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Pathogenic mutations identified by a multimodality approach in 117 Japanese Fanconi anemia patients

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ABSTRACT

Fanconi anemia is a rare recessive disease characterized by multiple congenital abnormalities, progressive bone marrow failure, and a predisposition to malignancies. It results from mutations in one of the 22 known *FANC* genes. The number of Japanese Fanconi anemia patients with a defined genetic diagnosis was relatively limited. In this study, we reveal the genetic subtyping and the characteristics of mutated *FANC* genes in Japan and clarify the genotype-phenotype correlations. We studied 117 Japanese patients and successfully subtyped 97% of the cases. *FANCA* and *FANCG* pathogenic variants accounted for the disease in 58% and 25% of Fanconi anemia patients, respectively. We identified one *FANCA* and two *FANCG* hot spot mutations, which are found at low percentages (0.04-0.1%) in the whole-genome reference panel of 3,554 Japanese individuals (Tohoku Medical Megabank). *FANCB* was the third most common complementation group and only one *FANCC* case was identified in our series. Based on the data from the Tohoku Medical Megabank, we estimate that approximately 2.6% of Japanese are carriers of disease-causing *FANC* gene variants, excluding missense mutations. This is the largest series of subtyped Japanese Fanconi anemia patients to date and the results will be useful for future clinical management.

Introduction

Fanconi anemia (FA) is a rare recessive disease characterized by multiple congenital abnormalities, progressive bone marrow failure, and predisposition to malignancies. It results from mutations in one of the 22 known *FANC* genes.[1] These genes are summarized in *Online Supplementary Table S1*. The proteins encoded by these genes participate in a DNA interstrand cross-link repair pathway that deals with DNA damage due to endogenous aldehydes, which are particularly deleterious to hematopoietic stem cells.[2] However, more recent studies have shown that biallelic mutations in *FANCM* cause infertility and early onset cancer but not a typical FA phenotype, and some of the FA genes are actually 'FA-like' since the patients with mutations in these genes do not display hematologic defects (*Online Supplementary Table S1*). Molecular subtyping is critical for the accurate diagnosis and clinical management of the FA patients. However, finding causative mutations for a FA patient is not an easy task.[3,4]

In this study, we successfully subtyped 113 of the 117 Japanese FA patients and identified 215 mutant alleles through a comprehensive strategy starting from a simple genome polymerase chain reaction (PCR)-direct sequencing approach, then progressing to next generation sequencing. The co-ordinated strategies included whole-exome sequencing (WES) and targeted exome sequencing (targeted-seq). In some cases in which we could not reach a conclusive diagnosis, additional methods, such as array-comparative genomic hybridization (aCGH) or RNA-sequencing (RNA-seq) and whole-genome sequencing (WGS) analysis, were extremely useful in detecting deletions or splicing abnormalities, respectively. Similar to other ethnic groups, we found that the FA-A and FA-G groups are the most prevalent in Japan. The *FANCC* mutation is rare and, a little surprisingly, FA-B is the third most prevalent subtype in Japan. The patients with the rare complementation groups, such as FA-D1, E, F, I, N, P, and T, were detected in less than 5% of the cases. We noted striking genotype-phenotype correlation in Japanese FA-B, D1, I, and N cases. In addition, we report the allele frequency of FA-associated deleterious genetic variations in the general Japanese population using the 3.5KJPNv2 database from the Tohoku Medical Megabank Organization (ToMMo).

Methods

Patients and samples

We studied 117 Japanese FA patients from 104 families in total. They overlap with previously reported cases (*Online Supplementary Table S2*)[5-10] and an additional 13 new FA patients were recruited. The diagnosis of FA was confirmed on the basis of chromosomal breakage tests and clinical features. Informed consent was obtained from the family for all subjects involved in this study, and the study was approved by the Research Ethics Committees of all participating hospitals and universities, including Tokai University, Kyoto University, and Nagoya University. Genomic DNA or total RNA was isolated from peripheral blood or cultured fibroblasts using Puregene (Qiagen) or RNAeasy (Qiagen) kit, respectively. cDNA was synthesized with a PrimeScript RT reagent kit (Takara).

Mutation screening for *FANCA* and *FANCG*, and *ALDH2* genotyping

Mutation analyses by PCR of *FANCA* or *FANCG* genes, Multiplex Ligation-mediated Probe Amplification (MLPA) tests for *FANCA* (Falco Biosystems), and *ALDH2* genotyping were performed as previously described.[11,12]

Targeted-sequencing and whole-exome sequencing

Ten and 67 patients were examined by targeted-seq and WES, respectively, as previously described.[8] In targeted-seq, 184 genes, including 15 FA genes (*FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, O* and *P*), were covered. All the mutation variants identified by targeted-seq or WES were verified by PCR and Sanger sequencing.

Array-comparative genomic hybridization analysis

For 10 patients, aCGH was performed as previously described.[6] The probes covered 19 FA genes (*FANCA, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P, Q, S, T, U*) as well as FA-related genes, including NBS1, three RAD51 paralogs (*XRCC3, RAD51B,* and *RAD51D*), *FAAP20, FAAP24,* and *FAAP100*.

RNA-sequencing

We performed RNA-seq for three patients (Cases 62, 98, and 104). Libraries for RNA-seq were prepared using the TruSeq RNA Sample Prep Kit (Illumina) at Macrogen, and sequenced using the Illumina HiSeq 2500 platform with a standard 126-bp paired-end read protocol. Exon skipping events were identified using Genomon-fusion[13] in which patient-specific spliced junctions were identified compared with those identified in a control sample.

Whole-genome sequencing

We performed WGS of DNA samples from one patient (Case 64) and his parents. The TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA) was used for library preparation. The prepared libraries were subjected to next-generation sequencing using a HiSeq X platform. We detected mutation variants as previously described.[8]

Estimating allele frequencies of the Fanconi anemia-associated deleterious genetic variations in the general Japanese population

We analyzed the 3.5KJPNv2 database, which was created with data generated by WGS of 3,554 individuals of the resident cohort of the ToMMo Project. The ToMMo project was established to develop a biobank that combines medical and genome information in the Tohoku area.[14] As of 5th November 2018, the allele frequencies, including indel variations, were released in the publicly accessible 3.5KJPNv2 database (*https://jmorp.megabank.tohoku.ac.jp/201811/*). Our analysis focused on nonsense mutations, frameshift mutations (indels) and splicing donor or acceptor site mutations with less than 1% allele frequencies.

Results

Genetic subtyping of 117 Japanese Fanconi anemia patients through a comprehensive mutation screening

We started mutation analysis of FA patients by direct sequencing of *FANCA* and *FANCG*, and MLPA analysis for *FANCA* in 2009. WES and targeted-seq analyses were initiated in 2012, and molecular diagnosis was successfully achieved in 107 (91.5%) of the 117 patients (Figure 1A). We also examined the *ALDH2* genotype which has been

Table 1. Allele frequency of FA-associated deleterious variants* in Japanese population.

Gene	Genomic location (hg19)	Reference allele/ Alternative allele	cDNA	Protein	Frequency
FANCA	chr16:89882954	CGGCCCAGGCCCTCCGGCGGCCCCCTG/C	c.77_102del	p.P15fs	0.0001
FANCA	chr16: 89833603	AG/A	c.2546delC	p.S849fs	0.0008
FANCA	chr16:89831476	T/A	c.2602-2A>T	aberrant splicing	0.0001
FANCA	chr16:89805357	TTTG/T	c.4189_4191del	p.T1397del	0.0001
FANCC	chr9:97897635	G/C	c.836C>G	p.S279X	0.0001
FANCC	chr9:97864024	G/A	c.1642C>T	p.R548X	0.0003
FANCD1 (BRCA2)	chr13:32903604	CTG/C	c.657_658del	p.Val220fs	0.0001
FANCD1 (BRCA2)	chr13:32911557	ATAACAT/A	c.3067_3072del	p.N1023_I1024del	0.0001
FANCD1 (BRCA2)	chr13:32911577	A/AT	c.3085_3086insT	p.M1029fs	0.0001
FANCD1 (BRCA2)	chr13:32913261	AGT/A	c.4770_4771del	p.C1591fs	0.0001
FANCD1 (BRCA2)	chr13:32914065	CAATT/C	c.5574_5577del	p.I1859fs	0.0003
FANCD1 (BRCA2)	chr13:32914209	ACT/A	c.5718_5719del	p.L1908fs	0.0001
FANCD1(BRCA2)	chr13:32914893	ATAACT/A	c.6402_6406del	p.N2135fs	0.0001
FANCD1 (BRCA2)	chr13:32920978	C/T	c.6952C>T	p.R2318X	0.0003
FANCD1 (BRCA2)	chr13:32930713	AG/A	c.7585delG	p.G2529fs	0.0001
FANCD1 (BRCA2)	chr13:32972800	C/T	c.10150C>T	p.R3384X	0.0004
FANCD2	chr3:10122879	T/TA	c.3072_3073insA	p.N1025fs	0.0001
FANCD2	chr3:10130510	A/C	c.3561-2A>C	aberrant splicing	0.0001
FANCE	chr6:35425734	GCTT/G	c.943_945del	p.L316del	0.0001
FANCG	chr9:35078714	AG/A	c.194delC	p.P65fs	0.0001
FANCG	chr9:35078601	C/G	c.307+1G>C	aberrant splicing	0.001
FANCG	chr9:35076439	G/A	c.1066C>T	p.Q356X	0.0004
FANCI	chr15:89801943	TCTC/T	c.94_96del	p.L33del	0.0001
FANCI	chr15:89803942	A/G	c.157-2A>G	aberrant splicing	0.0008
FANCI	chr15:89833476	G/GC	c.1854_1855insC	p.L619fs	0.0001
FANCI	chr15:89843085	GAA/G	c.2692_2693del	p.K898fs	0.0001
FANCI	chr15:89843605	C/CGGCAAT	c.2878_2879insGGCAAT	p.Q961_F962insWE	0.0004
FANCI	chr15:89850868	A/AC	c.3616_3617insC	p.L1208fs	0.0003
FANCJ (BRIP1)	chr17:59763487	G/C	c.2615C>G	p.S872X	0.0001
FANCJ (BRIP1)	chr17:59761334	AC/A	c.3072delG	p.S1025fs	0.0001
FANCJ (BRIP1)	chr17:59761166	C/CA	c.3240_3241insT	p.A1081fs	0.0003
FANCL	chr2:58456995	C/T	c.170G>A	p.W57X	0.0008
FANCL	chr2:58453870	ATCT/A	c.263_265del	p.K88del	0.0003
FANCL	chr2:58453867	AG/A	c.268delC	p.L90fs	0.0001
FANCL	chr2:58387305	C/CT	c.1044_1045insA	p.G349fs	0.0001
FANCM	chr14:45642287	A/ACT	c.2190_2191insCT	p.E735fs	0.0001
FANCM	chr14:45644477	TAAAC/T	c.2521_2522insAAAC	p.Q842fs	0.0001
FANCM	chr14:45650888	CGCAGA/C	c.4367_4371del	p.R1456fs	0.0001
FANCM	chr14:45658082	TGAA/T	c.4858_4860del	p.E1620del	0.0001
FANCM	chr14:45668139	G/A	c.6008+1G>A	aberrant splicing	0.0003
FANCN (PALB2)	chr16:23647568	AG/A	c.298delC	p.D101fs	0.0001
FANCN (PALB2)	chr16:23647395	G/A	c.472C>T	p.Q158X	0.0001
FANCN (PALB2)	chr16:23646369	AC/A	c.1497delG	p.L499fs	0.0001
FANCN (PALB2)	chr16:23646192	G/A	c.1675C>T	p.Q559X	0.0003
FANCN (PALB2)	chr16:23641004	CA/C	c.2470delT	p.C824fs	0.0001
FANCN (PALB2)	chr16:23635328	A/G	c.2834+2T>C	aberrant splicing	0.0001
FANCO (RAD51C)	chr17:56787352	G/C	c.837+1G>C	aberrant splicing	0.0001
FANCP (SLX4)	chr16:3651155	CAGA/C	c.985_987del	p.Ser329del	0.0001
FANCP (SLX4)	chr16:3647443	C/T	c.1620G>A	p.W540X	0.0001
FANCP (SLX4)	chr16:3644451	TA/T	c.2160+2delT	aberrant splicing	0.0003

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FANCP (SLX4)	chr16:3640407	CAGCTGG/C	c.3226_3231del	p.P1076_A1077del	0.0001
FANCP (SLX4)	chr16:3639742	CCT/C	c.3895_3896del	p.R1299fs	0.0001
FANCP (SLX4)	chr16:3639379	T/TG	c.4259_4260insC	p.I1421fs	0.0001
FANCQ (ERCC4)	chr16:14042182	C/G	c.2729C>G	p.S910X	0.0001
FANCR (RAD51)	chr15:40994106	C/T	c.328C>T	p.R110X	0.0003
FANCS (BRCA1)	chr17:41258497	A/T	c.188T>A	p.L63X	0.0003
FANCS (BRCA1)	chr17:41245553	G/GAAA	c.1995_1997insTTT	p.N665_L666insF	0.0001
FANCS (BRCA1)	chr17:41244748	G/A	c.2659C>T	p.Q887X	0.0001
FANCS (BRCA1)	chr17:41244333	AG/A	c.3214delC	p.L1072fs	0.0001
FANCS (BRCA1)	chr17:41226421	C/CA	c.4664_4665insT	p.E1556fs	0.0001
FANCT (UBE2T)	chr1:202304773	C/T	c.109+1G>A	aberrant splicing	0.0004
FANCU (XRCC2)	chr7:152346394	TA/T	c.175delT	p.T59fs	0.0001
FANCW (RFWD3)	chr16:74695317	G/A	c.31C>T	p.Q11X	0.0001
FANCW (RFWD3)	chr16:74685992	G/GA	c.546_547insT	p.Q183fs	0.0001
FANCW (RFWD3)	chr16:74678352	C/T	c.988-1G>A	aberrant splicing	0.0001
FANCW (RFWD3)	chr16:74660405	G/A	c.2017C>T	p.R673X	0.0001

These data were obtained from 3.5KJPNv2 database (*https://jmorp.megabank.tohoku.ac.jp/201811/*). *We focused on nonsense mutations, frameshift mutations, and splicing site mutations. Missense mutation variants were not included.

reported to affect FA phenotypes (see Discussion section) (*Online Supplementary Table S2*).[5,10] Unfortunately, mutations were found in only one allele in seven (six FA-A and one FA-G) of the 107 patients. Since the mutations in these patients were clearly pathogenic and rare, we assumed this was diagnostic, and did not perform further analysis.

For the remaining ten unclassified cases, we screened large deletions in FA and related genes using our custom-designed aCGH in 2014. It revealed large deletions in two FA-B cases and one FA-T case (Figure 1B). The *FANCB* deletions spanned the entire genic area of *FANCB* (complete loss), and the defects extended into neighboring genes *MOSPD2* and/or *GLRA2*. Reanalysis of the WES data suggested putative junctions, where were amplified and sequenced. While the junction in Case 60 had a 3 bp overlapping microhomology, implying microhomology-mediated end joining as the mechanism (see *Online Supplementary Figure S1* for further details), there was no such homologous sequence in the break point in Case 61, suggesting that the re-ligation was mediated by non-homologous end joining (Figure 1B).[15] Two cases of entire *FANCB* deletion have been described in the literature[16,17] without elucidation of the junctional sequence. All of these *FANCB* large deletions seem to be distinct, but uniformly accompany severe phenotypic malformations (see below). The FA-T case with a large deletion was previously described.[6]

After aCGH, seven FA cases remained unclassified. We performed WGS for Case 64, in which the parents' genome was available, and RNA-seq analysis was carried out for three cases (Cases 62, 98, and 104), in which the patients' fibroblast cell lines were available. Interestingly, these analyses identified three cases with aberrant splice site mutations. WGS revealed that Case 64 harbored a homozygous mutation (c.1154+5G>A) in intron 12 of the *FANCC* gene. Real-time PCR (RT-PCR) confirmed that the mutation caused a splicing abnormality, resulting in retention of 120bp of intron 12 and a subsequent in-frame nonsense codon (Figure 1C). In Case 62, RNA-seq analysis revealed skipping of *FANCB* exon 7 (Figure 1D). This was likely to be caused by a mutation in the first nucleotide of exon 7, which did not alter the encoded amino acid (p.Leu499Leu). This mutation was considered non-pathogenic when the WES results were originally evaluated. However, it has been increasingly recognized that similar synonymous mutations affect splicing and cause genetic disorders and cancer.[18,19] RNA-seq and WES also revealed that Case 98 had a homozygous mutation (c.3350+5G>A) in intron 12 of *PALB2/FANCN* gene, resulting in skipping of exon 12 (Figure 1D).

Collectively, 113 (97%) of 117 Japanese FA patients were subtyped, and a total of 215 mutant alleles were identified (*Online Supplementary Table S2* and Figure 2A and B). FA-A and FA-G accounted for 58% and 25% of FA patients, respectively (Figure 2A). Interestingly, *FANCB* was the third most common complementation group in our series (approx. 3%). In notable contrast to a previous report from the Rockefeller University Fanconi Anemia Mutation Database,[20] FA-C represented an extremely rare complementation group in Japan (*Online Supplementary Table S1*). In keeping with this, there was not a single record with an IVS4+4 mutation in the 3.5KJPN or the East Asian population represented in the Exome Aggregation Consortium (ExAC) database. In Europeans, the allele frequency of the mutation was relatively high (0.04%) in the ExAC database, which reflects a high frequency of the IVS4+4A>T mutation in Ashkenazi-Jewish FA-C cases.[21]

Characteristics of Japanese *FANCA* pathogenic variants

In 68 FA-A patients (from 59 unrelated families), 130 mutant alleles were identified that consisted of 55 different *FANCA* variants (listed in *Online Supplementary Table S3* and *Online Supplementary Figure S3A*). The mutant alleles included nine missense mutations, eight nonsense mutations, 16 small insertions/deletions (indels), 12 large

deletions, one large duplication, and nine splicing mutations. All of the nine missense mutations were rated as "damaging" by both SIFT and PolyPhen-2 prediction programs, including two novel variants (c.2723_2725TCT>GCC, p.LS908_909RP; c.3965T>G, p.V1322G). Three of the eight nonsense mutations, six of the 16 small indels, and four of the nine splicing mutations were novel (*Online Supplementary Table S3*). We consider that these 13 novel mutations are all pathogenic. The large duplication and all of the large deletions except one (c.3765+827_3814del) were detected by the MLPA assay. We did not identify the precise breakpoints of these *FANCA* deletions; therefore, it was unclear whether they were novel or not.

Similar to the previous reports from Western countries,[20,22,23] the mutational spectrum in Japanese FA patients was broad (Figure 2B). However, some mutations were recurrently detected. The *FANCA* c.2546delC mutation was the most frequent (41 of 130 alleles; 31.5%), and other mutations such as c.978_c.979delGA, c.2602-2A>T, and c.2602-1G>A were detected in at least three unrelated families. c.1303C>T, c.2170A>C, c.2840C>G, c.3720_3724del, c.4168-2A>G were each detected in two unrelated families. The 45 remaining mutation variants were unique and were detected in single patients. *FANCA* c.2546delC existed at 0.08% frequency among 3,554 individuals from 3.5KJPNv2 in the ToMMo (Table 1), but not in the ExAC database (0%). This mutation was also commonly identified in Korean FA-A patients,[24] and therefore seems to be a hotspot in the East Asian population.

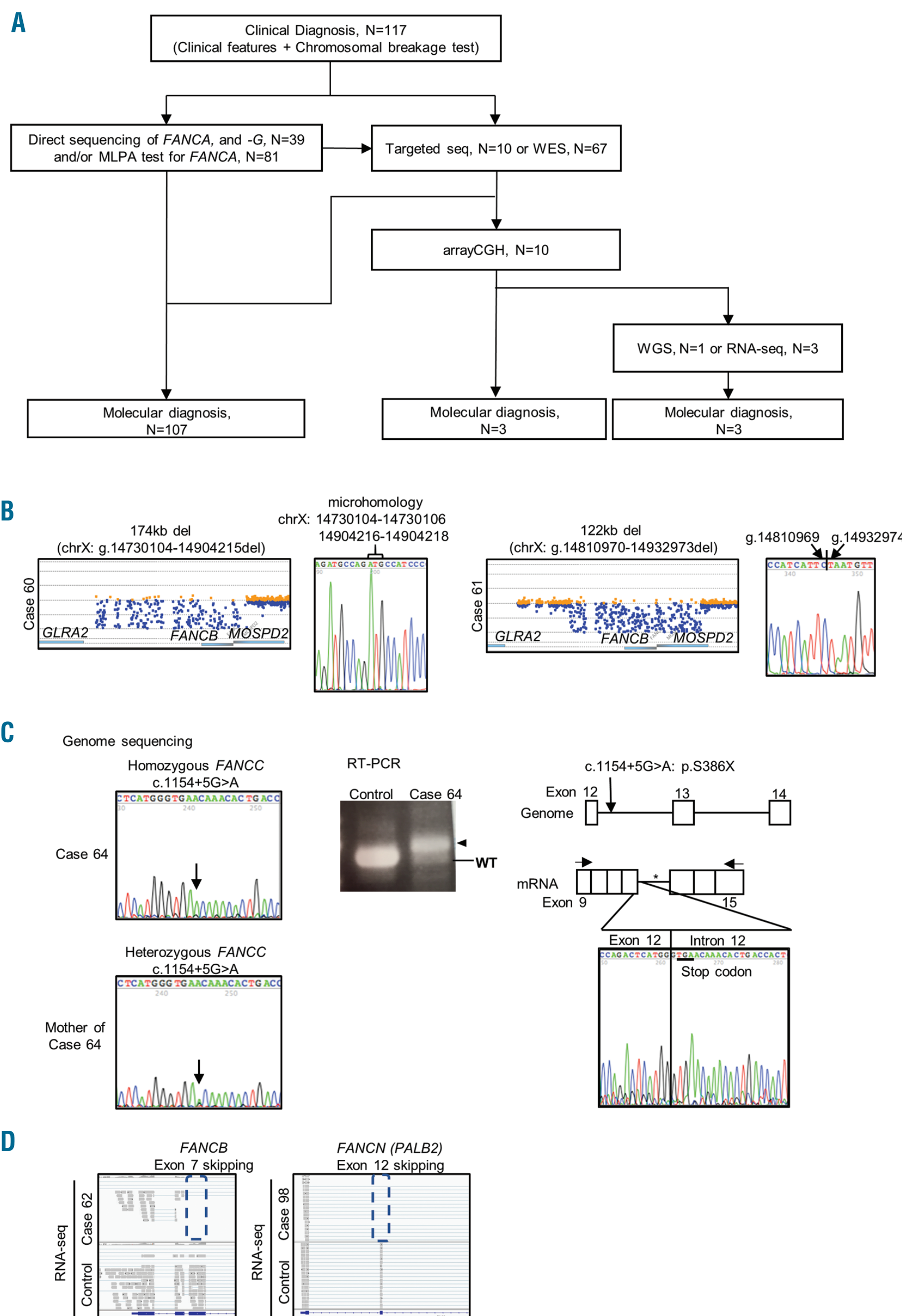

Figure 1. A comprehensive analysis successfully subtyped most of the Japanese Fanconi anemia (FA) patients. (A) Schematic presentation of the diagnostic strategy for the 117 FA patients. (B) The array-comparative genomic hybridization (aCGH) data displayed complete loss of the FANCB gene in Case 60 and Case 61. Sanger sequencing data identified the precise junctions in the two cases. (C) The whole-genome sequencing (WGS) analysis detected homozygous *FANCC* mutations in intron 12, resulting in a splicing defect. The Sanger sequencing data (left) identified the homozygous mutations in the patient (Case 64) and the heterozygous mutation in the patient's mother. The real-time polymerase chain reaction (RT-PCR) analysis showed a larger product (arrowhead) than the wild-type product, and sequencing analysis of the RT-PCR product (right) revealed the 120bp intron retention (*) after exon 12, resulting in a stop codon. (D) The RNA sequence reads of exon 7 in *FANCB* and exon 12 in *FANCN* were absent for Case 62 and Case 98, respectively. Corresponding whole-exome sequencing (WES) read alignments for Case 62 and Case 98 were diagnostic for the *FANCB* or *FANCN* mutations, as shown in *Online Supplementary Figure S2A* and *B*. N: number.

Table 2. Clinical phenotype of 10 Japanese Fanconi anemia patients with VACTERL-H association.

Individual	Affected gene	Mutation patterns	VACTERL-H features	FA-features	Family history of FA*	Birth weight SD score	DEB induced chromosome breakage (breaks /cell)	AL *** genotype
Case 18-1	*FANCA*	c.2546delC: p.S849FfsX40 c.4042_4043insC: p.I1348TfsX77	C: PDA R: Left renal agenesis L: Bilateral absent thumbs/ Bilateral radial hypoplasia	Short stature	+	-1.9	0.44	AA
Case 30	FANCA	c.2546delC: p.S849FfsX40 c.2546delC: p.S849FfsX40	V: scoliosis C: ASD/Persistent left superior vena E: Esophageal atresia	Skin pigmentation Deafness Right inguinal hernia Bicornuate uterus Short stature (-1.8SD)	–	-2.1	2.06	GG
Case 37	*FANCA*	c.2546delC: p.S849FfsX40 c.3295C>T: p.Q1099X	E: Esophageal atresia R: Right pelvic kidney L: Bilateral thumb hypoplasia	Jejunal atresia Strabismus Short stature (-4SD)	–	-2.3	0.12	GG
Case 60	*FANCB*	complete loss of *FANCB* gene (chrX g.14730104-14904216 del)	V: Spina bifida occulta/ Abnormal ribs A: Anal atresia C: PDA R: Right renal agenesis L: Right absent thumb/ Partial loss of left thumb	Skin pigmentation Microphthalmus/ Stenocephaly/Ptosis Duodenal stenosis Annular pancreas/ Hypospadias/ Undescended testis Short stature (-6SD)	–	-4.8	3.8	GG
Case 61	*FANCB*	complete loss of *FANCB* gene (chrX g.14810970-14932973 del)	V: Abnormal ribs/Scoliosis A: Anal atresia C: VSD/PS E: Duodenal atresia** R: Left renal agenesis L: Bilateral absent thumbs H: Hydrocephalus	Skin pigmentation Microphthalmus/ Deafness/ Ear canal stenosis Undescended testis (Short stature (-1SD))	–	-2.8	4.2	GA
Case 64	*FANCC*	c.1154+5G>A: p.S386X c.1154+5G>A: p.S386X	A: Anal atresia C: VSD, PDA E: Esophageal atresia	Skin pigmentation Deafness/Left aural stenosis/ Right aural atresia Cleft palate Short stature (-2SD)	–	-2.53	7.8	GG
Case 69	*FANCG*	c.307+1G>C c.1066C>T: p.Q356X	C: Coarctation complex R: Right renal agenesis/ Left renal cyst L: Bilateral absent thumbs/ Right radial hypoplasia	Skin pigmentation Short stature (-8SD)	–	-1.7	8.54	GA
Case 73-1	*FANCG*	c.307+1G>C c.307+1G>C	C: PDA R: Left renal agenesis L: Right absent thumb/ Bilateral radial hypoplasia	Skin pigmentation Bilateral aural atresia Short stature (-2.7SD)	+	-0.9	3.49	GA

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Case 96	*FANCI*	c.158-2A>G:p.S54FfsX5 c.288G>A:p.C56FfsX8	A: Anal atresia C: VSD/PDA R: Right renal agenesis/ Left renal hypoplasia L: Bilateral absent thumb/ Bilateral absent radius H: Hydrocephalus	Skin pigmentation Microphthalmus Hypogenitalia Short stature (-8SD)	–	-3.9	0.52	GA
Case 99-1	*FANCP*	c.343delA: p.S115AfsX11 c.343delA: p.S115AfsX11	C: ASD/VSD/PS R: horseshoe kidney L: Bilateral floating thumbs/ bilateral radial hypoplasia	Intestinal malrotation Duodenal stenosis Short stature (-5.8SD)	+	-2.3	0.91	AA

*Case 18-1, 73-1, and 99-1 had a sibling with Fanconi anemia (FA). ** Duodenal atresia is considered to be a part of the VACTERL association by some reports.[27] ****ALDH2* wild type and the inactivating mutation (p.Glu504Lys) allele is referred to as G and A, respectively. ALDH2: aldehyde dehydrogenase-2; ASD: atrial septal defect; BM: bone marrow; DEB: diepoxybutane; PDA: patent ductus arteriosus; PS: pulmonary stenosis; SD: Standard Deviation; VACTERL-H: vertebral anomalies, anal atresia, cardiac anomalies, tracheal-esophageal fistula, esophageal atresia, renal structural abnormalities, limb anomalies, and hypocephalus; VSD: ventricular septal defect.

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Characteristics of Japanese *FANCG* pathogenic variants

In 29 FA-G patients (from 27 unrelated families), 57 mutant alleles were identified, and seven different *FANCG* variants were detected (*Online Supplementary Table S4* and *Online Supplementary Figure S3B*). There were fewer unique mutation variants in FA-G compared with FA-A (Figure 2B). Three of the seven *FANCG* variants were novel. Of the three novel variants, two (c.907_908del and c.1386delC) were clearly pathogenic, whereas one mutation in intron 12 (c.1637-15G>A) was of uncertain significance. As previously reported, c.307+1G>C and 1066C>T accounted for most of the *FANCG* mutant alleles (49 of 57; 86%) in the Japanese FA-G patients.[25,26] Thirteen of the 29 FA-G patients were homozygous for c.307+1G>C, and eight were compound heterozygous with one c.307+G>C allele. Five of the eight remaining FA-G patients had homozygous c.1066C>T mutations. Four cases were compound heterozygous for the c.307+G>C and c.1066C>T mutations. In the 3.5KJPNv2 data, *FANCG* c.307+1G>C and c.1066C>T mutation variants were present with frequencies of 0.1% and 0.04%, respectively (Table 1). These mutations were similarly detected in Korean FA-G patients[24] but hardly ever observed in the other ethnic populations according to the ExAC database.

VACTERL-H phenotype caused by *FANCB, FANCI,* and other Fanconi anemia gene variants

We identified *FANCB* mutations in four affected males. The *FANCB* gene maps to the X-chromosome. Two of the four FA-B patients had a complete loss of the *FANCB* gene, as detected by aCGH (Figure 1B). In the remaining two patients, one harbored a nonsense mutation (c.516G>A/p.W172X) and one had a synonymous mutation (c.1497G>T/p.L499L) resulting in exon 7 skipping (Figure 1D and *Online Supplementary Figure S4A*). All four mutations were unique. The two FA-B cases with complete loss of *FANCB* displayed severe somatic abnormalities, consistent with VACTERL-H association (Table 2). The VACTERL-H association is defined as having three or more of the following defects: vertebral anomalies, anal atresia, cardiac anomalies, tracheal-esophageal fistula, esophageal atresia, renal structural abnormalities, limb anomalies, and hydrocephalus.[27] This set of anomalies has been reported in rare cases of FA, and is particularly associated with FA-B, I, J, N, or O cases.[28] The most frequent combination patterns in these patients with VACTERL-H association were cardiac-renal-limb anomalies (CRL), anal-renal-limb anomalies (ARL), and vertebral-renal-limb anomalies (VRL), which accounted for more than half of the patients. Cases 60 and 61 had five and seven features of the VACTERL-H anomalies, respectively.

Compared with these two FA-B cases, Case 62 with C-terminally truncated FANCB protein showed a less severe phenotype and experienced later onset of bone marrow failure (*Online Supplementary Figure S4A*). A recent biochemical study revealed that FANCB together with FAAP100 and FANCL are the central subcomplex components of the FA core complex, which is essential for ID2 complex monoubiquitination, a key activation event in the FA pathway. The FANCB:FAAP100 subunits form a scaffold that drives dimer formation of FANCL,[29] which is the E3 ligase component in the FA core complex. The truncated FANCB protein in Case 62 might, to some extent, maintain the ability to interact with FAAP100 or FANCL protein.[30] We were unable to obtain clinical information from another FA-B patient (Case 63).

Two FA-I cases were identified, and both had compound heterozygous mutations (*Online Supplementary Figure S4B*). Case 96, with N-terminal premature termination codons, had the five features of the VACTERL-H anomalies and died within two months after hematopoietic stem cell transplantation (HSCT) (Tables 2 and 3). On the other hand, Case 97, with C-terminal mutations, had only two features of the VACTERL-H and survived for more than 17 years after HSCT. In Case 96, a c.158-2A>G mutation in intron 3 and a c.288G>A mutation in the last codon of exon 4 caused splicing defects that resulted in a

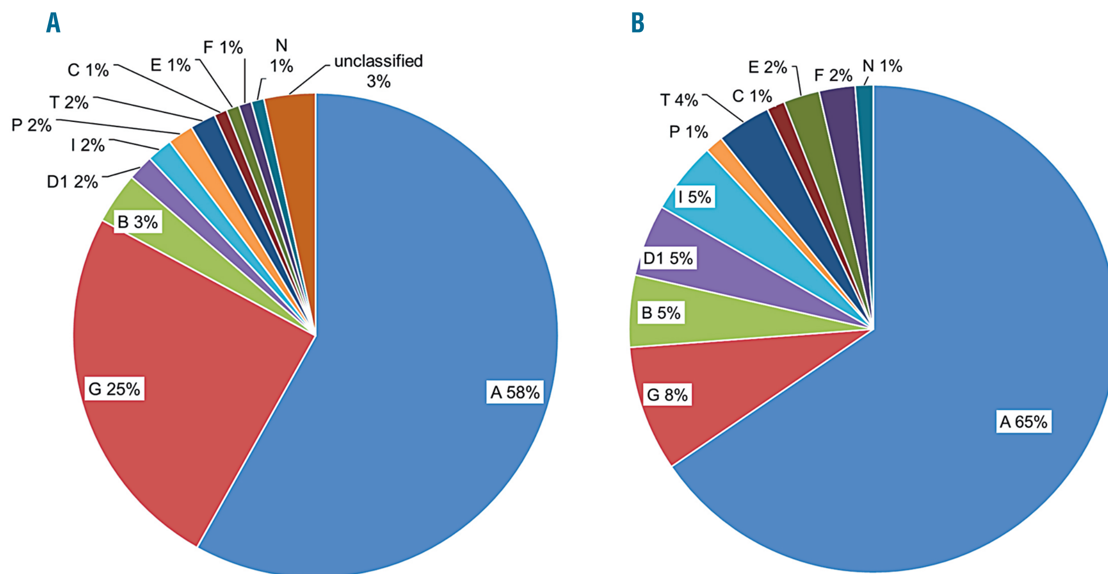

Figure 2. Frequency distribution of total (A) *versus* unique (B) Fanconi anemia (FA) gene mutations in the 117 Japanese FA patients. The frequency of the total FA gene mutation was based on subtyping of 117 FA cases, while frequency of unique FA gene mutations was derived from 84 genetic variants detected in the 117 FA patients.

Table 3. **Hematologic findings and outcome of 10 Japanese Fanconi anemia patients with VACTERL-H association.**

Individual	Onset of BMF (months)	BM status at HSCT	Karyotype of BM	Age at HSCT (months)	Outcome after HSCT (months)
Case 18-1	0	RCMD	46,XY,add(2)(q33)	13	Alive (105)
Case 30	70	SAA	46,XX	153	Dead/Esophageal cancer (165)
Case 37	49	RAEB1	46,XX, complex	192	Alive (66)
Case 60	58	SAA	46,XY	72	Alive (167)
Case 61	24	RCMD	46,XY,add(5)(p15)	51	Alive (160)
Case 64	40	SAA	46,XX	61	Alive (73)
Case 69	12	RCMD	46,XY	62	Alive (144)/Tongue SCC at 14 years old
Case 73-1	48	SAA	46,XY	88	Dead/Oral SCC (111)
Case 96	7	SAA	46,XY	45	Dead (2)
Case 99-1	0	RCMD	46,XY,+del(3)(q12)	13	Alive (59)

BM: bone marrow; BMF: bone marrow failure; FA: Fanconi anemia; HSCT: hematopoietic stem cell transplantation; RAEB: refractory anemia with excess of blasts; RCMD: refractory cytopenia with multilineage dysplasia; SAA: severe aplastic anemia; SCC: squamous cell carcinoma.

single nucleotide (guanine) insertion after exon 3 and skipping of exon 4, respectively (Figure 3). For Case 97, cells were not available and we could not verify the actual splicing defect caused by the c.3006+3A>G mutation. The patient's mother had only the c.3346_3347 insT mutation, while the father's genome was unavailable. The mutation at the +3 splice donor position was indicative of a potential splice defect[31] and we therefore considered that c.3006+3A>G would be a pathogenic mutation. This mutation was very rare and not reported as an SNV in the 3.5KJNv2 and ExAC database.

We also revisited available clinical data from 103 additional FA patients, and identified seven more cases with VACTERL-H (Tables 2 and 3). These include three FA-A, one FA-C case, two FA-G cases, and one FA-P case. All these seven cases met with VACTERL-H criteria with only three features. Four of the seven cases showed the CRL defect combination pattern. Compared with these cases, FA-B and FA-I cases with VACTERL-H association appeared to have higher number of malformations (from 5 to 7). We were unable to obtain detailed clinical information from the remaining nine patients. Thus, altogether there were ten VACTERL-H cases out of 108 cases with clinical data in our series, which seems slightly high compared to the previous report by Alter and Rosenberg (108 cases out of 2,245).[28]

Early-onset malignancies associated with the *FANCD1* (*BRCA2*) or *FANCN* (*PALB2*) complementation group

We identified two FA-D1 patients and one FA-N patient in our series. To the best of our knowledge, no FA-N cases and only one FA-D1 case (AP37P in Table 4) have been previously reported from Japan.[32,33] The two FA-D1 cases in our study had compound heterozygous mutations, of

which one was an N-terminal splice site mutation and the other was a nonsense or missense mutation (*Online Supplementary Figure S4C*). Both of the two *FANCD1* (*BRCA2*) splice site mutations (c.475+1G>A, c.517-2A>G) were regarded as deleterious. The one missense mutation (*FANCD1* c.7847C>T/p.S2616F) was rated as "damaging" by both SIFT and PolyPhen-2 prediction programs. It is notable that this missense mutation falls into the region termed "FA cluster" (amino acid position 2336-2729) where all of the five FA-D1-associated *BRCA2* missense mutations are found.[34] One FA-N patient had a homozygous splice mutation (c.3350+5C>T), resulting in skipping of exon 12 and C-terminal truncation (Figure 1D and *Online Supplementary Figure S4D*). This truncation may

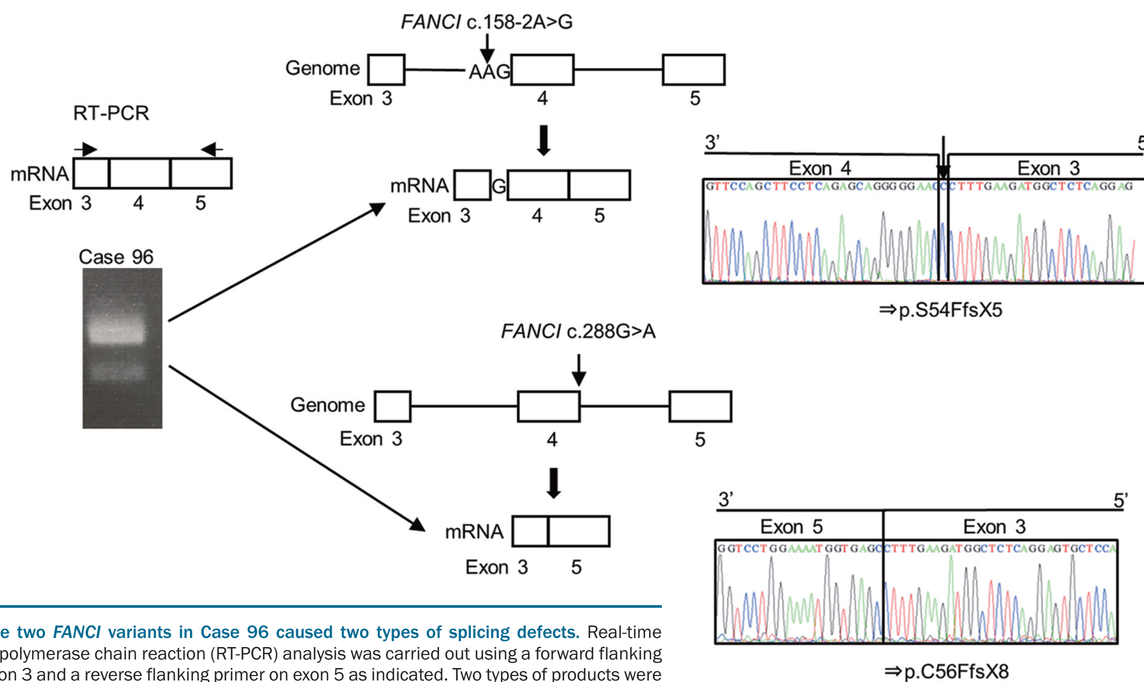

Figure 3. The two *FANCI* variants in Case 96 caused two types of splicing defects. Real-time quantitative polymerase chain reaction (RT-PCR) analysis was carried out using a forward flanking primer on exon 3 and a reverse flanking primer on exon 5 as indicated. Two types of products were obtained, and the sequencing analyses revealed a single nucleotide insertion (top) and exon 4 skipping (bottom).

Table 4. Clinical features of Japanese Fanconi anemia (FA)-D1 and FA-N cases.

Individual	Case 65	Case 66	Case 98	AP37P*
Sex	Female	Male	Male	Male
FA mutations	*FANCD1* c.517-2A>G, c.6952C>T: p.R2318X	*FANCD1* c.475+1G>A c.7847C>T:p.S2616F	*FANCN* c.3350+5C>T c.3350+5C>T	*FANCD1* c.-40+1G>A, c.8504C>A: p.S2835X
FA-features	Short stature Left thumb polydactyly Right renal agenesis Microphthalmus Microcephaly	Short stature Microcephaly	Short stature ASD, PDA Congenital absence of inferior vena cava, Congenital tracheal stenosis Microcephaly	Short stature Mid-face hypoplasia Sprengel's deformity Multiple café-au-lait spots
Chromosome breakage test	Positive (MMC)	Positive (MMC)	Positive (DEB)	Positive (MMC)
ALDH2 genotype	GG	GA	GA	GG
Hematologic abnormality (onset)	None	None	None	Acute myeloid leukemia (2 years old)
Solid tumors (onset)	Immature teratoma (9 months old)	T-lymphoblastic lymphoma, Adenosquamous lung carcinoma (23 years old)	Wilms tumor (1 year old)	None
Outcome	Alive with progressive teratoma at 1.7 years old	Died of lymphoma at 25.5 years old	Died of Wilms tumor at 1.5 years old	Died of leukemia at 2 years old

*a previously reported case.[32] MMC, mitomycin C, Other abbreviations are explained in Table 2 and 3.

affect *PALB2* interaction with *RNF168* or *BRCA2* which is mediated by the *PALB2* C-terminal WD40 domain.[35,36]

The three FA-D1 patients (including the previous Japanese case), as well as the one FA-N patient, all developed early-onset malignancies; this is in line with previous reports from Western countries (Table 4).[34,37,38] Although it is important to note that the first clinical manifestation in such cases could be onset of malignancy without prior clinical problems, Cases 65 and 98 had severe physical anomalies as well. Their malformations did not fully meet VACTERL-H criteria (Table 2). Alter *et al*. had previously reported that FA-D1 and FA-N patients were characterized by frequent VACTERL-H association and early-onset tumors, such as Wilms tumor, or acute myeloid leukemia (AML), with a cumulative incidence of malignancy as high as 97% by the age of 5.2 years.[34] Thus, Case 66 was highly unusual as a FA-D1 patient. He developed T-lymphoblastic lymphoma at 23 years of age, with a relatively short stature, and severe microcephaly (*Online Supplementary Appendix*). He received standard chemotherapy for the lymphoma, which caused prolonged pancytopenia. Then a mitomycin C-induced chromosome breakage test was performed, and he was diagnosed as FA. We list Case 66 as FA-D1, since he had biallelic, likely deleterious, *BRCA2* variants but no other FA gene mutations. This case may expand the clinical spectrum of FA-D1. Alternatively, for the moment, the possibility that hidden FA gene variants caused his FA phenotype cannot be excluded.

Allele frequency of pathogenic variations in 22 Fanconi anemia genes in the Japanese population

To estimate the frequency of pathogenic FA gene variations in the Japanese population, we analyzed WGS data for 22 FA genes from the 3.5KJPNv2 database. We identified 66 deleterious genetic variations (nonsense, frameshifts, and splicing site mutations) in 19 FA genes (Table 1). In addition to the three common *FANCA* [c.2546elC (0.08%)] and *FANCG* mutations [c.307+1G>C (0.1%); c.1066C>T (0.04%)], carriers with *FANCA* c.2602-2A>T, *FANCD1* c.6952C>T, *FANCG* c.194delC, or FANCI c.157-2A>G mutations were detected at low percentages (0.01-0.08%), and these variants were identified as causative mutations in Japanese FA patients. Allele frequencies of *FANCL* c.170G>A (p.W57X) variants were relatively high (0.08%); however, no patients with these variants were identified in our FA collection.

Monoallelic mutations in some FA genes, such as *BRCA1, BRCA2, BRIP1, PALB2* and *RAD51C*, cause adult-onset cancer predisposition[39-41] and we identified 25 deleterious variants in these genes (5 in *BRCA1*, 10 in *BRCA2*, 3 in *BRIP1*, 6 in *PALB2*, and 1 in *RAD51C*). *BRCA1* c.188T>A (p.L63X) and *BRCA2* c.6952C>T (p.R2318X) are well-known mutations in hereditary breast and ovarian cancer (HBOC) in Japan.[42] The *BRCA2* c.10150C>T (p.R3384X) was more prevalent than p.R2318X, but it has been classified as non-pathogenic because of its location near the 3'-end.[43] The *PALB2* c.2834+2T>C was recently identified in a Japanese female with bilateral breast cancer.[44]

From these analyses of allele frequency of FA-associated deleterious variants in 3,554 individuals, we estimated that approximately 2.6% of the Japanese could be considered to be carriers of pathogenic variations in FA genes.

Discussion

In this study, we report the largest series of subtyped Japanese FA patients to date by updating our previously reported cases with an additional 13 new cases (*Online Supplementary Table S2*). We employed various methods, including PCR-direct sequencing and next generation sequencing. WES and targeted exome sequencing were extremely useful in identifying mutations, as reported previously.[8] However, approximately half of the cases were undiagnosed even after these procedures.[8] When combined with the data generated by FANCA-MLPA, the diagnosis rate was much enhanced, since *FANCA* deletion was frequent, and WES/target-seq is not necessarily effective in identifying deletions. We also noted that mutations affecting splicing, such as intronic or synonymous variants, were difficult to detect by WES or targeted-seq. The former weak point was complemented by the use of aCGH, while RNA-seq was useful in detecting splicing abnormalities. We think the identification of two synonymous mutations affecting splicing is of great significance, since this type of mutations could have been easily overlooked. Thus, our approach ultimately achieved molecular diagnosis in most of the cases, and many private and novel mutations were identified in 11 of the 22 known FA genes.

Given the present results, we suggest that a molecular work-up of Japanese FA patients should start with screening for the three most-common mutations (*FANCA* c.2546delC, *FANCG* c.307+1G>C, and *FANCG* c.1066C>T) along with an MLPA assay for *FANCA*. As a next step, targeted-seq or WES analysis should be considered. For the remaining unclassified cases, aCGH, WGS, and RNA-seq analysis may be useful to identify large indels or splicing defects. Through these combined and comprehensive efforts, correct genetic diagnosis may be obtained in more than 90% of the Japanese FA patients.

Aldehyde dehydrogenase 2 (*ALDH2*) converts acetaldehyde to acetate, and potentially catalyzes other aldehydes as well. In East Asian countries, including Japan, a significant fraction (approx. 50%) of the population carries *ALDH2* variant *ALDH2*504Lys* which is encoded by the so-called A allele, and affects alcohol tolerance and some aspects of human health.[45] We have previously described a subset of severe FA cases that were homozygous for the *ALDH2*504Lys* variant (the AA genotype), and who experienced bone marrow failure and/or myelodysplastic syndrome (MDS) immediately after birth.[5,7] We also found several FA-B and FA-I cases that were accompanied by severe physical abnormalities, termed VACTERL-H. Two (Cases 18-1, 99-1) of the six previously reported FA cases carrying a homozygous *ALDH2* AA genotype also displayed these severe malformations[7] (Table 2), but, interestingly, their siblings (Cases 18-2, 99-2) with *ALDH2* GG genotype displayed relatively minor physical abnormalities (*Online Supplementary Table S2*). We note here that the FA-B or FA-I patients with VACTERL-H anomalies were carriers of the *ALDH2* GG or the GA genotype. The impact of endogenous aldehyde catabolism on bone marrow stem cells is very clear, and this effect also extends to the role of the FA proteins in preventing severe malformations. It has been suggested that the extent of physical abnormalities and severity of hematologic defects tend to be correlated.[46] In any event, FA-B and FA-I groups often exhibit severe malformations, as described previously[47,48] and confirmed here in Japanese cases. Since many of our

cases were referred to us in order to carry out HSCT, our data could be biased toward a proportion of patients with more severe malformations and may not reflect all individuals carrying FA gene variants. The relatively high incidence of VACTERL-H anomalies in our series could reflect this[46] and/or this may be due to the impact of the *ALDH2* genotype.

An important issue is how prevalent the FA-causing variants in the Japanese population are. We estimate that at least approximately 2.6% of the Japanese population might carry pathogenic variants in FA genes, using the 3.5KJPNv2 database. In Japan, approximately ten individuals with FA are born per one million births each year according to the report from the Japanese Society of Pediatric Hematology/Oncology.[49] FA-G accounted for 25% of Japanese FA patients according to our study and approximately two FA-G patients are estimated to be born each year in Japan. Our estimated allele frequency for *FANCG* (0.16%) from the 3.5KJPNv2 database is a reasonable one given the birth rate of the FA-G patients. Rogers *et al*. reported that at least one FA disease-causing variant among 16 FA genes (nonsense, splice altering, frame shifts, and a subset of missense variants that are judged to be highly deleterious) was identified in 4.3% of individuals from the ESP and 1KGP studies.[50] This estimate was substantially higher than ours, but our numbers may increase if we include deleterious missense mutation data in the future.

In conclusion, the molecular diagnostic strategy and data described in this study provide a basis for future molecular work-ups and clinical management for Japanese FA patients. In four cases, we failed to achieve a definitive subtyping; this could be due to technical problems or due to novel FA genes awaiting discovery. These remain as "unclassified", and could be of particular interest in further attempts to elucidate FA etiology.

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