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ORIGINAL ARTICLE

Droplet Digital PCR-Based Chimerism Analysis for Primary Immunodeficiency Diseases

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Abstract

Objective In the current study, we aimed to accurately evaluate donor/recipient or male/female chimerism in samples from patients who underwent hematopoietic stem cell transplantation (HSCT).

Methods We designed the droplet digital polymerase chain reaction (ddPCR) for *SRY* and *RPP30* to detect the male/female chimerism. We also developed mutation-specific ddPCR for four primary immunodeficiency diseases.

Results The accuracy of the male/female chimerism analysis using ddPCR was confirmed by comparing the results with those of conventional methods (fluorescence in situ hybridization and short tandem repeat-PCR) and evaluating dilution assays. In particular, we found that this method was useful for analyzing small samples. Thus, this method could be used with patient samples, especially to sorted leukocyte subpopulations, during the early post-transplant period. Four mutation-specific ddPCR accurately detected post-transplant chimerism.

Conclusion ddPCR-based male/female chimerism analysis and mutation-specific ddPCR were useful for all HSCT, and these simple methods contribute to following the post-transplant chimerism, especially in disease-specific small leukocyte fractions.

Keywords Droplet digital PCR · chimerism · severe combined immunodeficiency · hematopoietic stem cell transplantation

Abbreviations

APDS1	Activated PI3K-delta syndrome type 1
CIs	Confidence intervals
ddPCR	Droplet digital PCR
FISH	Fluorescence in situ hybridization
GVHD	Graft versus host diseases
HSCT	Hematopoietic stem cell transplantation
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked
NMA	Non-myeloablative
PIDs	Primary immunodeficiency diseases
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RIC	Reduced intensity conditioning
SCID	Severe combined immunodeficiency
STR	Short tandem repeat
VNTR	Variable number tandem repeat
WAS	Wiskott-Aldrich syndrome
XLP1	X-linked lymphoproliferative syndrome type 1

Tsubasa Okano and Yuki Tsujita contributed equally to this work.

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Introduction

In hematopoietic stem cell transplantation (HSCT) for treating non-malignant diseases that include primary immunodeficiency diseases (PIDs), inherited metabolic disorders and bone marrow failures, non-myeloablative (NMA) conditioning, or reduced intensity conditioning (RIC) are chosen for

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engraftment with minimal adverse complications. Such low-intensity regimens have contributed to improved survival of patients with such non-malignant diseases [1]; however, insufficient ablation of the recipient cells is liable to cause engraftment failure and donor-recipient mixed chimerism [2, 3], which lead to graft rejection and graft versus host diseases (GVHD) [4]. Consequently, physicians must follow the donor-recipient chimerism longitudinally, and depending on the results, their post-transplant immunosuppressive therapies must be modified.

Short tandem repeats (STR)/variable number tandem repeat (VNTR)-polymerase chain reaction (PCR)-based genotyping or fluorescence in situ hybridization (FISH) are commonly used for the detection of post-transplant or infant-maternal chimerism [5, 6]; however, the sensitivity of STR/VNTR-PCR depends on the genotypes of every donor and recipient, and FISH takes longer, needs larger samples, and is relatively high cost. Therefore, these methods present some difficulties under specific clinical conditions, particularly in the early post-transplant period, when physicians must assess microchimerism in each lineage fractions but can obtain only a small amount of sample.

In this study, we developed a new method by which to analyze chimerism using droplet digital PCR (ddPCR), which allowed us to accurately detect the target sequences, even in small amounts of sample, from the sorted target cells.

Materials and Methods

Cell Sorting and Genomic DNA Extraction

Peripheral blood mononuclear cells and granulocytes were isolated from whole blood by Lymphoprep (STEMCELL technologies, Vancouver, Canada), and target leukocyte subsets were sorted by magnet activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Genomic DNA was extracted from whole blood or leukocyte subsets using the QIAamp DNA Mini Kit and Micro Kit (Qiagen, Venlo, Netherlands).

Droplet Digital PCR

Target genomic loci were quantified by ddPCR (Bio-Rad Laboratories, Hercules, CA). Target-specific primers and probes are listed in Table 1. Thermal cycling was performed as follows: step 1, 95 °C for 10 min (1 cycle); step 2, 94-95 °C for 30 s followed by 50-52 °C for 2 min (40 cycles); step 3, 98 °C for 10 min (1 cycle); and step 4, hold at 4 °C.

Calculations

The concentration ratio of the target genes was converted into a cell ratio using the following calculations (square brackets indicate concentration):

SRY/RPP30 ddPCR-based sex chromosomal chimerism analysis.

$$[\text{Male cells}]/[\text{total cells}] = [\text{SRY}]/([\text{RPP30}]/2)$$

Genes inherited as X-linked recessive, when male patients received HSCT from male donor.

$$[\text{Wild-type cells}]/[\text{total cells}] = [\text{Wt}]/([\text{Wt}] + [\text{Mut}])$$

Genes inherited as X-linked recessive, when male patients received HSCT from female donor.

$$[\text{Wild-type cells}]/[\text{total cells}] = [\text{Wt}]/2/([\text{Wt}]/2 + [\text{Mut}])$$

Genes inherited as autosomal dominant.

$$[\text{Wild-type cells}]/[\text{total cells}] = ([\text{Wt}]-[\text{Mut}])/([\text{Wt}] + [\text{Mut}])$$

Statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA).

Results

Evaluation of ddPCR-Based Male/Female Chimerism Analysis

Using ddPCR, we quantified the absolute copy numbers of *SRY* on chromosome Y and *RPP30* on chromosome 10 and converted them to a male/female cell ratio. This analysis enabled us to correctly distinguish male and female samples from healthy donors (i.e., male cells were 100% of XY cells and female cells were 0%; Fig. 1a). We then applied the method to clinical samples from post-transplant patients and compared the results with those obtained after FISH and STR-PCR analyses. Significant correlation was identified between ddPCR and FISH ($R^2 = 0.9997$; Fig. 1b), and correlation between ddPCR and STR-PCR was found to be less significant ($R^2 = 0.8995$; Fig. 1c).

We then assessed the sensitivity of the ddPCR-based method by evaluating two types of serial dilution samples. First, we prepared a series of mixtures that were composed of male genomic DNA that was serially diluted with female samples (range, 0.78 to 100% of male DNA). The results obtained by ddPCR showed a remarkable correlation with the estimated male/female chimerism ($R^2 = 0.99938$; Fig. 2a).

Next, to determine assay sensitivity with reducing amounts of the template, two samples (containing 5 and 90% of male

Table 1 Target-specific primers and probes

Target gene	Chromosome	Mutation	Probe	Primer 1	Primer 2
SRY	*Chr.Y*	Wt	CCGATTGTCCTACAGC	CTAGGTAGGTCTTTGTA	CACACACTCAAGAATG
RPP30	*Chr.10*	Wt	TTCTGACCTGAAGGCTCTGCG	GATTTGGACCTGCGAGCG	GCGGCTGTCTCCACAAGT
IL2RG	*Chr.X*	c.865C>T Wt	TGGGAATTCaGGGCATC TGGGAATTCGGGGCA	CCTCTTTCTCCCCTGTC	CAGTAACAAGATCCTCTAGG
SH2D1A	*Chr.X*	c.207_208insC Wt	AGACAGCACCcTGGG AGACAGCACCTGGGG	AATAATTTGCTTGGCCTTTT	TCTGAAATGCTGAAATGAGA
PIK3CD	*Chr. 1*	c.3061G>A Wt	TTTAACaAAGCCCTCCGT TTTAACGAAGCCCTCCG	GAGGCACTGAAGCACT	TGCCTGTTGTCTTTGGAC
WAS	*Chr.X*	c.931+2T>C Wt	TCTCgCCCTGGCGC TCTCACCCTGGCGCC	CTGTGCGGCAGGAGA	CTAGAGAAGGGAGCGTATGG

Lowercases in probe indicate mutations in genome DNA

DNA, respectively) were serially diluted with water (range, 0.25 to 50 ng/assay). Interestingly, the accuracy varied depending on the sample's male DNA concentration. Namely, the smaller the male/female ratio in the samples, the smaller the confidence intervals (CIs) shown in this assay. As a result, the male DNA-dominant (90% male) samples had larger CIs than the female DNA-dominant (5% male) samples; however, we could have obtained ideal mean values using as little as 0.5 ng, which would have been from approximately 100 cells, from both samples (Fig. 2b).

ddPCR-Based Male/Female Chimerism Analysis for Clinical Samples

The ddPCR-based method was applied in the post-transplant clinical samples. A 3-year-old male (P3 in Table 2) with X-linked lymphoproliferative disease type 1 (XLP1), which is a disorder that causes fatal hemophagocytic lymphohistiocytosis owing to excessively activated T and NK cells [7], received HSCT from an unrelated female donor along with a RIC regimen. Donor-type engraftment was smoothly established on day 23; FISH detected 92% female cells. However, after modulating his immunosuppressive therapies against skin GVHD, the female cell chimerism suddenly decreased to 13% on day 150. In order to consider further treatment, we needed to assess which cell fraction contained the 13% of female cells; therefore, we performed ddPCR-based male/female chimerism analysis for sorted cell fractions. The results revealed that donor-type chimerism also decreased in whole blood (24%) and particularly in the granulocyte fraction (12.8%); however, it was preserved in XLP1's therapeutic targets (i.e., T cells (95.5%) and NK cells (65%); Fig. 3). Therefore, we could have removed re-transplantation and additional donor cell infusion from the treatment choices and reduced immunosuppressive therapies and followed up the chimerism using both FISH and ddPCR. Finally, donor-type chimerism was recovered to 98% by day 400.

Mutation-Specific ddPCR-Based Chimerism Analysis

Given that ddPCR-based male/female chimerism analysis could be applicable to all sex-mismatched HSCT, we prepared mutation-specific ddPCR analysis for several sex-matched HSCT. By using *SRY/RPP30* and *SH2D1A* mutation-specific ddPCR, we could accurately discriminate the droplets into two single-positive (blue and green) and double-positive (orange) droplets, and we obtained comparable results between these two methods ($R^2 = 0.9939$; Fig. 4a, b).

We also designed mutation-specific ddPCR for X-linked severe combined immunodeficiency (X-SCID), activated PI3K-delta syndrome type 1 (APDS1), and Wiskott-Aldrich syndrome (WAS) (Table 2).

Discussion

Several methods are now used to detect post-transplant chimerism; these methods include erythrocyte phenotyping, G-banding, FISH, and PCR-based genotyping [6]. FISH-based sex chromosomal analysis and STR/VNTR-PCR-based genotyping are the most commonly used methods for post-transplant chimerism detection in clinics; however, these two methods have some limitations, particularly in pediatric patients. FISH requires a relatively large quantity of samples, and it is difficult to obtain enough from patients, particularly in the early engraftment periods, or sorted small cell fractions.

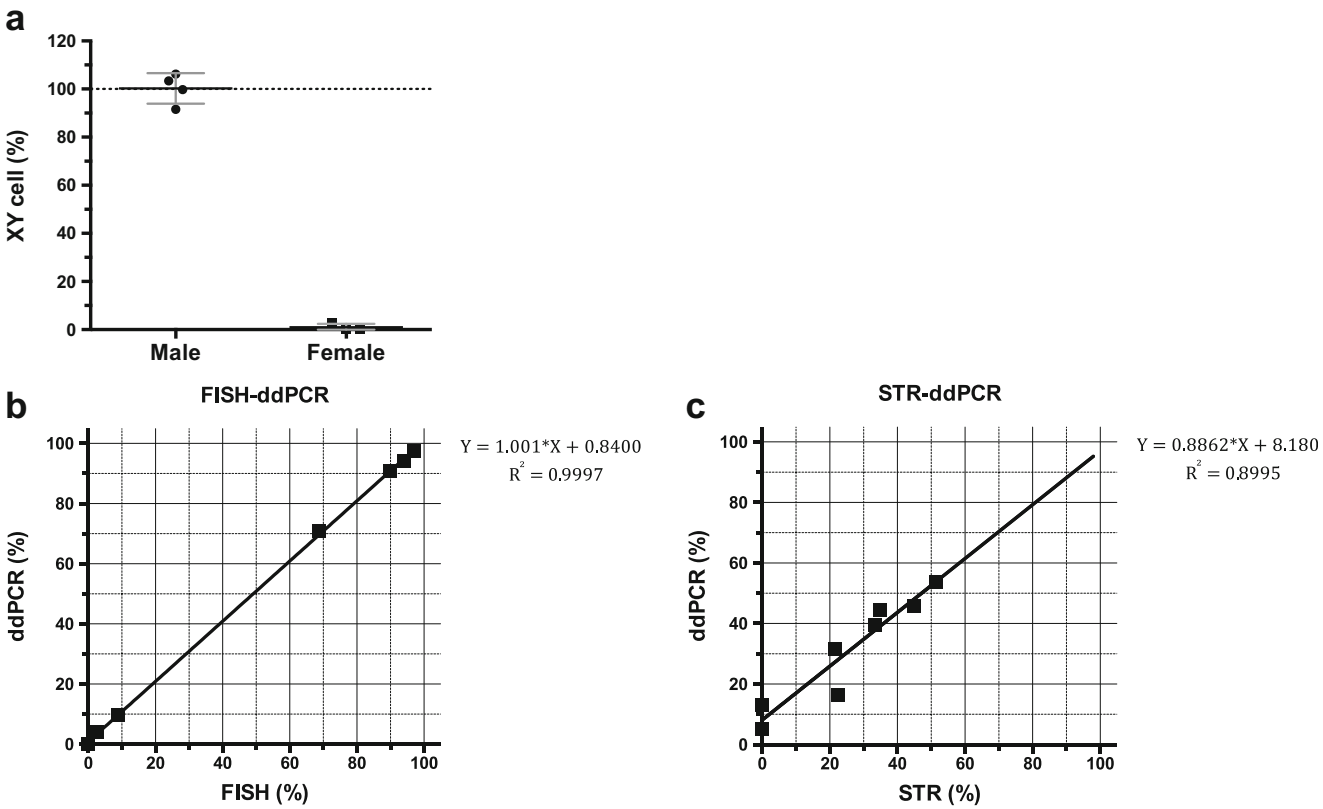

Fig. 1 Accuracy of droplet digital polymerase chain reaction (ddPCR)-based male/female chimerism analysis. **a** Control samples from healthy males ($n = 4$) and females ($n = 3$) were assessed using the ddPCR-based male/female chimerism analysis. The *Y*-axis indicates the percentage of male cells (i.e., cells with *SRY* signal). Error bars represent 95% confidence intervals. **b**, **c** Genomic DNA samples from post-transplant patients were assessed using ddPCR, fluorescence in situ hybridization (FISH), or short tandem repeat (STR)-PCR-based male/female chimerism analysis, and the results were compared

The STR/VNTR-PCR-based method has difficulties with sensitivity [8] depending on the combinations of recipients and donors.

Quantitative PCR (qPCR) with TaqMan probe technology has already been applied in selected clinical samples and has the advantage of having high sensitivity [9]; however, qPCR is highly influenced by the efficiency of every amplification reaction, and calibration curves and replicate assays are required. Recently, the ddPCR-based method was put to practical use to overcome these issues with qPCR. Because ddPCR

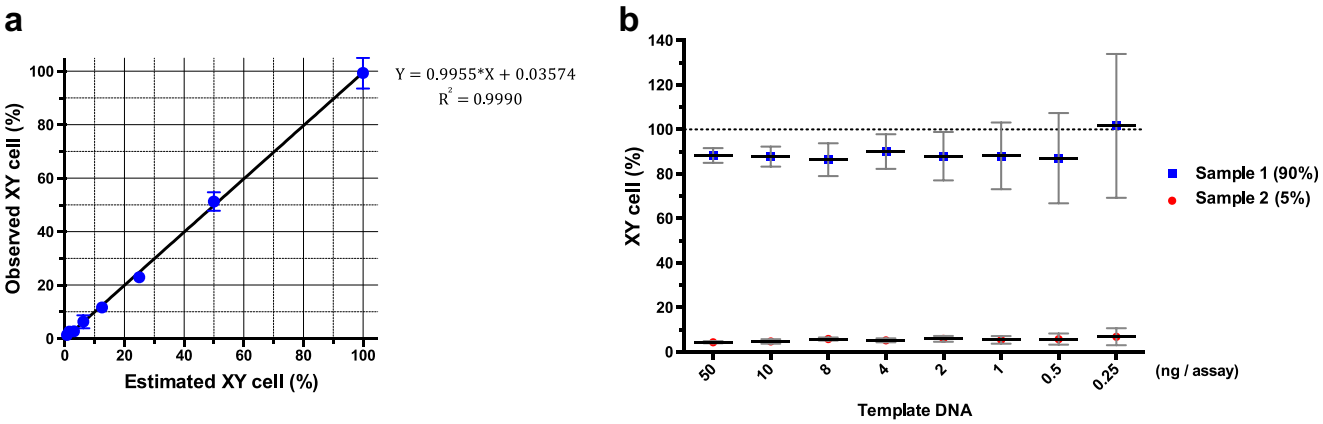

Fig. 2 Assay sensitivity assessed by sample dilution assays. **a** Genomic DNA samples from male donors were serially diluted with female samples, and the observed results (*Y*-axis) were compared with the estimated (*X*-axis) percentage of male cells. $R^2 = 0.99938$. **b** Two control samples were serially diluted with water and assessed by ddPCR. The estimated male cell ratio was 90% (sample 1) and 5% (sample 2), respectively, and dilution ranged them from 50 to 0.25 ng/assay. Error bars represent 95% confidence intervals

Table 2 Mutation-specific ddPCR-based chimerism analysis

Case	Disease	Gene	Mutation	Inheritance	Donor type chimerism (%)		
					Fraction	Before HSCT	After HSCT
P1	X-SCID	*IL2RG*	c.865C>T, p.Arg289Ter	XR	Whole blood	0	59.8
					Granulocytes	–	19.1
					PBMC	–	78.6
					T cells	–	99
					B cells	–	3.7
P2	XLP1	*SH2D1A*	c.207_208insC	XR	Whole blood	0	99.7
					Granulocytes	–	99.4
					PBMC	–	99.8
					T cells	–	99.9
					NK cells	–	99.8
P3	XLP1	*SH2D1A*	c.207_208insC	XR	Whole blood	0	28.4
					Granulocytes	–	18.3
					PBMC	–	56.2
					T cells	–	95.4
					NK cells	–	66.1
P4	APDS	*PIK3CD*	c.3061G>A, p.Glu1021Lys	AD	Whole blood	0	99.7
					Granulocytes	–	99.8
					$CD4^+$ T cells	–	99.3
					$CD8^+$ T cells	–	100
					Monocytes	–	100
P5	APDS	*PIK3CD*	c.3061G>A, p.Glu1021Lys	AD	Whole blood	0	77.7
					Granulocytes	–	67.1
					T cells	–	35.6
					Monocytes	–	45.3
P6	APDS	*PIK3CD*	c.3061G>A, p.Glu1021Lys	AD	Whole blood	0	97.1
P7	WAS	*WAS*	c.931+2T>C	XR	Whole blood	0	98.2
					Granulocytes	–	96.8
					PBMC	–	98.1
					Monocytes	–	98.8
					Lymphocytes	–	97.9

X-SCID X-linked severe combined immunodeficiency, *XLP1* X-linked lymphoproliferative disease, *APDS* activated PI3K-delta syndrome, *WAS* Wiskott-Aldrich syndrome, *XR* X-linked recessive, *AD* autosomal dominant, *HSCT* hematopoietic stem cell transplantation, *PBMC* peripheral blood mononuclear cell

detects the target sequences at the end point of amplification, amplification efficiencies have the least effect on the results. This helps in accurately determining absolute copy numbers of target sequences without any calibration.

We applied ddPCR to the clinical samples and noticed that the advantages of this method are not only its accuracy and simplicity but also that it requires only a small number of samples; it is beneficial especially for the diseases which have disease-specific treatment targets, such as XLP1. We also applied this method to a case of immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome to analyze post-transplant chimerism in $CD4^+CD25^{high}$ regulatory T cells, the target of HSCT for IPEX [10]. In addition, we used this method to analyze atypical X-SCID patients with hypergammaglobulinemia. We identified female cells in the $CD27^+$ memory B cell fraction by ddPCR and revealed that maternal B cells, engrafted in SCID patients, produced massive amount of immunoglobulin [11].

In the current study, we designed five pairs of primers and probes, all of which could accurately detect their targets. ddPCR-based male/female chimerism analysis is useful for all sex-mismatched HSCT, which corresponds to approximately one half of all HSCT [12]; moreover, it is also useful

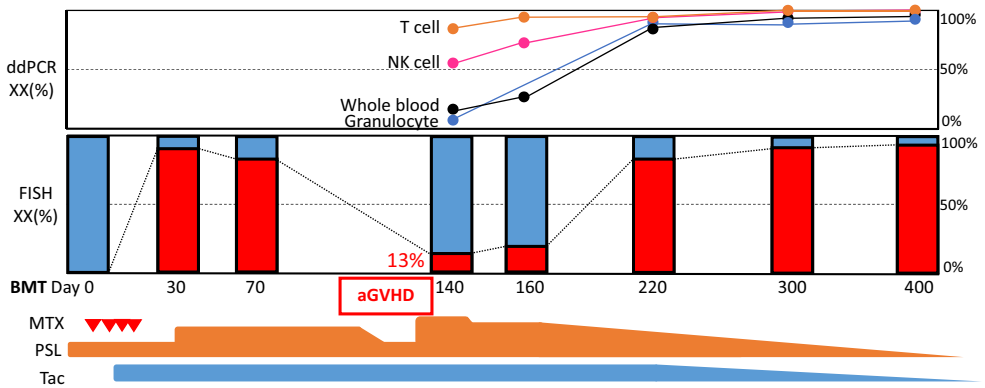

Fig. 3 Applying the ddPCR-based male/female chimerism analysis for post-transplant patient. ddPCR-based male/female chimerism analysis was performed for a male with XLP1 (P3), who underwent hematopoietic stem cell transplantation (HSCT) from a female donor. The percentage of female donor cells was assessed by ddPCR (top panel) and FISH (middle panel). Analysis for sorted cell fractions (T cells; orange, NK cells; magenta, granulocytes; blue) was performed using only ddPCR. The treatment course and symptoms are summarized at the bottom. Notes: FISH fluorescence in situ hybridization, BMT bone marrow transplantation, aGVHD acute graft versus host disease, MTX methotrexate, PSL prednisolone, Tac tacrolimus (color figure online)

in mutation-specific ddPCR for all sex-matched HSCT. In particular, mutation-specific ddPCR is useful for family cases, such as XLP1, and hereditary diseases that have recurrent mutations, such as APDS1.

In conclusion, ddPCR-based male/female and donor/recipient chimerism analyses are useful for all sex-mismatched and sex-matched HSCT. This method will clearly improve the outcomes of HSCT.

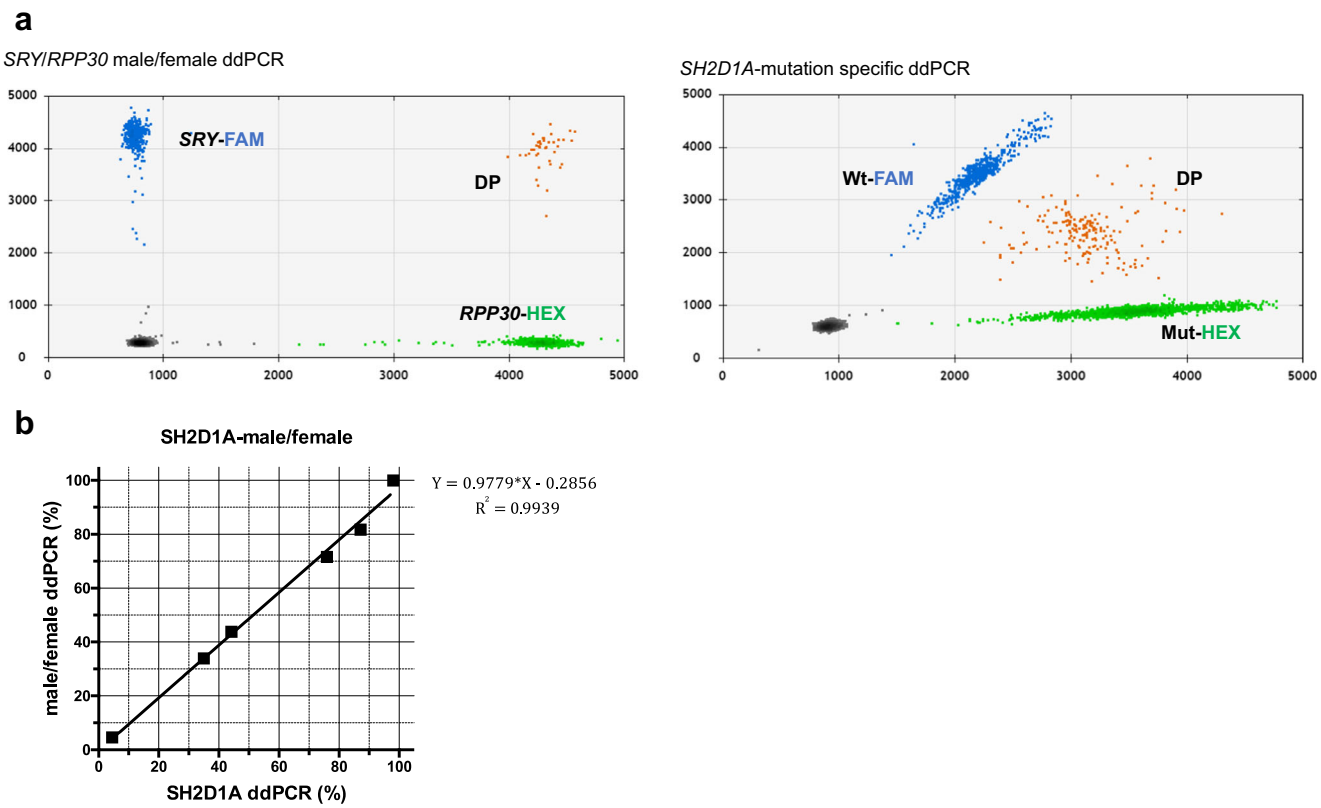

Fig. 4 Comparison of male/female and mutation-specific ddPCR. **a** Two-dimensional distribution of droplets. *X*-axis indicates the HEX signal, generated by the presence of *RPP30* or mutant *SH2D1A* (Mut) sequences. *Y*-axis indicates the FAM signal, generated by the presence of *SRY* or wild-type *SH2D1A* (Wt) sequences. Fluorescence-positive droplets are distinguished into HEX single positive (green), FAM single-positive (blue), and double-positive (orange). **b** Comparable results of male/female and *SH2D1A* mutation-specific ddPCR performed for the same samples from P3 (color figure online)

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Author Contributions T.O. and Y.T. performed the experiments and wrote the manuscript. H.K. designed the study and wrote the manuscript. K.M-S., K.T., S.M., T-W.Y., M.Y., N.T., and Y.O performed the experiments. M.T., K.I., S.N., and T.M. provided critical discussion.

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Compliance with Ethical Standards

We obtained written informed consent from the parents of patients and healthy controls. The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics boards of Tokyo Medical and Dental University and National Defense Medical College.

Conflict of Interest The authors declare that they have no conflict of interest.

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