomic mutation ID COSV52736313 in Catalogue Of Somatic Mutations In Cancer (COSMIC)[7], but it has not been a germline mutation associated with LFS, to the best of our knowledge.

Previously, conventional Sanger sequencing had not identified any genetic variant in the *P53* gene in this family. This demonstrates the limitations of conventional Sanger sequencing for detecting pathologic genetic variants even in a targeted gene. There are many possible reasons for the failure to detect pathologic variants by conventional Sanger sequencing including human error, PCR amplification errors for both of the two alleles, and an inability of PCR to amplify large base deletions. A previous study has demonstrated that the quality of targeted next-generation sequencing of a disease-specific subset of genes is equal to the quality of Sanger sequencing and also that it has 100% reproducibility[8]. We conclude that exome sequencing is a viable alternative to conventional sequencing for genetic diagnosis even in the case of a targeted gene.

p53 protein is a transcription factor that binds to DNA as a tetramer. P53 monomer comprises a transactivation domain (TAD), a proline-rich domain (PRD), a cen-

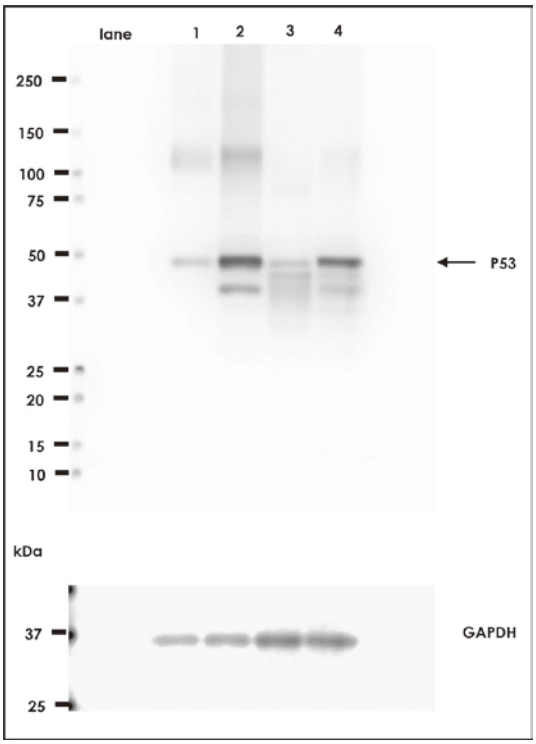


Fig. 7. Immunoblotting analyses of wild-type and mutant-type P53 expressed in COS-7 cells.
Expressions of wild-type and mutant-type T53 protein in COS-7 cells were investigated by immunoblotting analyses. lane 1 : COS-7 cell without transfection, lane 2 : COS-7 cells transfected with wild-type P53, lane 3 : COS-7 cells transfected with mutant-type P53, lane 4 : COS-7 cells co-transfected both with wild-type and mutant-type P53. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was immunoblotted as inner protein contents of COS-7cells.

tral DNA-binding domain (DBD), an oligomerization domain (OD), and a C-terminal domain (CTD)[1,2] (Fig. 5). The c.997delC mutation is predicted to encode a prematurely truncated P53 protein, designated as p.R333Vfs*12. As demonstrated in Fig. 5, the c.997delC mutation causes truncation of the C-terminus of P53 protein following residue 333 of the tetramerization domain. To understand the molecular basis for loss-of-function in this truncated P53 protein, we transiently expressed the truncated P53 protein in COS-7 cells for subsequent analysis using confocal microscopy for localization and immunoblotting for expression analysis.

The ability of P53 to shuttle between the nucleus and the cytoplasm is important to P53 function and this ability is controlled by both a nuclear localization sequence (NLS) and a nuclear export sequence (NES)[2,9]. The NLS of P53 is present in the CTD. The major NLS, NLSI, is the sequence motif (Pro^{316}-Gln^{317}-Pro^{318}-Lsy^{319}-Lys^{320}-Lys^{321}-Pro^{322}), while the minor NLS involves an interaction between a basic motif (Lys^{305}-Arg^{306}) and the CTD (residues 326-356). Because p.R333Vfs*12 P53 is truncated in the CTD and this could have a negative effect on nuclear localization, we performed a subcellular localization study of the p.R333Vfs*12 P53 in COS-7 cells. However, the results showed appropriate localization of p.R333Vfs*12 P53 within the nucleus. Notwithstanding any limitations of using the pAcGFP system, the c.997delC mutation did not affect subcellular localization of the mutant P53 into the nucleus.

In the immunoblotting study of the p.R333Vfs*12 P53, mutant P53 showed weak staining, compared to experiments with wild-type P53 protein, and several additional weak lower molecular weight bands were observed. This result is compatible with the finding that cells from the tumor of the patient were partially positive for p53 immunostaining. Together, these results suggest that p.R333Vfs*12 P53 may be unstable and consequently degraded into lower molecular weight non-functional proteins.

The p.R333Vfs*12 P53 is truncated after residue 333 of the CTD, which is essential for oligomerization. A malfunction in oligomerization may affect P53 function in the nucleus. Some missense mutations in the CTD of P53 (for example, L344P and R337C) have been reported to interfere with oligomerization[10]. The L344P mutant P53 was shown to be monomeric, apparently unfolded, and unable to interact with wild-type P53.

In summary, a heterozygous germline mutation of the *P53* gene, c.997delC, was identified in a three-generational Japanese family with Li-Fraumeni syndrome. The mutated gene yields a truncated P53 protein, p.R333Vfs*12, which may be unstable in cells and may have oligomerization defects.

Conflict of Interest

The authors have no conflict of interest to declare.

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