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## **Heterozygous *TGFBR2* Mutations in Marfan Syndrome**

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**Marfan syndrome (MFS) is an extracellular matrix disorder with cardinal manifestations in the eye, skeleton, and cardiovascular systems and associated with defects in the fibrillin gene (*FBNI*) at 15q21.1 (ref. 1). We previously mapped the second locus for MFS (MFS type 2, *MFS2*, OMIM \*154705), at 3p24.2-p25 in a large French family (MS1)<sup>2</sup>. Identification of a 3p24.1 chromosomal breakpoint disrupting the TGF- $\beta$  receptor 2 gene (*TGFBR2*) in a Japanese MFS patient led us to consider *TGFBR2* as the *MSF2* gene. We found a Q508Q mutation of *TGFBR2* that resulted in abnormal splicing and segregated with *MFS2* in MS1. Three other missense mutations were found in four unrelated probands and were shown by luciferase-assays to lead to loss of function of the TGF- $\beta$  signaling activity on extracellular matrix formation. These results show that heterozygous mutations in *TGFBR2*, a putative tumor suppressor gene implicated in several malignancies, are also associated with inherited connective-tissue disorders.**

We encountered a Japanese MFS patient with *de novo* complex chromosomal rearrangements, 46,XY,t(1;5;4)(p35;q33.2;q35),ins(3)(q11.2;p24.1p14.2). As one of these chromosomal breakpoints, 3p24.1, was consistent with the *MFS2* locus and the patient carried no *FBNI* mutation (data not shown), it was hypothesized that disruption of a gene at 3p24.1 by the insertion caused the disease. FISH mapping revealed that BAC clones, RP11-479I10, RP11-775G14, and RP11-1056A20, spanned the breakpoint (**Fig. 1**). The *TGFBR2* gene was the only gene in the common region overlapped by the three BACs and was disrupted at the breakpoint according to the detailed FISH analysis using cosmid subclones derived from RP11-775G14 (**Fig. 1**).

*TGFBR2* was analyzed in a large French family (MS1) that provided mapping of *MFS2* at 3p24.2-p25 (ref. 2) (**Fig. 2a**). The nucleotide substitution 1524G>A was found in 3 out of 3 affected subjects initially tested. This transition was synonymous (amino acid substitution Q508Q), but located at the last nucleotide of exon 6, implying that a splicing process could be affected. RT-PCR analysis with a primer set in exons 5 and 7 using cDNA of fibroblasts from two affected members (III-37 and IV-10) revealed a stable larger product together with the expected normal band (**Supplementary Fig. 1** online). Sequencing analysis confirmed an abnormal splicing by which 23 nucleotides of intron 6 were added to exon 6 creating a premature stop codon at amino acid position 525 (**Fig. 2b**). Quantitative experiments demonstrated equal expression of the two alleles (**Supplementary Fig. 1** online). This abnormal fragment was not found in fibroblast cDNA from III-41 (healthy) or an unrelated normal control. All 74 members of MS1 were then analyzed by PCR-direct sequencing of exon 5 using genomic DNA as a template. The 1524G>A substitution was found in 25 members (12 affected and 13 with an ambiguous status) but absent in 32 healthy sibs, one “ambiguous” (IV-53), and 16 unrelated spouses. The substitution was also absent in 60 French, 92 American, and 267 healthy Japanese controls. Molecular data for MS1 members were consistent with clinical data except for one subject (IV-53)<sup>3</sup>. This “ambiguous” patient, presented with borderline skeletal features and an aortic diameter of 32 mm (+ 1.98 SD) at age 16 (ref. 3). He is now 32 years old, has a normal aortic diameter and should be considered as unaffected. A common haplotype of *D3S1609*, *D3S3727* (intragenic *TGFBR2* marker), *D3S3567* and *D3S1619* was shared by carriers of the Q508Q mutation (**Fig. 2a**).

We subsequently studied 9 MFS probands from 9 unrelated French families, as well as 10 unrelated Japanese MFS patients, in which no *FBN1* mutation or no linkage to *FBN1* was recognized. After bi-directional sequencing of all the 7 exons of *TGFBR2*, we identified three

missense mutations: 923T>C (L308P), 1346C>T (S449F) and 1609C>T (R537C). The 923T>C was found only in the proband of French family MS57 but not in her unaffected parents (paternity confirmed) or in two healthy sibs, thus demonstrating *de novo* occurrence. The 1346C>T was identified in a French family (MS382), and 609C>T in another French family (MS587) and in one Japanese patient. In MS587, the proband (III-7) and two affected members (III-1 and IV-1) as well as a child with an ambiguous status (IV-3) were shown to carry 1609C>T (**Fig. 3**). All the missense mutations are located within the serine/threonine kinase domain of TGF- $\beta$  receptor 2 protein, and each affects an amino acid that is highly conserved or chemically similar in the homologous mouse and rat *Tgfb $\beta$ 2* genes, mouse and fly *Acvr2*, and nematode *daf* genes (**Fig. 3**). Among 267 unrelated Japanese healthy controls (534 chromosomes) and 92 unrelated American healthy controls (184 chromosomes), the mutations were not found. We also investigated 10 French probands presenting with thoracic aortic aneurysms and dissection (TAAD), since a putative locus has been mapped for this disease at 3p24-25 (ref. 4), but no mutation was identified.

We used an *in vitro* luciferase assay to evaluate an effect of the mutations on TGF- $\beta$  signaling. A pTARE-Luc cis-reporter plasmid and a pRL-TK vector were transfected into HEK 293 and relative luciferase activity (RLA) was determined. Basal activity was detected that increased 3 fold after exposure to exogenous TGF- $\beta$ 1 (**Fig. 4a**). Transfection with wild-type (WT) *TGFBR2* cDNA showed about 15 of RLA even in the absence of exogenous TGF- $\beta$ 1 (approximately 12 RLA) as previously reported<sup>5</sup>. In contrast, a truncated mutant,  $\delta$  cyt, lacking the kinase domain, showed a significant drop of RLA even with exogenous TGF- $\beta$ 1 compared to WT. In transfection with other missense-type mutants, L308P, S449F, and R537C, a significant decrease of RLA was also recognized, indicating their negative effect on TGF- $\beta$  signaling.

TGF- $\beta$ s are cytokines that regulate a large spectrum of cellular processes including proliferation, cell cycle arrest, apoptosis, differentiation and extracellular matrix formation through a heteromeric complex of type I and type II receptors which have serine/threonine kinase activities in their cytoplasmic domains. It is well established that defective TGF- $\beta$  signal transduction plays an important role in tumorigenesis and that *TGFBR2*, *MADH4* and *MADH2* act as tumor suppressors in several cancers<sup>6</sup>. The majority of *TGFBR2* gene mutations found in tumor cells are clustered in the poly A repeat in exon 3 (ref. 7) (**Fig. 3**). Many missense mutations have also been reported in the gene in various cancers and have been suggested to result in loss of function<sup>5,8-10</sup>. Only one germline mutation [944C>T (T315M) located in the kinase subdomain IV of the receptor] has been reported in a kindred with hereditary nonpolyposis colorectal cancer (HNPCC)<sup>11</sup>. During our analysis of *TGFBR2* in different populations, heterozygous 944C>T was unexpectedly detected in 7 of 492 normal Japanese controls (7/984=0.71%) and 6 of 228 patients with sporadic colorectal cancer (6/456=1.32%). As the frequency in the two populations was not statistically different ( $P>0.05$ ,  $\chi^2$  analysis), 944C>T could in fact be a rare polymorphism. Functional studies by Lu et al.<sup>12</sup> indicated that 944C>T showed a defect in growth inhibition in response to TGF- $\beta$  despite maintenance of the ability to induce extracellular matrix proteins, suggesting that two divergent TGF- $\beta$  signaling transduction pathways may exist. The p3TP-Lux reporter system using *Tgfbr2*-negative DR-26 cells revealed that the three missense mutations found in MFS suppressed RLA drastically, compared to WT, suggesting downregulation in MFS of an extracellular matrix protein, plasminogen activator inhibitor type 1 (PAI-1), which was not shown by the germline mutation, T315M (944C>T) reported in HNPCC<sup>12</sup> (**Fig. 4b**). These diverging effects of the different mutations may explain why malignancies were not observed in MFS families with the *TGFBR2* mutations. Furthermore, co-transfection of WT and R537C

with different ratios (25:75, 50:50, and 75:25) in DR-26 showed serial increase of RLA in parallel with WT cDNA quantity, suggesting that the mutation was less likely to result in a dominant negative effect (**Fig. 4b**). The L308P and S449F also showed similar results in co-transfection with WT cDNA (data not shown). Since we have identified three different classes of *TGFBR2* mutations (gene disruption, splicing and missense mutations disturbing TGF- $\beta$  signaling), it is most likely that loss-of-function accounts for the dominant inheritance of MFS.

By a study of *Fbn1* deficient mice, Neptune et al. demonstrated an excessive activity of TGF- $\beta$  that likely underlies their tendency to develop emphysema and could explain other manifestations of the disease<sup>13</sup>. We previously reported that domain-specific germline mutations of *TGFBI* cause Camurati-Engelmann syndrome (OMIM #131300) with which patients usually have Marfanoid habitus, i.e., long slender extremities and vertebral deformation<sup>14</sup>. TGF- $\beta$  possibly regulates the extracellular matrix<sup>12, 15-17</sup>. Our results provide further evidence that perturbation of TGF- $\beta$  signaling contributes to the pathogenesis of extracellular matrix disorders, but by a mechanism different from that reported for the *Fbn1* deficient mice.

Among the 10 French MFS probands examined, *TGFBR2* mutations were found in only 4 patients who share a common clinical picture: prominent aortic, skeletal and skin/integument anomalies, mild ocular features (with the exception of subject MS1-IV-83 who has ectopia lentis), infrequent dural ectasia and pulmonary feature. However, these findings are not specific since 100 *FBNI* mutations have been identified in MFS probands with comparable clinical features<sup>18</sup>. Furthermore, only a small number of MFS probands presenting with complete clinical features and not carrying a *FBNI* gene mutation were screened for *TGFBR2* mutations. Therefore, further data must be collected to assess the complete clinical spectrum

associated with mutations in the latter gene. Other overlapping pathologies should also now be investigated such as MASS syndrome (Mitral valve prolapse, Aortic dilatation, and Skin and Skeletal manifestations syndrome, OMIM #604308), isolated familial mitral valve prolapse (OMIM#157700), and autosomal dominant TAAD (OMIM # 132900). A locus for TAAD was mapped to 3p24-25, overlapping or close to the *MFS2* locus (termed *TAAD2*)<sup>4</sup>. Although no mutation was found in the 10 TAAD probands that we tested, the disease is heterogeneous and further investigations are warranted before ruling out that *TGFBR2* and *TAAD2* are allelic.

In conclusion, MFS is associated with mutations in at least two genes: *FBNI* and *TGFBR2*. In effect, the sample of MFS families contained 6 French and 9 Japanese probands for which no mutation has been identified in either of these two genes. This observation could indicate a higher degree of genetic heterogeneity for MFS.

## METHODS

### Subjects and clinical evaluation.

***A Japanese patient with 46,XY,t(1;5;4)(p35;q33.2;q35),ins(3)(q11.2;p24.1p14.2) de novo:*** The patient was a 13-year old boy. Severe pectus excavatum was operated at age of 10. He was clinically diagnosed as MFS<sup>19</sup> because of his high arched palate, arachnodactyly (positive wrist and thumb signs), atlantoaxial subluxation, scoliosis (>20 degree), reduced extensions at the elbow, dilatation of the ascending aorta (30 mm at age 12) involving the Valsalva sinus, mitral valve prolapse, incomplete right bundle-branch block, bilateral inguinal hernia, dural ectasia by lumbo-sacral CT, and increased axial length of eye globe. He presented with short stature (127.0 cm height [-2.9 SD] and 21.0 kg weight [-2.2 SD]) at age of 12, which was evaluated as hypophyseal because insulin or L-DOPA stimulation test showed low growth hormone (GH) response (12.8 ng/mL or 10.3 ng/mL at the maximum GH level), and blood serum IGF-I, TSH, T3, and T4 were 31.4 ng/mL (normal range, 144-924 ng/mL), 0.7 µU/mL (normal range, 0.436-3.78 µU/mL), 129 ng/dL (normal range, 76-177 ng/dL), and 12.9 µg/dL (normal range, 4.8-11.2 µg/dL), respectively. GH treatment was started since then. At age of 13, his height was 135.2 cm (-2.8 SD) and weight 24.8 kg (-2.5 SD). No mutation was found after PCR and direct sequencing of *FBN1* coding regions and exon-intron boundaries with the described method<sup>20</sup>.

***MS1 family:*** A large French family was ascertained following the death of a 39-year old man from aortic dissection. Complete individual clinical features for the first part of the family study were described previously<sup>3</sup>. The second step of family investigation was undertaken in the Marfan Clinic of Hôpital Ambroise Paré. Subjects at risk underwent careful physical examination, echocardiography and slit-lamp examination. Twelve new family members were investigated and sampled (IV-40, IV-43, IV-48, V-6, V-7, V-8, V-9, V-10, V-

11, V-12, V-13, and V-14). As a result, a total of 12 members (III-4, III-36, III-37, III-40, III-49, III-52, IV-10, IV-44, IV-49, IV-51, IV-54, and IV-83) were diagnosed as MFS, 14 as carrying ambiguous status (III-13, III-16, IV-30, IV-32, III-37, IV-53, IV-84, IV-86, IV-88, V-1, V-8, V-9, V-10, and V-11), 16 unrelated spouses, and 32 sibs without abnormality in any of the skeletal, ocular and cardiovascular systems (as well as the lung, skin-integument and central nervous system), or with only isolated minor findings in these organs/tissues, were considered as unaffected. Extensive clinical data for most subjects can be found in Boileau et al.<sup>3</sup>. New members from generation V with an ambiguous status carried minor skeletal features and borderline aortic root diameter. Recent clinical data are given in the **Supplementary table 1** online.

**MS57 family:** MS57-II-2 presented with dolichostenomelia, pectus carinatum, arachnodactyly, mild scoliosis, spondylolisthesis, protrusio acetabulae, hyperlaxity of joints, dysmorphic face, narrow arched palate and dental crowding, striae distensae, flat cornea, aortic dilation (+8 SD) with mild regurgitation, mitral valve prolapse and myxoid valves, aneurysm of interatrial septum, and small atrial septal defect, and was diagnosed as MFS. She died suddenly at aged 18 of unknown cause. Her brother and parents were unaffected. Her sister MS57-II-1 presented with only arachnodactyly, mild pectus carinatum but no other feature of MFS. She was considered as unaffected.

**MS382 family:** II-2 is a 13-year old girl who had been diagnosed in infancy. Her father (I-1) died suddenly at the age of 39 of unknown cardiovascular cause, he was tall and had hyperlaxity. For II-2 pronounced skeletal symptoms of MFS were noted including asymmetric pectus carinatum, arachnodactyly with positive wrist sign, dolichocephaly, severe dorso-lumbar scoliosis, hyperlaxity, joint hypermobility at ankles and knees, clear muscular hypotonia and umbilical hernia. Radiological examination revealed major coxa valga and absence of dural ectasia. She presented with patent ductus arteriosus, foramen ovale,

interventricular septal defect and aortic root dilation (50 mm at age 10) requiring surgery at age 12. No ocular symptom was noted except for very slow dilation. As only two major criteria for MFS were found, she did not fulfill the revised diagnostic criteria for MFS. Only her mother (I-2) and sister (II-1) were available for clinical examination and showed no feature of MFS. Blood samples were available for subjects I-2 and II-2 and were tested.

**MS587 family:** III-7 is a 33-year old female who had been examined after her father's sudden death at the age of 26 (II-4). Aortic dilation and mitral valve regurgitation were noted in III-7 at age of 5 and dilation was + 6 SD at age 31 with minimal regurgitation. She presented with a history of lung disease and pleurisy, joint hyperlaxity, narrow palate with dental crowding, scoliosis, striae distensae, mild myopia with mild astigmatism. She was diagnosed as MFS. Family history revealed two other sudden deaths: her father's brother (II-3) at age 22 and her father's sister (II-2) at age 32. III-1 (aged 36) showed aortic dilation, reduced upper to lower segment ratio, dolichostenomelia, pectus excavatum, narrow arched palate, and scoliosis. III-4 (aged 30) had mild scoliosis, pectus excavatum, hyperlaxity and hypermobility of joints, high narrow palate with dental crowding, pes planus, striae distensae, and aortic dilation (+6 SD) with moderate aortic valve regurgitation. IV-1 (aged 4.5 years) presented with aortic dilation (+6 SD) without regurgitation, increased body length, dolichocephaly, narrow arched palate, and mild scoliosis. III-1, III-4, IV-1 were also diagnosed as MFS. Detailed clinical information of IV-3 with an ambiguous status was not available. Blood samples were only available for subjects III-1, III-5, III-7, IV-1 and IV-3 and were tested.

**Japanese MFS patients:** Ten Japanese MFS patients who were shown not to carry *FBNI* mutations by PCR direct sequencing of *FBNI* coding regions and exon-intron boundaries using genomic DNA as previously described<sup>20</sup> were screened for *TGFBR2* mutations. Seven of them were reported<sup>20</sup>. All 10 patients fit the revised criteria for MFS<sup>19</sup>,

although detailed clinical information or parental DNA was not available because patient materials were treated as anonymous MFS DNAs according to an IRB approved-protocol.

***French TAAD patients:*** Ten French patients presenting with TAAD were tested. All belong to families with vertical transmission of documented aortic aneurysm/dissection without risk factor for atheroma including hypertension. Inclusion criteria were ascending aortic diameter above mean +2 SD according to Roman et al.<sup>21</sup> or aortic diameter > 40 mm, or operation because of dissection or dilation of the ascending aorta. Proband and family members were also systematically investigated to rule out MFS and Ehlers-Danlos syndrome as well as secondary aneurysm/dissection.

The French and the Japanese research protocols were approved by Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Boulogne Billancourt Hôpital Ambroise Paré and the ethics committee of Nagasaki University, respectively. Informed consent was obtained for all subjects included in this study in agreement with the requirements of French and Japanese regulations.

### **FISH analysis**

Clone DNA was labeled with SpectrumGreen<sup>TM</sup>-11-dUTP or SpectrumOrange<sup>TM</sup>-11-dUTP (Vysis, Downers Grove, IL, USA) by nick translation, and denatured at 76 °C for 10 min. Probe-hybridization mixtures (10 µl) were applied on the chromosomes, incubated at 37 °C for 16 hrs, then washed. Fluorescence photomicroscopy was performed under a Zeiss Axioskop microscope equipped with a quad filter set with single band excitation filters (84000, Chroma Technology Corp., Brattleboro, VT, USA). Images were collected and merged using a cooled CCD camera (TEA/CCD-1317-G1, Princeton Instruments, Trenton, NJ, USA) and IPLab/MAC software (Scanalytics, Inc., Fairfax, VA, USA).

After isolation of a BAC clone spanning the 3p24.1 breakpoint, a cosmid library was prepared from the BAC DNA for a detailed FISH mapping. Purified BAC DNA was isolated using Qiagen Midi-Prep columns (Qiagen, Chatsworth, CA), partially digested with *Sau3AI*, and ligated to SuperCos1 cosmid vector according to a manufacturer's instruction (Stratagene, La Jolla, CA). Cosmid DNA was extracted with Qiagen Mini-Prep columns (Qiagen) and a total of 182 cosmid clones were screened by PCR.

### **Mutation analysis.**

Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Exons 1-7 covering the *TGFBR2* coding region (GenBank accession number, NT\_022517) were amplified by PCR for direct sequencing. PCR amplification and sequencing primers are available on request. PCR was cycled 35 times at 95 °C for 30 sec, at 50 °C for 30 sec, and at 72 °C for 30 sec in a 50 µL mixture, containing 1x PCR buffer with 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 1 µM each primer and 2.5 U TaqGold polymerase (Applied Biosystems, Foster city, CA). PCR products were purified with ExoSAP-IT (Amersham-Pharmacia, Cleveland, OH), and sequenced on both strands with BigDye Terminator chemistry version 3 by the standard protocol (Applied Biosystems). Sequencing reaction was performed at 96 °C for 10 sec, at 50 °C for 5 sec, and at 60 °C for 4 min (25 cycles) either on Gene Amp PCR System 2700, or 9700 (Applied Biosystems). The reaction mixture was purified using Centri-Sep Columns<sup>TM</sup> (Princeton Separation Inc., Adelphia, NJ) and analyzed on the ABI Genetic Analyzer 3100 (Applied Biosystems) according to the supplier's protocols with the sequence analysis software (Applied Biosystems) and the AutoAssembler ver. 2.1.1 software (Applied Biosystems).

Total mRNA was extracted from fibroblasts of 2 affected (III-37 and IV-10), and one unaffected member (III-41) of family MS1, and a normal control person, using RNA B<sup>TM</sup>

(Bioprobe Systems, Montreuil-sur-bois, France) according to the manufacturer's instructions. Samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). Total mRNA was reverse-transcribed using Superscript<sup>TM</sup> II RNase Reverse Transcriptase (Gibco BRL/Invitrogen, Cergy-Pontoise, France). PCR was performed with a primer set in exons 5 and 7 and subsequently sequenced as previously described. Screening was performed in all subjects from French families by PCR on genomic DNA and subsequently sequenced. All primer sequences are available on request.

### Genotyping of family MS1

Microsatellite markers were selected from public genetic databases: Généthon: <http://www.genethon.fr/>, and CHLC: <http://www.chlc.org/>. Genomic DNA was genotyped as previously described<sup>2</sup>. Regional haplotypes for 4 marker loci were constructed from tel-*D3S1609* to *D3S1619*-cen [**Fig. 2** (*D3S1609*, *D3S3727*, *D3S3567*, and *D3S1619*)]. *D3S3727* is an intragenic *TGFBR2* marker. Physical mapping allowed us to determine the marker order.

### DNA constructs

A wild-type *TGFBR2* cDNA (WT) (GenBank accession number, NM 003242; amino acids 1-567) and a truncated-type cDNA ( $\delta$  cyt) (amino acids 1-222 lacking the entire kinase domain) were generated by RT-PCR using human fetal brain BD Marathon-Ready cDNA (BD Biosciences, Palo Alto, CA) as a template, and subcloned into the pcDNA3.1(-) expression vector (Invitrogen, Carlsbad, CA). Site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol to generate three variant types of *TGFBR2* in pcDNA3.1(-), L308P, S449F, and R537C, which were found in MFS families we studied. All variant cDNAs were verified by sequencing. Sequences of all primers used are available on requests.

### Cell culture, transfection and luciferase assay

HEK 293, Mv1Lu (a wild-type mink lung epithelial cell line), and DR-26 (a chemically mutagenized Mv1Lu cell line in which *Tgfb $\beta$ 2* is defective)<sup>12</sup> were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> incubator. HEK 293 cells were transiently transfected with a *TGFBR2* construct (WT,  $\delta$  cyt, L308P, S449F, or R537C), a reporter plasmid (pTARE-Luc cis-reporter or pCIS-CK negative control) and a pRL-TK vector (an internal control for standardization) in Dual-Luciferase Reporter Assay System (Promega) using Transfast Transfection Reagent (Promega). The pTARE-Luc cis-reporter plasmid contains the basic promoter element (TATA box) and TGF- $\beta$ /Activin response element (TARE). This reporter plasmid expresses firefly luciferase under control of these elements, whereas the pCIS-CK negative control plasmid contains no inducible cis-enhancer element to evaluate whether effects are TGF- $\beta$  signaling-specific. The p3TP-Lux containing the luciferase reporter gene under control of a portion of the plasminogen activator inhibitor type 1 (*PAI-1*) gene promoter region and three consecutive TPA response elements was used to evaluate extracellular matrix protein production by TGF- $\beta$  signaling<sup>12</sup>. After transfection, HEK 293, Mv1Lu, or DR-26 cells were incubated in DMEM for 36 hours and medium was replaced to DMEM containing 10ng/ml TGF- $\beta$ 1. Eight hours later, cells were harvested and assessed for luciferase activity in quadruplicate. Luciferase activity was measured using TD-20/20 Luminometer DLRReady (Turner Designs Instrument, Sunnyvale, CA). Statistical analysis was performed by Post-hoc test using StatView (SAS institute Inc, Cary, NC, USA) and  $P < 0.05$  were considered to be statistically different.

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## FIGURE LEGENDS

**Figure 1: *TGFBR2* was isolated from the 3p24.1 breakpoint of a Japanese patient with complex chromosomal abnormalities.**

The 3p24.1 breakpoint was analyzed because the *MFS2* locus was also mapped to 3p24.2-p25. In the patient, a chromosomal segment 3p24.1-p14.2 was inserted into 3q11.2. FISH analysis confirmed that RP11-775G14 spans the 3p24.1 breakpoint. A summarized physical map covering the 3p24.1 breakpoint is indicated. Horizontal bars show BAC and cosmid clones: green depicts clone spanning the breakpoint, and arrow shows gene. *TGFBR2* exons are indicated as purple squares. Cosmid subclones, c1C and c3G spanned the breakpoint. c1C contains 35.2-kb genomic region of *TGFBR2* (through intron 3 to the end of 3' UTR) out of the 39.4 kb insert-DNA according to its end sequence information. The gene must have been disrupted at the breakpoint because signals on the 3q would have not been seen if the breakpoint existed outside *TGFBR2*.

**Figure 2: Haplotype analysis of a large French family (MS1) and a mutation causing abnormal splicing.**

(a) Pedigree of family MS1 and segregation of 3p24.1 markers in members affected with MFS (filled symbol), having an ambiguous status (gray symbol) or unaffected (open symbol). Orange symbol indicates member with a mutation, 1524G>A (Q508Q), and green, member without the mutation. The haplotype co-segregating with the disease is shown in red.

(b) Normal (green) and abnormal splicing (orange) caused by 1524G>A (Q508Q). The consensus value on splicing<sup>22</sup> was modified from 95.6 to 82.3 by 1524G>A and another good consensus sequence (AGgt xxg, value 90.5) was located 23 bp away in the intronic sequence.

In the mutated allele, 23 nucleotides of intron 6 were newly added after exon 6 and connected to regular exon 7 as predicted, resulting in a premature stop codon at amino acid 525.

**Figure 3: Genomic structure of *TGFBR2* and mutations found in MFS families.**

*TGFBR2* consists of seven exons. Square indicates exon. A transmembrane domain, a kinase domain, and UTRs are shown in light blue, pink, and gray, respectively. (A)10 and (GT)3 are genomic instability sites. Three other missense mutations, 923T>C (L308P), 1346C>T (S449), and 1690C>T (R537C) are found in families MS57, MS382, and MS587, respectively.

Symbols (filled, open, green, and orange) are depicted in the same manner as in **Fig. 2**. Each mutation occurred at an amino acid that is evolutionally conserved or chemically similar (red square) in a kinase domain among mouse and rat *Tgfb $\beta$ 2*, mouse and fly *Acvr2*, and nematode *daf* genes. Multiple sequence alignment was done using the web-based software, CLUSTALW (<http://clustalw.genome.ad.jp/>).

**Figure 4: Impairment of TGF- $\beta$  signaling activity by *TGFBR2* missense mutations found in patients with MFS.**

(a) Relative luciferase activity (RLA) of pTARE-Luc cis-reporter in HEK 293 cells after transient transfection with various *TGFBR2* constructs was calculated by normalization using the activity of a co-transfected control vector, pRL-TK, containing distinguishable *R. reniformis* luciferase. The pCIS-CK negative control plasmid containing no inducible cis-enhancer element was also used to evaluate whether effects are TGF- $\beta$  signaling-specific. Data represent mean + SD. Basal activity only by pTARE-Luc cis-reporter is probably due to endogenous TGF- $\beta$ 1 in HEK 293. Transfection with wild-type (WT) *TGFBR2* cDNA showed a significant high value (about 15) of RLA, while that with a truncated mutant,  $\delta$  cyt, lacking the kinase domain, showed significantly low RLA. Transfection with other missense-type

mutants, L308P, S449F, and R537C, also showed significantly low RLA, similar to that with  $\delta$  cyt. \*: Statistically different ( $P<0.0001$ ) by Post-hoc test.

**(b)** RLA of pT3P-Lux reporter in DR-26 (a *Tgfb $\beta$ 2* negative cell line) after transient transfection. Mv1Lu (a *Tgfb $\beta$ 2* positive cell line) was also used to show normal response of the pT3P-Lux reporter to TGF- $\beta$ 1. Transfection with wild-type (WT) *TGFBR2* cDNA showed a significant high value (about 20) of RLA, while that with an empty vector [pcDNA3.1(-)] and the truncated mutant,  $\delta$  cyt, showed significantly low RLA. Transfection with other missense-type mutants, L308P, S449F, and R537C, also showed significantly low RLA, similar to that with  $\delta$  cyt. Co-transfection in DR-26 of WT and R537C with different ratios showed serial increase of RLA in parallel with WT cDNA quantity, suggesting that the mutation was less likely to cause a dominant negative effect. \*: Statistically different ( $P<0.0001$ ) by Post-hoc test.

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