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Identification of a single base insertion in the COL4A5 gene in Alport syndrome

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Identification of a single base insertion in the COL4A5 gene in Alport syndrome. We identified a novel mutation in the COL4A5 gene of a Japanese patient with Alport syndrome. A combination of *in vitro* amplification of the exons with single strand conformation polymorphisms (SSCP) analysis suggested the presence of a mutation in exon 48. Sequencing of the amplified DNA revealed a single base (T) insertion which was between nucleotides T 4750 and G 4751 within the methionine 1516. This mutation caused a shift in the reading frame of nine amino acids and introduced a premature termination signal that would be expected to lack about two-thirds of the noncollagenous (NC1) domain. This mutation may interfere with type IV collagen assembly leading to increased permeability and play a causative role in the glomerular basement membrane abnormality of this patient with typical Alport syndrome. Gene tracking by restriction enzyme *Nla*III digestion revealed that the patient's mother is heterozygous whereas the patient's brother and one sister are normal, albeit they have hematuria and proteinuria. Without gene analysis, they would have been misdiagnosed. We propose that the diagnosis of Alport syndrome should be made on the basis of both clinical phenotypes and molecular defects.

Alport syndrome [1] is an inherited, progressive hematuric kidney disease often accompanied by high-tone sensorineural deafness and eye lesions (lenticonus and macula flecks). Carrier females have a variable and generally milder clinical course [2]. Alport syndrome is characterized by abnormalities in the glomerular basement membrane (GBM) as diffuse irregularly thickening with splitting of the lamina densa. Ultrastructural findings and immunological evidence led to the suggestion that the principal defect in Alport syndrome was due to a complete absence of, or alterations in an α chain of type IV collagen, a major structural component of the GBM [3–6]. The COL4A5 gene mapped to Xq22, encoding a newly found chain (α5) [7, 8], was confirmed to be the Alport syndrome locus, based on findings of several mutations, single base mutations [9–14] and major rearrangements such as large deletions [13, 15–17] and duplication [16]. Full-length cDNA clones providing the complete amino acid sequence of the α5(IV) collagen have been isolated and characterized [11].

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In our analysis of part of the 3′-end of the COL4A5 gene in Japanese patients with Alport syndrome, we noted a novel mutation of a single base insertion in exon 48 of the gene.

Methods

Patient

Patient OYM was a 15-year-old Japanese male with Alport syndrome accompanied by sensorineural deafness. At age 18 months, he experienced gross hematuria, upper respiratory infection and high fever. The proteinuria and hematuria had been persistent and as it advanced gradually, nephrotic syndrome was suspected (proteinuria, 2 g/day; serum albumin, 2.3 g/dl; serum cholesterol, 225 mg/dl) at age five years. At this time, electron microscopic examination of glomeruli obtained by the renal biopsy revealed that the lamina densa of GBM had widened and split into several layers, which were findings compatible with the GBM of Alport syndrome (Fig. 1A). The GBM did not react with Goodpasture serum by immunofluorescence microscopy. Bilateral high tone sensorineural deafness was also present, but the eyes were unaffected. Renal function further deteriorated when he was 12 years old; over 1.2 mg/dl of serum creatinine (current data, 4.0 mg/dl).

Hematuria, proteinuria, and renal dysfunction are present in the mother of the patient. Three maternal uncles had had renal failure at about 18 years of age; two died at age 20 years and one underwent transplantation. One of two maternal aunts had had renal failure and was treated with hemodialysis, while the other one is healthy. In the patient's sister, age 16 years, hematuria and proteinuria were first evident when she was six years old. Renal biopsy specimens obtained then revealed minimal glomerular abnormalities (light microscopy) and no splitting of GBM indicating Alport syndrome (electron microscopy; Fig. 1B). At present, she has hematuria (1+) and mild proteinuria (+/−) by a dipstick, and serum creatinine is 0.4 mg/dl. The patient's brother, 12 years old, was found to have hematuria at age two years and developed proteinuria later. Renal histopathological examination could not be done as the mother refused permission (because we told her findings of his gene analysis, as discussed later). At present, he has hematuria (1+) and proteinuria (1+) by a dipstick, and serum creatinine is 0.3 mg/dl. Sensorineural deafness and eye lesions are absent in two siblings. The father was not examined.

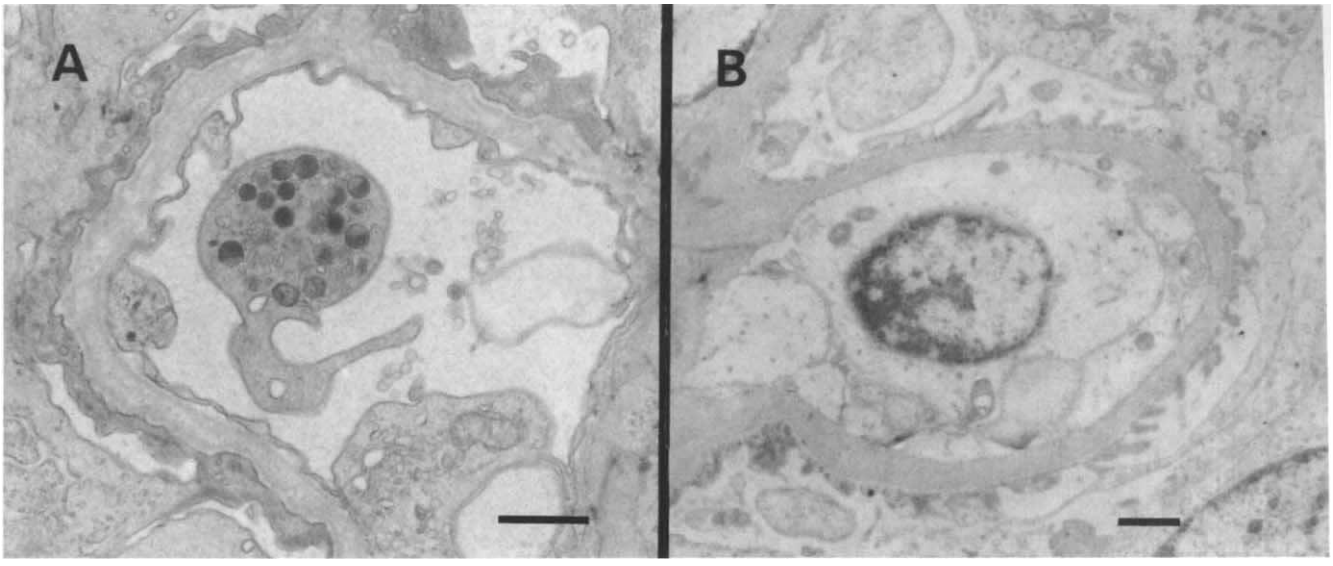
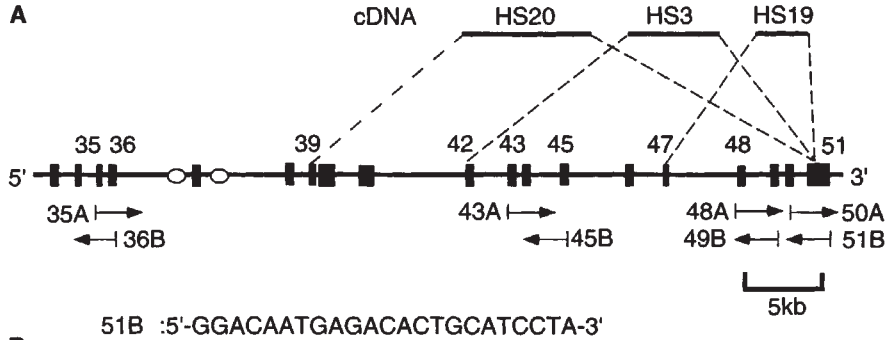


Fig. 1. *Electron micrograph of renal biopsy specimens.* **A.** Patient's GBM shows diffuse irregular thickening with splitting and splintering with small round electron-dense granulation of the lamina densa, compatible with GBM of Alport syndrome (×12,600). **B.** GBM of patient's sister has a normal lamina densa and foot process, and with no evidence of Alport syndrome (×8,100).



B

51B :5'-GGACAATGAGACACTGCATCCTA-3'
50A :5'-ACAACTGCAGGGGCAGGAAGGCTCA-3'
49B :5'-CATCATGAAGGAATAACCAATCCACAG-3'
48A :5'-AGCTGCCTTCGTCGCTTTAGTACCATG-3'
45B :5'-CTGGTAAGCCTTGAGGTCCTTGGG-3'
43A :5'-CCGGGTCTCAATGGAATGAAAGGA-3'
36B :5'-CTTTGGACCAGGAAGACCTGGAA-3'
35A :5'-GTCCCAAAGGTAACCCTGGTCTCC-3'

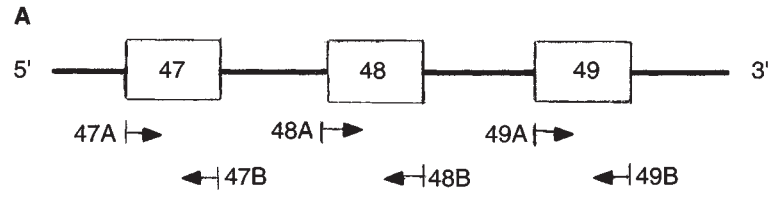
Fig. 2. *Structure of the 3' end of the COL4A5 gene and cDNA clones and synthetic oligonucleotide primers.* **A.** Diagram of the gene with exons indicated by shaded boxes and introns indicated by a solid line. The exons are numbered from the 5' end. The location of 8 oligonucleotide primers are indicated by arrows. Three types of cDNA clones HS19 (0.8 kb), HS3 (1.3 kb), and HS20 (1.7 kb) obtained by screening are indicated above. Scale of 5 kb in the gene is below. **B.** Sequences of eight oligonucleotide primers.

Methods

Oligonucleotide primers were synthesized on a 391 DNA Synthesizer (Applied Biosystems, USA) based on the sequence of the COL4A5 gene [18]. A human placental cDNA library in λgt11 (Clontech) was screened using, as the probe, a polymerase chain reaction (PCR) products mixture amplified with four pairs of primers; 35A and 36B, 43A and 45B, 48A and 49B, and 50A and 51B (Fig. 2). Human placental and leukocyte genomic DNA libraries (Clontech) were screened using, as a probe, amplified DNA with primers 48A and 48B (Fig. 3). The probes were labeled with ^{32}P-dCTP using the random priming method [19]. Positive clones were isolated, and subcloned into the pUC18 vector. Double stranded DNA was sequenced by the dideoxy chain termination method [20] using Sequenase Version 2.0 DNA Sequencing Kits (USB).

Genomic DNA was extracted from heparinized peripheral blood cells from the patient using the standard method [21]. Genomic DNA was amplified *in vitro* by PCR [22, 23]. PCR was carried out on a thermal cycler (Perkin Elmer Cetus) using 0.1 μg of genomic DNA as template and primers at 50 pmol concentration. Taq DNA polymerase (2.5 U, Boehringer Mannheim) was added and 30 cycles were carried out as follows: denaturation at 94°C for one minute, annealing at 55°C for one minute, and extension at 72°C for three minutes.

For Southern blot analysis, genomic DNA (10 μg) was digested with *Eco*RI, electrophoresed on a 0.5% agarose gel, transferred to a nitrocellulose filter, and hybridized with the ^{32}P-labeled normal human COL4A5 cDNA that was the 0.8 kb fragment spanning exons 47 to 51 (Fig. 2). Conditions were as described elsewhere [24].



B

49A : 5'-ATGTGCAGTATGTGAAGCTCCAGCT-3'
48B : 5'-CGACTAATGAATGGCTGGATGCTCT-3'
47A : 5'-ATAGGTACCCGTGGTTTGGATGGTCC-3'
47B : 5'-ACCCAAGTCTTGACCGTGGGCTCTT-3'

C

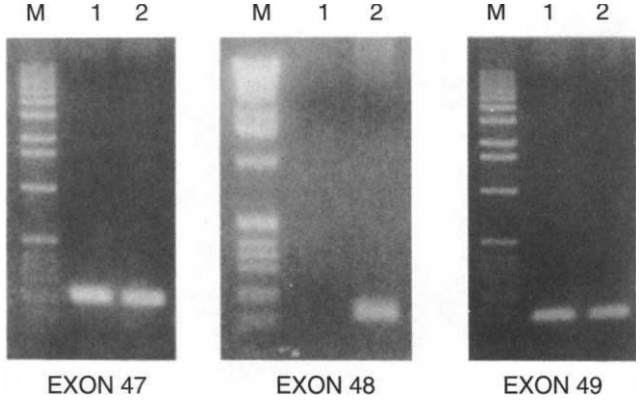


Fig. 3. *Agarose gel electrophoresis of PCR amplified DNAs from normal and patient OYM.* **A.** Strategy for PCR. Amplifications were performed for detection of exon 47, exon 48, and exon 49. **B.** Sequences of oligonucleotide primers. See Fig. 2 in primers 48A and 49B. **C.** Agarose gel (1.5%) electrophoresis of amplified DNAs. Left, exon 47; middle, exon 48; right, exon 49. Lane 1, patient; lane 2, normal male control; M, 1 kb ladder marker. Amplified DNA of only exon 48 in the patient was not detected.

We isolated the genomic clone (1.5 kb) containing exon 48 from genomic libraries and determined the flanking sequence (data not shown). A new oligonucleotide primer IV1 (5′-AGG-TACCTCTGTTCTACCTAACCTAGA-3′) was synthesized based on nucleotides 81 to 105 bp upstream of exon 48, which was used for PCR with primer 48B (3′ end primer).

For a single strand conformation polymorphisms (SSCP) analysis [25], the amplified DNA fragments were denatured and electrophoresed under non-denaturing conditions on a 7.5% polyacrylamide gel with 6% glycerol at 4°C at 100 volts for 10 hours. The DNA was detected after silver staining of the gel.

For family tracking, amplified DNAs from the patient and family members were digested with restriction enzyme *Nla*III, and electrophoresed on a 15% polyacrylamide gel.

Results

We screened one million plaque-forming units of cDNA and obtained three duplicate positive signals. The three types of the COL4A5 cDNA isolated were named HS19, HS3, and HS20, spanning exons 47 to 51 (0.8 kb), exons 42 to 51 (1.3 kb), and exons 39 to 51 (1.7 kb), respectively (Fig. 2). Southern blot

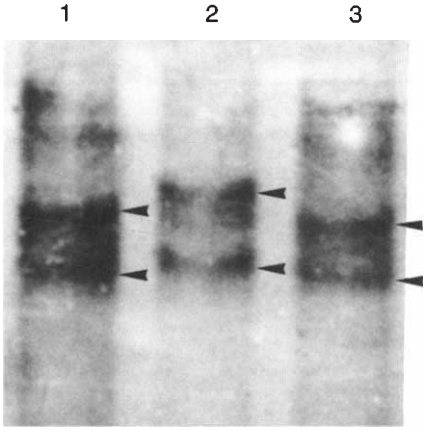


Fig. 4. *PCR-SSCP analysis of exon 48 by silver staining.* The amplified DNA fragments with primers IV1 and 48B were denatured and electrophoresed under non-denaturing condition on a 7.5% polyacrylamide gel with 6% glycerol at 4°C at 100 volts for 10 hours. The DNA was detected after silver staining of the gel. Lane 1 and 3, normal male control; lane 2, patient OYM. Compared to normal control, the mobility of two single strand DNAs, indicated by arrowheads, was altered.

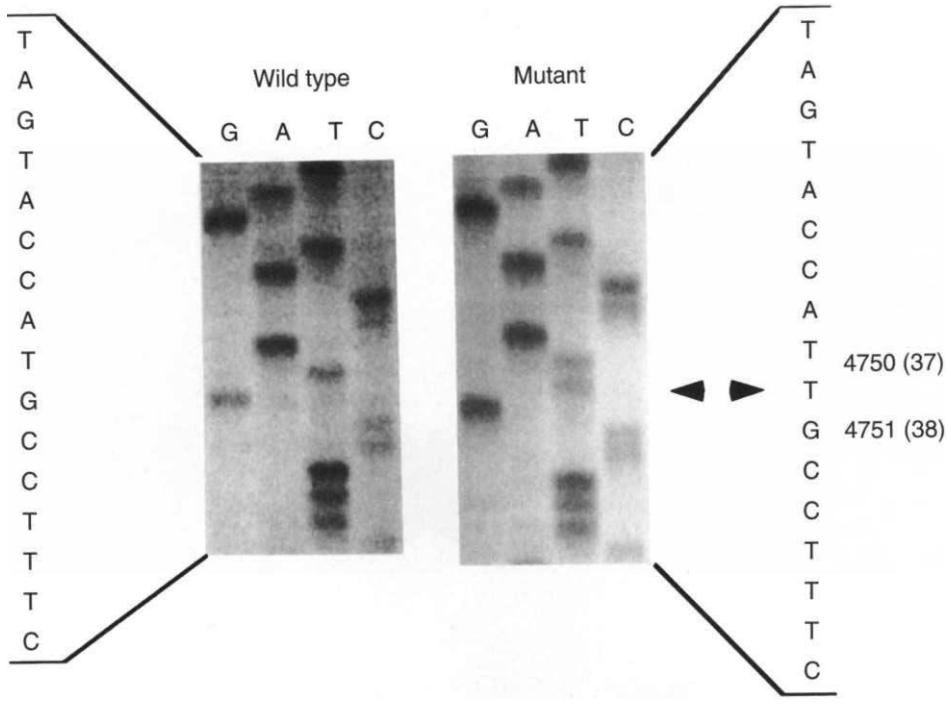


Fig. 5. *Partial nucleotide sequence of PCR amplified DNA with primers IV1 and 48B.* Left, normal male control; right, patient OYM. The mutation in the patient proved to be a single base insertion indicated by arrows between nucleotides 4750 (37) and 4751 (38). The sequence is labeled 5′ and 3′ in reference to orientation of the COL4A5 cDNA (parentheses: nucleotides in the exon 48).

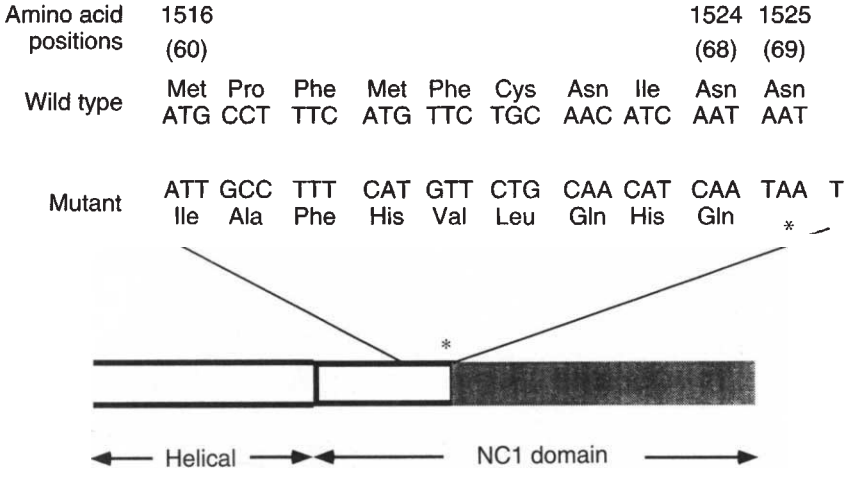


Fig. 6. *The amino acid sequence and deduced protein of the α5(IV) collagen chain of patient OYM.* Top, amino acid numbers start at the translation initiator methionine [11]. Parentheses: those in the NC1 domain. Middle, nucleotide and deduced amino acid sequences in wild type and mutant are shown by inner and outer lines, respectively. A single base insertion is indicated by an arrowhead, and the premature stop codon by an asterisk. Bottom, illustration of deduced protein of the α5(IV) collagen chain of patient OYM. The insertion alters the reading frame and changes 9 amino acids 1516 to 1524, and introduces a premature terminal signal at codon 1525. The deduced protein would lack about two-thirds of the NC1 domain shown by the gray box.

analysis after digestion with *Eco*RI revealed no loss or change in the restriction fragments when hybridized with cDNA HS19, thereby indicating the absence of any major rearrangement in the gene from exons 47 to 51 (data not shown). PCR analysis of part of exons showed that exon 48 was not amplified, while exons 47 and 49 were normal (Fig. 3).

To obtain more information of exon 48 and adjacent regions, we screened one million plaque-forming units of genomic DNA and obtained four positive signals containing exon 48. We isolated one genomic clone (1.5 kb) and determined the flanking sequence (data not shown). With a combination of *in vitro* amplification of exon 48 with primers IV1 and 48B and SSCP analysis, the presence of a mutation in exon 48 was suggested. Compared to control samples, the mobility of the amplified fragment of the patient was clearly altered (Fig. 4).

DNA sequencing analysis of the amplified DNA product revealed a single base insertion (T) between nucleotides T 4750 and G 4751 [11] (between nucleotides 37 and 38 in exon 48) within methionine 1516 (60 in the NC1 domain) (Figs. 5, 6). This finding was confirmed by repeated sequencing. The insertion was localized between nucleotides T and G at the 3′ end of primer 48A (Fig. 2B). Thus, the failure to amplify exon 48 from genomic DNA of the patient could be due to a mismatch in the primer 48A and template (Fig. 3C).

When the PCR amplified DNA (282 bp) from the control was digested with *Nla*III (restriction site; CATG/), five fragments

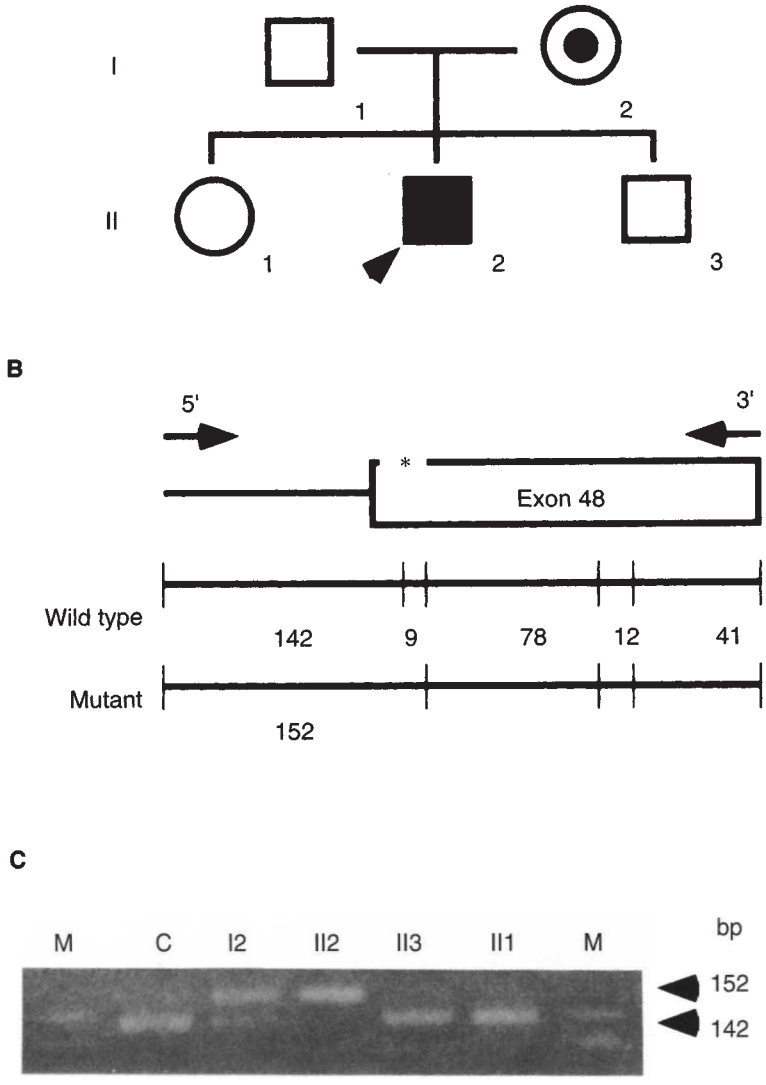


Fig. 7. *Pedigree of patient OYM and gene tracking by restriction enzyme* Nla*III digestion of amplified DNA.* **A.** Pedigree of patient OYM. Obligate female carrier is shown as an open circle with a dot. Unaffected female is shown as an open circle and unaffected male as an open box. Affected male is shown as a closed box. The patient (II2) is indicated by an arrowhead. **B.** Region of amplified DNA with primers IV1 (5'-end) and 48B (3'-end) indicated by arrows. *Nla*III restriction sites in wild type and mutant are indicated by vertical lines, and fragment sizes by numbers (bp). The insertion point is indicated by an asterisk. **C.** Digestion of amplified DNA with *Nla*III and electrophoresed on a 15% polyacrylamide gel. Four fragments of 78 bp and less ran off the gel. Lane 2 (C), normal male control; lane 3 (I2), mother; lane 4 (II2), patient; lane 5 (II3), brother; and lane 6 (II1), sister; lane 1 and 6 (M), 1 kb ladder marker. The patient (II2) is missing the 142 bp fragment and has a variant 152 bp fragment. His mother (I2) is heterozygous with the normal 142 bp fragment as well as the variant 152 bp fragment.

(142, 78, 41, 12, and 9 bp) were produced. For the patient, two fragments (142 and 9 bp) were absent and a new fragment (152 bp) appeared because of the missing first *Nla*III site by a change from CATG to CATTG in the insertion point (Fig. 7). The patient's mother was heterozygous with the normal 142 bp fragment and the variant 152 bp fragment, whereas the patient's brother and one sister had the normal 142 bp fragment. DNA samples from other members of the family could not be obtained.

Discussion

We identified a single base (T) insertion in exon 48 (NC1 domain) of the COL4A5 gene located between nucleotides 4750 and 4751. We did not detect the same insertion in 24 unrelated Japanese patients. Several mutations have been reported [9–17], but none was the same as ours. We propose that the mutation we identified is a novel one in Alport syndrome. This insertion alters the reading frame and introduces a premature termination signal, TAA (nucleotides 4775-4778) after amino acid 1524 (68 in the NC1 domain). This mutation would be expected to result in synthesis of the truncated α5(IV) collagen chain that differs from the normal protein in the nine amino acids at its carboxy terminus and that lacks about two-thirds of the NC1 domain (Fig. 6). Lesions such as this mutation in the globular NC1 domain of α5(IV) collagen chain would have a direct effect on the type IV collagen network at lamina densa in the GBM. This part of the molecule is needed to align the three α chains involved in the triple-helical domain to enable correct folding [26, 27]. In addition, the NC1 domain is an integral part of the cross-linking between two adjacent triple-helical molecules during formation of the type IV collagen network [28, 29]. Massive proteinuria with splitting of the lamina densa was considered to be due to an increased permeability of GBM, because of abnormal networks of the type IV collagen as a result of this mutation. Rumpelt reported that the degree of proteinuria and splitting of the lamina densa were correlated positively in patients with Alport syndrome [30].

Gene tracking by *Nla*III digestion revealed that the patient's mother was a heterozygote of the mutant allele and wild type allele (Fig. 7), therefore, the mutation was inherited from the

mother. The patient's sister and younger brother carried the wild type allele alone, though both had hematuria and proteinuria but no audiometry and ocular abnormalities. Renal biopsy findings of the sister showed no splitting of the lamina densa in all fields examined. Although histological examination could not be done in case of the brother, our observation did suggest that a disorder other than Alport syndrome was present. Takebayashi identified a patient with IgA nephropathy among a large kindred with Alport syndrome (personal communication). Without gene analysis, they would be misdiagnosed as Alport syndrome on clinical and familial basis. We propose that diagnosis of the disease should be made on the basis of both clinical phenotypes and molecular defects.

Acknowledgments

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